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ATG7 contributes to plant basal immunity towards fungal infection

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Autophagy has an important function in cellular homeostasis. In recent years autophagy has been implicated in plant basal immunity and assigned negative ("anti-death") and positive ("pro-death") regulatory functions in controlling cell death programs that establish sufficient immunity to microbial infection. We recently showed that Arabidopsis mutants lacking the autophagy-associated (ATG) genes *ATG5*, *ATG10* and *ATG18a* are compromised in their resistance towards infection with necrotrophic fungal pathogens but display an enhanced resistance towards biotrophic bacterial invaders. Thus, the function of autophagy as either being pro-death or anti-death depends critically on the lifestyle and infection strategy of invading microbes. Here we show that ATG7 contributes to resistance to fungal pathogens. Genetic inactivation of *ATG7* results in elevated susceptibility towards the necrotrophic fungal pathogen, *Alternaria brassicicola,* with *atg7* mutants developing spreading necrosis accompanied by production of reactive oxygen intermediates. Likewise, treatment with the fungal toxin fumonisin B1 causes spreading lesion formation in the *atg7* mutant. We conclude that ATG7-dependent autophagy constitutes an "anti-death" ("pro-survival") plant mechanism to control the containment of cell death and immunity to necrophic fungal infection.

Key words: autophagy, *ATG7*, basal immunity, fungal resistance, arabidopsis

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Plants have evolved a bipartite plant immune system to cope with microbial infections. The first layer of defense relies on the recognition of pathogen-associated molecular patterns (PAMP) by pattern-recognition receptors (PAMP-triggered immunity, PTI).[1,2] To overcome this defense strategy, successful pathogens deliver so-called effector proteins into plant cells to modify host cellular processes and to suppress immune responses to enhance virulence. The presence or activities of these microbial effectors is sensed by plant resistance proteins and triggers the second layer of defense, the effector-triggered immunity (ETI).[1,2] In contrast to PTI, ETI is most often accompanied by programmed host cell death (PCD) at the site of attempted microbial invasion; however the molecular basis of this apoptosis-like hypersensitive response (HR) is largely unknown.

In recent years evidence accumulated that a non-apoptotic form of cell death called autophagy is not only involved in animal PCD and innate immunity[3] but is also an important component in the plant basal immune response.[4] Generally, autophagy (auto, meaning "self" and phagy, "to eat") is a cytoplasmic bulk degradation process in which cellular components are targeted to lysosomal or vacuolar degradation. This process is ubiquitous in eukaryotic organisms and is considered to aid cellular survival, differentiation, development and homeostasis by nutrient recycling or removal of damaged or toxic materials.[5-7]

Autophagy in Plant Innate Immunity—Pathogen Life Style Makes the Difference

Autophagy has a key role in protecting animal organisms against diverse pathologies, including microbial infection.[3] Numerous autophagy (ATG)-associated

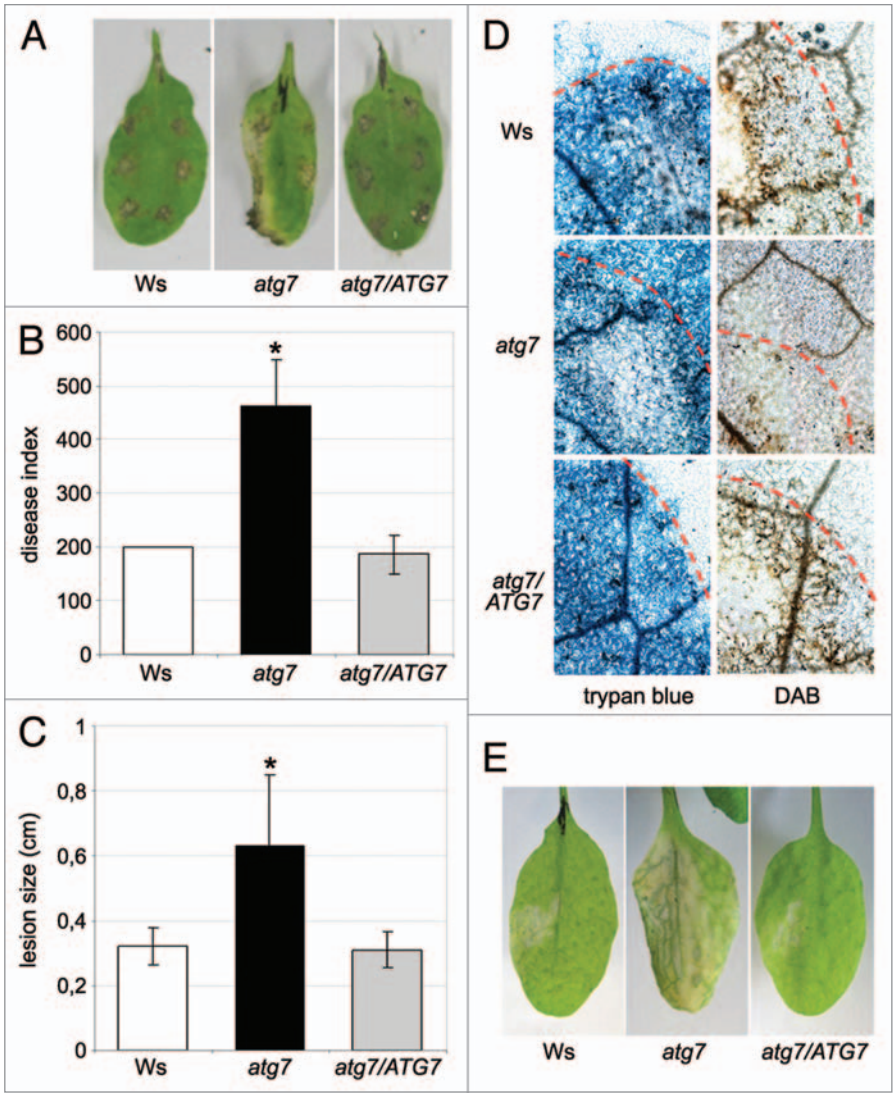

Figure 1. *atg7* mutants are more susceptible to infection with necrotrophic *A. brassicicola* and treatment with fumonisin B1. Wild-type Ws plants, *atg7* mutants and the corresponding complemented *atg7/ATG7* line were drop-inoculated with 10^6 spores/ml of *Alternaria brassicicola*. (A) Disease symptoms on representative infected leaves were monitored on day 11 after infection. (B and C) From leaves shown in (A) the disease indices (B) and lesion sizes (C) were determined. Results represent means ± SD (n ≥ 12) and statistical significance compared to the wild-type ($p \leq 0.05$, Student's t-test) is indicated by asterisks. (D) Fungal structures and dead plant cells were stained with trypan blue at day 7 after infection (left parts). H_2O_2 production in infected leaves was detected 7 days post infection using 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining (right parts). Leaves were analyzed by light microscopy. Dotted lines indicate borders of spore inoculation sites. (E) The fungal toxin fumonisin B1 causes spreading necrosis in *atg7* mutants. Wild-type Ws plants, *atg7* mutants and the *atg7/ATG7*-complementation line were infiltrated with 10 μM fumonisin B1 on one leaf side and development of necrotic cell death was monitored 6 days after treatment. All shown experiments were performed at least in triplicate with similar results.

genes have been identified in metazoans and yeast, most of which also have counterparts in plants.[8-10] These proteins regulate the formation of double-membrane-coated autophagosomes and subsequent fusion of these vesicles with lysosomal or vacuolar compartments for cargo degradation.

Recent reports have shown that plant autophagy is essential for the restriction, and conversely, also for the promotion of HR-PCD at the site of pathogen infection.[11-14] In tobacco mosaic virus (TMV)-infected *Nicotiana benthamiana*, knock-down of *ATG6* and *ATG7* genes resulted in spreading of N-mediated HR to adjacent uninfected tissue.[12] Likewise, Arabidopsis plants silenced for *ATG6* and *atg5* mutant plants were not able to contain effector-triggered HR-PCD upon infection with avirulent *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 bacteria carrying the AvrRpm1 effector protein.[13,14] Additionally, the disease-associated cell death induced by the infection of virulent *Pto* DC3000 bacteria was also partially mis-regulated in *AtATG6*-silenced plants.[13] Notably, it was also reported that autophagy-deficiency does not result in unrestricted HR-PCD but is rather required for HR-PCD execution.[11] Mutants in *ATG7* and *ATG9* did not develop the typical HR after infection with avirulent strains of *Pto* DC3000 expressing either the effector protein AvrRps4 or AvrRpm1. Likewise, *atg7* and *atg9* mutant genotypes also did not respond with an HR to infection with an avirulent isolate of the oomycete *Hyaloperonospora arabidopsidis*, indicating that autophagy may have a death-promoting function in some plant-pathogen interactions.

Recently, we conducted a comparative analysis including three Arabidopsis *atg* genotypes (*atg5*, *atg10*, *atg18-1*, in Col-0 background) to assess the role of autophagy in plant disease-associated cell death and basal immunity.[15] We showed that genetic inactivation of *ATG5*, *ATG10* and *ATG18a* resulted in enhanced fungal growth of the necrotrophic fungus *Alternaria brassicicola*, accompanied by a spreading necrosis and increased production of reactive oxygen species (ROS). In contrast, *atg5*, *atg10* and *atg18a* mutants showed reduced bacterial growth rates when infected with the virulent biotrophic bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto*DC3000), suggesting that autophagy may support infection by biotrophic bacteria. The increased resistance towards *Pto* infection was solely based on subtly elevated SA levels in the *atg* mutants and all other assayed innate immune responses proved to be unaltered in the autophagy-deficient plants.

Based on the above described reports we concluded that autophagy controls plant basal immunity to microbial infection in a pathogen lifestyle-dependent manner in either a positive or negative way.[16] Moreover, during ETI the function

of autophagy as death-promoting or death-containing cellular mechanism seems to be dependent on the nature of the immune receptor involved.[11]

ATG7 Functions in Immunity Towards Necrotrophic Pathogens

During autophagy cytoplasm containing cellular components is sequestered within double-membrane vesicles called autophagosomes that are subsequently transported to lytic compartments, such as the lysosome or the vacuole.[17] At the final step of autophagosome formation two ubiquitin-like conjugates are required. The first is the phosphatidylethanolamine (PE)-conjugated ATG8 which is produced by the ATG7 (E1-like) and ATG3 (E2-like) enzymes. In the second conjugation system, ATG5 is conjugated to ATG12, again relying on ATG7 E1-like activity, followed by ATG10 E2-like processing.[4,18] Mutations in the E1-like enzyme ATG7, which in the Arabidopsis genome is encoded by a single gene (*At5g45900*), results in accelerated, pre-mature senescence and in hypersensitivity to nutrient-limiting growth conditions.[19,20] Moreover, ATG7 has been implicated in plant immune responses towards viral and bacterial infections.[11,12]

Here we investigated if ATG7 is also involved in the plant immune response towards infection with *Alternaria brassicicola*, a necrotrophic fungal pathogen that causes black spot disease on cruciferous plants.[21] Wild type plants of the ecotype Wassilewskija (Ws), *atg7* mutants and corresponding complemented *atg7/ATG7* plants[19] were infected with spore preparations of *A. brassicicola* and disease symptom development was determined. Necrotic spots on wild type Ws plants appeared approximately 3–4 days after infection (data not shown) and were restricted to inoculations sites up to day 11 (**Fig. 1A**). However, *atg7* mutation resulted in spreading necrosis beyond infection loci, in increased lesion size and increased disease indices when compared to infected wild-type or *atg7/ATG7* complemented plants (**Fig. 1B and C**). Cell death progression, as detected by trypan-blue staining and cell death-associated production of reactive oxygen intermediates, as detected by diaminobenzidine-staining, spread beyond fungal infection loci in the *atg7* mutant line, but was limited to infection sites in control plants (**Fig. 1D**). Likewise, necrotic cell death progression induced by the fungal toxin fumonisin B1 was enhanced in *atg7* mutants (**Fig. 1E**). Thus, our previous observation using *atg5*, *atg10* and *atg18a* mutant genotypes[15] could be extended to the *atg7* mutant. As the genetic background of the *atg7* mutant was the ecotype Ws, we further introduced a novel ecotype in our studies in addition to the previously used Col-0 ecotype (*atg5*, *atg10* and *atg18a*).[15]

Our results are in agreement with a recent report that infection of *atg7* mutant lines resulted in a 2–3-fold higher spore production rate of *A. brassicicola* compared to the respective control plants.[22] Likewise, *atg7* mutants exhibited enhanced susceptibility to another necrotrophic fungal pathogens, *Botrytis cinerea*. Additionally, ATG7 gene expression was upregulated upon infection with Botrytis, and *atg7* mutants lacked Botrytis-induced formation of autophagosomes.[22]

These findings are indicative of an essential role of ATG7-dependent autophagy in the plant immune response towards necrotrophic pathogens, most likely by restricting chlorosis and cell death evoked upon infection by these destructive pathogens. Failure to contain necrotic collapse of the host tissue thus favors growth of necrotrophic pathogens on autophagy-deficient mutants.

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