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ARTICLE ADDENDUM

Processing of AtBAG6 triggers autophagy and fungal resistance

Yurong Li and Marty Dickman

Institute for Plant Genomics and Biotechnology, Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX, USA

ABSTRACT

The Bcl-2–associated athanogene (BAG) family is an evolutionarily conserved, multifunctional group of cytoprotective co-chaperones. Using structural bioinformatic approaches we identified 7 homologs of the *Arabidopsis* BAG family. Evaluating knockouts in Arabidopsis of individual BAG family members, we noted that Arabidopsis BAG6 (AtBAG6) knockout lines exhibited a pronounced enhancement of susceptibility to the necrotrophic fungal pathogen *Botrytis cinerea*. Moreover, we identified a single predicted caspase-1 site that was cleaved by an aspartyl protease (AtAPCB1). Finally, we showed AtBAG6 forms a complex with AtAPCB1 via coupling to a C2 GRAM domain protein (AtBAGP1). This complex and its activation is necessary for triggering pathogen mediated autophagic cell death and host resistance.

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In response to stress, multicellular organisms have developed various strategies for cytoprotection. Among those, altruistic cellular suicide cell or programmed cell death (PCD) involves the orchestration of cellular demise that under non-stressed conditions is a beneficial cell death to the organism, maintaining cell homeostasis for growth and development. While purported to be a conserved regulatory circuit, core regulators or functional equivalents of apoptotic-like PCD (e.g. Bcl-2 family and caspases), while well characterized in mammalian cells, have in general, not been identified in plants; at least at the primary sequence level. We reasoned that if some degree of conservation occurs between plant and animal PCD, it might be evident at the structural level, independent of sequence. Based on structural homology and computational searches, we uncovered the Bcl-2 athanogene (BAG) family in Arabidopsis, a family of co-chaperone regulators distinguished by a common conserved region known as the BAG domain which can mediate direct interaction with the ATPase domain of heat-shock protein 70 (Hsp70)/heat-shock cognate 70 (Hsc70).[1]

Although the mammalian BAGs localize either to the nucleus or cytoplasm, the 7 Arabidopsis BAGs are individually differentially localized to the nucleus, mitochondria, cytoplasm, endoplasmic reticulum (ER), and vacuole.[2,3] Several plant BAGs were evaluated and shown to be cytoprotective in response to biotic and abiotic stresses.[1-3] In particular, *AtBAG6* knockout lines and no others, exhibited an enhanced susceptibility phenotype when challenged by the necrotrophic fungus *Botrytis cinerea*, suggesting AtBAG6 may impact basal resistance.[1] We recently uncovered the role by which AtBAG6 effects plant immunity.[4] We found that AtBAG6 is processed at a single caspase 1-like cleavage site through association with protein partners that include a C2-GRAM protein (AtBAGP1), and an aspartyl protease (AtAPCB1). Both AtBAGP1 and AtAPCB1 are required for AtBAG6 cleavage and the cleavage is required for subsequent host resistance. Knock-out of AtBAGP1 or AtAPCB1 resulted in the blocking of AtBAG6 processing coincident with loss of resistance. Expressing cleavage site-mutated AtBAG6 led to inhibited autophagy and unimpeded fungal growth.

To determine the subcellular location of AtBAG6, YFP-tagged AtBAG6 was co-transformed with different RFP-tagged organelle markers into Arabidopsis protoplasts. Our results indicated YFP-AtBAG6 particularly merged with the vacuole signal (Vac-RFP) (Fig. 1). The result suggests the plant vacuole is a relevant location for AtBAG6 and accords with the occurrence of autophagy.

Autophagy is an evolutionary conserved catabolic process originally noted during yeast starvation, and is known to trigger pathways that non-selectively degrade cytosolic molecules and maintain homeostasis when resources are limiting. Autophagy is characterized by double membrane-bound vesicles or autophagosomes that sequester cytoplasmic components and damaged organelles which are then delivered to plant vacuoles for degradation and recycling.

Numerous studies have demonstrated important roles for autophagy in the regulation of nutrient starvation, cell differentiation and aging as well as abiotic as well as biotic stresses.[5-9] Like a double edged sword, autophagy has been shown to activate pro-death or pro-survival pathways, leading to much discussion as to whether autophagy is pro-life, pro-death, or both. Consistent with our observations, autophagy correlated with basal resistance to necrotrophs.[10-12] In contrast to biotrophs, necrotrophic pathogens kill host cells relying on a range of virulence factors, including toxins, reactive oxygen species (ROS) and hydrolytic enzymes for nutrient acquisition.[11-14]

CONTACT Marty Dickman mbdickman@tamu.edu
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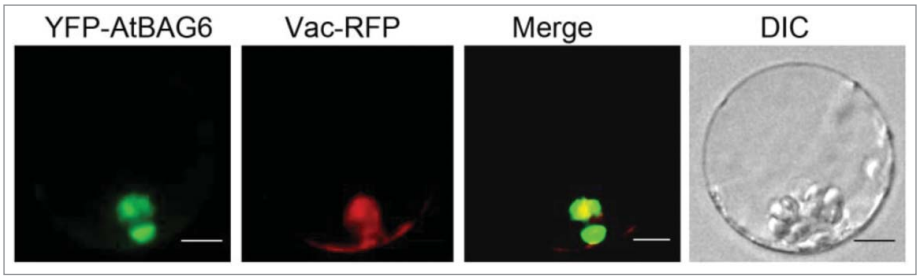

Figure 1. AtBAG6 localizes to the plant vacuole. Arabidopsis protoplasts were co-transfected with the fluorescent constructs YFP-AtBAG6 (yellow) and Vac-RFP (red). Fluorescence was visualized using an Olympus IX81 inverted fluorescence confocal microscope All images were collected using an Olympus DP controller and processed using Olympus FLUOVIEW software. Scale bar = 5 μm.

Dead tissue via autophagy could be construed to promote and enhance invasion and colonization by necrotrophic pathogens. However, Arabidopsis plants defective in autophagy genes (*atg*) were more susceptible to *B. cinerea* and *Alternaria brassicicola*, suggesting autophagy promotes host resistance (pro-survival) to necrotrophic pathogens.[11,12] In accordance, *atbag6* mutants have a similar phenotype to those described in other autophagy defective systems.[4,11,12] We investigated whether a functional autophagy system was present in *atbag6* mutants. Importantly, *atbag6* mutants retained functional autophagic responses when treated with chemical autophagy inducers (Trehalose, Tunicamycin)[15] in an *atbag6* backgound; thus *atbag6* was dispensable for general autophagy and the observed phenotype is a direct result of *B. cinerea* challenge. The chemical inducers restored the ability of the plant to respond successfully to fungal attack.[4] These results indicate AtBAG6 is not directly required for the process of autophagy. Thus we consider this to be a pathogen-induced autophagy (PIA) and AtBAG6 is likely a link between pathogen perception and the execution of PCD resulting in restricted fungal growth and resistance.[4] As a result of such specificity, we suggest the PIA may be involved with selective autophagy rather than a non-specific bulk degradation of cell components. For example, starvation-induced autophagy is a non-selective process that degrades undefined cellular materials providing the cell building blocks. Conversely, selective autophagy removes specific cytoplasmic constituents, including damaged organelles, protein aggregates and bacteria. Selectivity is regulated via cargo receptors and ATG8, a ubiquitin like protein involved in autophagosome formation.[16] While well studied in mammals, the relatively large family of ATG8 proteins[8,17] may represent an evolutionary adaptation by plants to promote flexibility in selective autophagy for a range of stress stimuli. We thus suggest that ATG8 harbors more complex and/or additional specialized functions specifically associated with plant selective-autophagy. This may be needed due to the sessile nature of plants and unpredictable environmental conditions.[18] If and how this complex selectively captures appropriate cargo material and the identity of cargo materials that in this case triggers autophagy induced resistance is a topic of keen interest.

To date, bona fide strictly defined caspase genes have not been identified in plants. However, caspase-like protease

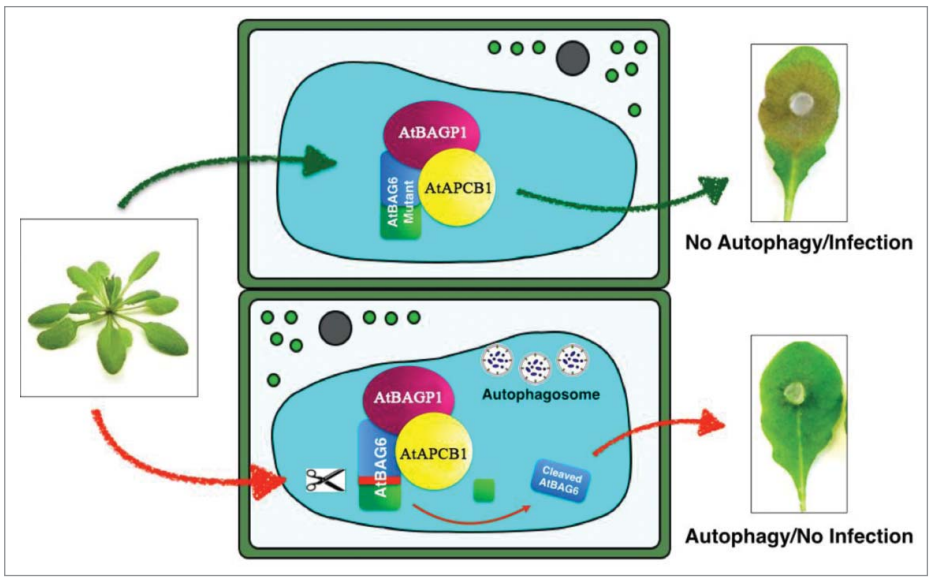

Figure 2. Autophagy induced by processed AtBAG6 is required for plant defense. In response to the fungal phyopathogen *B. cinerea*, a complex (AtBAG6-AtBAGP1-AtAPCB1) is formed, and processing of AtBAG6 occurs. Autophagy is then triggered fungal growth is restricted and plant resistance occurs. If cleavage is blocked, autophagy is suppressed and PCD runaway cell death occurs.

activities are widely detected in plants.[19-22].The aspartyl protease AtAPCB1 cleaves the single caspase 1-like cleavage site in AtBAG6.[4] Caspase-1 enzymes are inflammatory; thus it is unclear how the aspartyl proteases are regulated in this system. In addition, aspartyl proteases are associated with plant development[23] and systemic acquired resistance.[24] AtAPCB1 appears to be AtBAG6 specific as 2 unrelated Arabidopsis aspartyl proteases were unable to complement the *atacpb1* mutant line with respect to AtBAG6 cleavage. The C2-GRAM proteins are only found in plants and are typically endomembrane localized; thus far their functions are largely unknown. The C2 domain is a Ca^{2+}-dependent membrane targeting module found in many cellular proteins involved in signal transduction or membrane trafficking[25] and is coupled to enzymatic domains.[26] AtBAG6 is a calmodulin (CaM)-binding protein (CaMBP) which is selectively induced by Ca^{2+} but not other divalent cations and modulated by CaM through transducing Ca^{2+} signals.[27] It is reasonable to hypothesize that the C2-GRAM protein AtBAGP1, recruits AtBAG6 as a consequence of Ca^{2+}-regulation, and couples the enzymatic domain of AtAPCB1 via the C2 domain to degrade the substrate AtBAG6 under specific stimulation (Fig. 2).

In summary, Arabidopsis AtBAG6 is a cytoprotective co-chaperone that we suggest is involved with selective autophagy. As a result of fungal interaction with the necrotrophic pathogen *B. cinerea*, a complex is formed, and processing of AtBAG6 occurs. The processed AtBAG6 subsequently triggers autophagy, resulting in restricted fungal growth and resistance. If this process is blocked, autophagy is suppressed and PCD runaway cell death occurs (Fig. 2).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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