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The plant cell death suppressor Adi3 interacts with the autophagic protein Atg8h

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ABSTRACT

The tomato AGC protein kinase Adi3 is known to function as a suppressor of PCD and silencing of Adi3 leads to spontaneous cell death on leaves and stems. In an effort to isolate Adi3 interacting proteins, a yeast two-hybrid screen was carried out and identified the autophagy protein Atg8h as an Adi3 interactor. This interaction occurred independent of the kinase activity status of Adi3. Silencing of genes involved in autophagy is known to eliminate the restriction of pathogen-induced PCD to a few cells and leads to run away PCD. Cosilencing Adi3 with several autophagy genes lead to the same run away cell death suggesting Adi3 may be involved in autophagic regulation of PCD.

1. Introduction

Programmed cell death (PCD) is a genetically encoded, highly regulated process in multi- and single cell eukaryotic organisms [1–4] and bacteria [2,5]. In multicellular organisms, PCD often occurs during developmental processes, imparting a positive effect by killing specific cells in the organ connected with the process [1]. Without PCD, proper development is not achieved. In plants, flower and embryo development, seed coat generation, senescence, establishment of leaf shape, xylem formation, and resistance to pathogens all involve PCD [1]. Thus, PCD plays a central role in many aspects of maturation and survival of plants.

Despite the many processes in plants that require PCD, identification of genes and signaling pathways involved in plant PCD has been difficult compared to mammalian systems [1,6–8]. However, in recent years, the number of genes identified to be involved in plant PCD control has increased and includes homologues of mammalian genes [9,10], MAPKs [11–15], transcription factors [16], lipid biosynthetic genes [17–20], and ubiquitin E3 ligases [21]. The pathways associated with these genes for the most part remain to be determined.

My lab studies the tomato Ser/Thr AGC protein kinase Adi3, which we have identified as a suppressor of PCD [22,23]. Adi3 was initially identified through its interaction with the effector protein AvrPto from the tomato pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) and the host resistance protein Pto [24]. The interaction of Pto and AvrPto leads to cell death associated with the hypersensitive response (HR) and resistance to *Pst* [25]. We have shown that silencing of *Adi3* by virus induced gene silencing (VIGS) leads to the formation of cell death lesions on stems and leaves, reduced plant stature, and ultimately whole plant death [22]. Additionally, Adi3 functions in the nucleus to suppress PCD and prevention of Adi3 nuclear entry leads to cell death by eliminating its PCD suppression activity [23]. Thus, we predict the Adi3/Pto/AvrPto interaction prevents Adi3 nuclear entry and leads the HR cell death [23,26].

In an effort to identify Adi3-interacting proteins, a Y2H screen was carried out in this study and identified Atg8h as an Adi3 interactor. Atg proteins are involved in autophagy, a process by which cellular contents are enveloped in an autophagic vesicle and transported to the vacuole for degradation [27]. Atg8 is critical for the formation of the autophagic vesicle and fusion to the vacuolar membrane [28,29]. In plants there are nine different *Atg8* genes designated *Atg8a* to *Atg8i* [30]. Recently, autophagy has been shown to be important for controlling the spread of HR cell death, and VIGS of autophagy genes leads to uncontrolled spread of HR cell death [31]. Here it is shown that Adi3 specifically interacts with tomato Atg8h and that cosilencing of several autophagy genes with *Adi3* leads to uncontrolled spread of the *Adi3* VIGS cell death phenotype. This suggests that *Adi3* may work in coordination with autophagy to control cell death.

2. Materials and methods

2.1. Yeast two-hybrid assays and Adi3 interactor screen

For the Y2H screen and follow up assays, the pEG202 vector was used for bait constructs and the pJG4-5 vector for prey constructs.

Abbreviations: HR, hypersensitive response; MAPK, mitogen activated protein kinase; ORF, open reading frame; PCD, programmed cell death; *Pst*, *Pseudomonas syringae* pv. *tomato*; VIGS, virus induced gene silencing; Y2H, yeast two-hybrid assay.

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Constructs were transformed into yeast strain EGY48 containing the pSH18-34 reporter vector. The Y2H screen utilized a prey vector previously constructed from RNA isolated from tomato plants treated with *Pst* [32] and this library was screened against the *Adi3* bait by standard protocols [33]. For Y2H interaction assays with Adi3, the *Atg8h*, *Atg8a*, and *Atg8f* cDNAs (see below) were cloned into the *Eco*RI (5′) and *Xho*I (3′) sites of both pEG202 and pJG4-5. The *Adi3* ORF was cloned into the *Eco*RI (5′) and *BamH*I (3′) sites of pEG202 and the *Aid3* prey vector was previously described [22]. The *Drosophila Bicoid* bait and *Dorsal* prey vectors were described previously (Tang et al., 1996; [34]). Protein expression was confirmed by western blot. All other procedures for the yeast two-hybrid assays followed standard protocols [33].

2.2. Cloning of the tomato Atg8a, Atg8f, and Atg8f cDNAs

The tomato *Atg8h* Y2H clone contained a full length cDNA and the ORF was amplified by PCR using the following primers: forward, 5′-**ATG**GGGAAGACCTTCAAAGATG-3′ (start codon bold) and reverse, 5′-**CTA**AGAGTGACCACCAAAGGT-3′ (stop codon bold). The *Atg8h* sequence has been deposited in GenBank (accession # JF261157). The tomato *Atg8a* and *Atg8f* genes were identified using the tomato Atg8h ORF to screen the tomato EST library (http://sol-genomics.net/) for all Atg8-like proteins. Unigene SGN-U578682 and SGN-U584702 were identified as containing the *Atg8a* and *Atg8f* cDNAs, respectively. The cDNAs for these genes were amplified by standard RT-PCR using Superscript III reverse transcriptase (Invitrogen) and an oligo dT primer for first strand cDNA production. The ORF of each gene was amplified using the following primers: *Atg8a* forward, 5′-**ATG**GCCAAAAGCTCCTTCAAATTG-3′ (start codon bold) and reverse, 5′-**TCA**GAAGGATCCGAAGGTATTCTC-3′ (stop codon bold); *Atg8f* forward, 5′-**ATG**GCTAAGAGCTCATTCAAG-CAAG-3′ (start codon bold) and reverse, 5′-CTACAGTTCGCTCAG-GACC (stop codon bold). The *Atg8a* and *Atg8f* sequences have been deposited in GenBank (accession # JF304784 and JF304785, respectively).

2.3. Virus induced gene silencing

For silencing experiments, Rio Grande PtoR tomato plants were grown as previously described [22]. The TRV system was used for VIGS [35] and the VIGS vectors for *Atg6*, *PI3K*, *Atg7*, and *Atg3* were obtained from the Dinesh-Kumar lab [31]. The *Adi3* VIGS vector was previously described [22]. Agrobacterium containing the VIGS constructs were syringe infiltrated into cotyledons of one-week-old tomato seedlings before the first leaves were visible. All other conditions for tomato VIGS are previously described [22,36] and silencing was confirmed by RT-PCR.

2.4. Protein sequence alignment

The protein sequences for *Arabidopsis* Atg8h (AtAt8g8h; NM111517) and tomato Atg8h (SlAtg8h) were aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

3. Results and discussion

3.1. Yeast two-hybrid screen to identify Atg8h interaction with Adi3

The Adi3 ORF was cloned into the bait vector pEG202 for use in the LexA Y2H system and was shown to not auto-activate [22]. The Adi3 bait was then screened against a cDNA prey library previously developed from *Pst* exposed tomato leaves [32]. Approximately 15 million yeast transformants were screened for Adi3-interacting proteins using selection on Leu- plates and 1366 transformants were followed-up in a *LacZ* screen. Prey inserts from 85 random positive clones were sequenced and screened against GenBank for identification and the tomato homologue of *Atg8h* was identified 11 independent times as an Adi3 interactor. The *Atg8h* Y2H clone contained the full length cDNA, which was cloned, sequenced, and the encoded protein analyzed (Fig. 1).

3.2. Analysis of the tomato Atg8h protein

The tomato Atg8h protein contains 119 amino acids and alignment of the *Arabidopsis* and tomato Atg8h proteins indicates the two proteins have 67.2% amino acid identity and 99.0% amino acid similarity (Fig. 1). One interesting difference between the two proteins is in the C-terminal sequence. Atg8 proteins are processed by the Cys protease Atg4 by cleavage of any amino acids after the last C-terminal Gly residue [37]. In *Arabidopsis*, Atg8a to Atg8g all contain between two to five amino acids after this C-terminal Gly, while Atg8h and Atg8i contain no amino acids after this Gly [30]. In contrast, tomato Atg8h contains three additional amino acids after this Gly, one of which is an additional Gly (Fig. 1). It remains to be determined if the tomato Atg8h C-terminus is cleaved by Atg4 and if it is, which Gly becomes the terminal residue.

3.3. Interaction of Adi3 activity mutants with Atg8h

Next, the *Atg8h* ORF was cloned into the bait and prey Y2H vectors and tested for interaction with Adi3 kinase activity mutants. The two proteins were shown to have a stronger Y2H interaction with Atg8h as a bait and Adi3 as a prey (Fig. 2A). This is not uncommon as the strength of Y2H interactions can vary when switching interacting proteins between the bait and prey [38]. Adi3 Lys337 is the amino acid that binds ATP and mutation to Gln ($Adi3^{K337Q}$) eliminates kinase activity [22]. Adi3 Ser539 is phosphorylated by the upstream kinase Pdk1 and mutation to Asp ($Adi3^{S539D}$) produces a constitutively active Adi3 [22]. The Adi3/Atg8h interaction was not affected when using either of these Adi3 kinase activity mutants (Fig. 2A) suggesting Adi3 kinase activity is not required for the interaction.

3.4. Adi3 interaction with Atg8a and Atg8f

The specificity of the Adi3/Atg8h interaction was analyzed by testing the interaction of Adi3 with other Atg8 proteins. The tomato EST database was screened for all Atg8 genes and ESTs for *Atg8a*, *Atg8c*, *Atg8d*, *Atg8e*, *Atg8f*, and *Atg8i* were identified. However, only

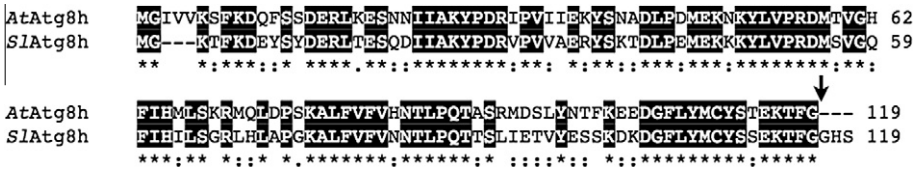

Fig. 1. Alignment of Atg8h proteins from *Arabidopsis* (*At*Atg8h) and tomato (*Solanum lycopersicum*; *Sl*Atg8h). Identical amino acids are boxed in black. In the consensus line "∗" = identical amino acids, ":" = conserved substitutions. "." = semi-conserved substitution. Arrow indicates the position of cleavage by Atg4 in *Arabidopsis* Atg8 proteins.

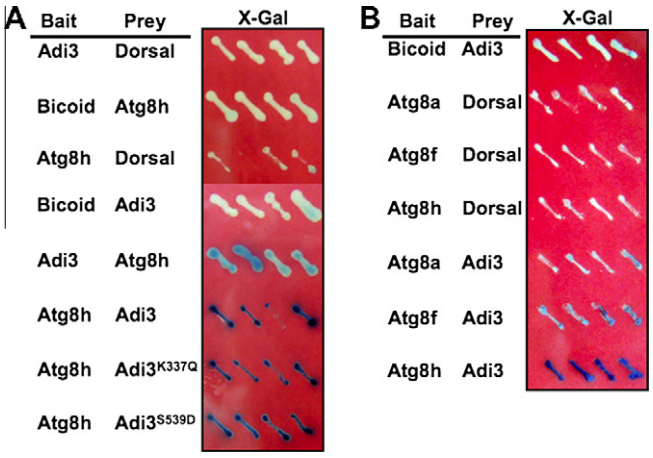

Fig. 2. Yeast two-hybrid interaction of Adi3 with Atg8h, Atg8a, and Atg8f. (A) Adi3 and Atg8h interaction as identified in Y2H screen (Adi3 as bait, Atg8h as prey) and in reverse combination (Atg8h as bait, Adi3 as prey) with Adi3 kinase activity mutants. (B) Y2H interaction of Adi3 with Atg8a and Atg8f.

Atg8a and *Atg8f* were successfully amplified from tomato by RT-PCR (Supplemental Fig. 1). In the yeast two-hybrid assay, these two Atg8 proteins interacted weakly with Adi3 (Fig. 2B) suggesting there is some specificity to the Adi3/Atg8h interaction.

3.5. Cosilencing of Adi3 with autophagy genes

The classic view of autophagy is that it is induced during nutrient starvation in order to recycle proteins and organelles for supplying energy requirements. During this process autophagic vesicles are formed *de novo* around the items to be degraded [39]. This induction of autophagic vesicle formation is initiated by a protein complex containing Atg6 (aka Beclin-1/Vps30) and class III phosphoinositide 3-kinase (PI3K; aka Vps34) [40]. Downstream of this event and part of initiating autophagic vesicle formation is the processing of Atg8. As stated above, the Cys protease Atg4 will cleave Atg8 at the C-terminal Gly. This Gly is conjugated to phosphatidylethanolamine (PE) in a ubiquitin-like conjugation process [41]. The autophagy proteins Atg7 and Atg3 function as E1 and E2 ubiquitin-like enzymes, respectively, during

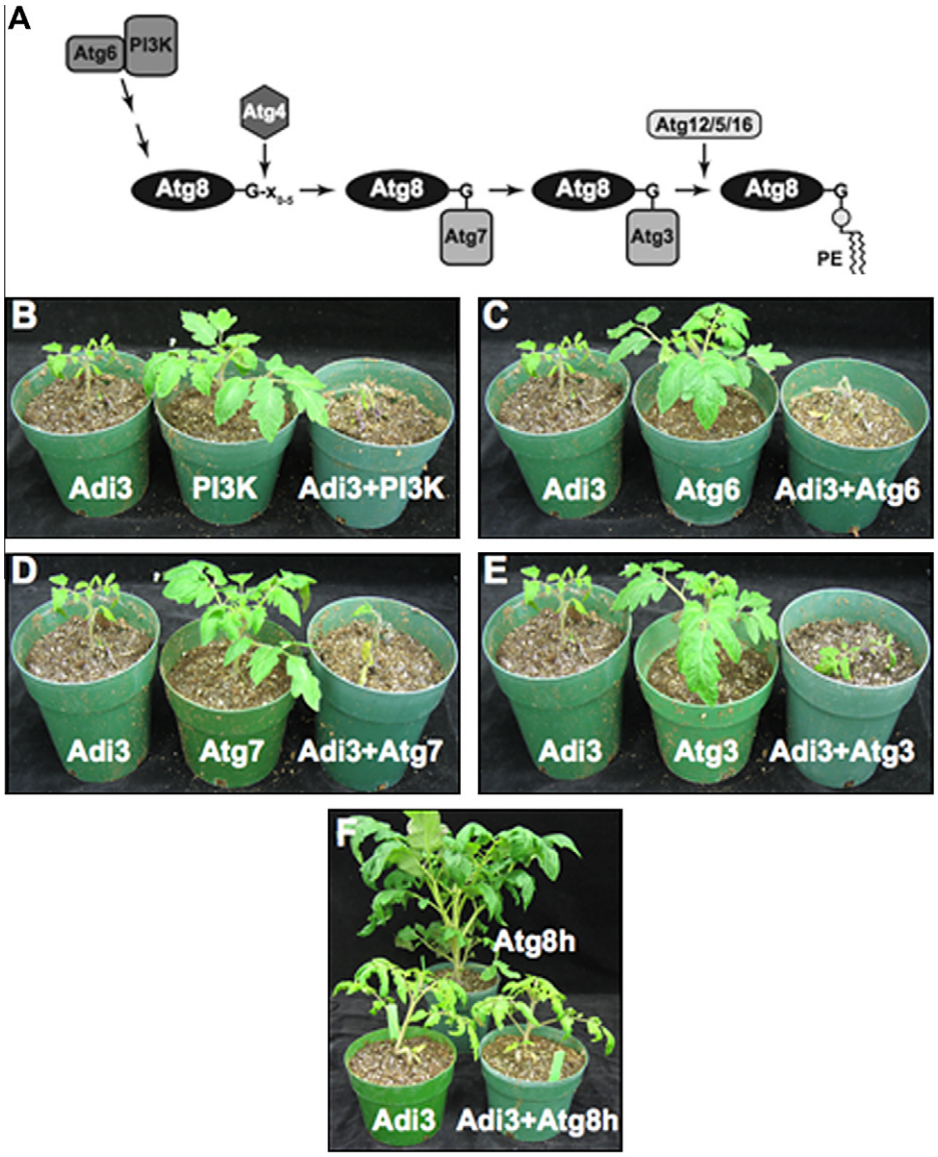

Fig. 3. Cosilencing of Adi3 and *Atg* genes leads to whole plant death. (A) Pathway of Atg8 conjugation to PE showing position of the other Atg genes involved in this process. (B) to (F) cosilencing of *Adi3* and *PI3K* (B), *Adi3* and *Atg6* (C), *Adi3* and *Atg7* (D), *Adi3* and *Atg3* (E) at 3 weeks, and *Adi3* and *Atg8h* at 5 weeks (F).

Atg8-PE conjugation and a complex of Atg12, 5, and 16 is thought to function as the E3 ubiquitin ligase-like enzyme transferring the PE to Atg8 [40] (Fig. 3A). Atg8-PE is then used as an anchor during autophagosome formation [28,29].

In plants, individual silencing by VIGS of *Atg6*, *PI3K*, *Atg7*, and *Atg3* leads to the uncontrolled spread of HR cell death [31]. Since Adi3 interacts with Atg8h (Fig. 2) and silencing of *Adi3* leads to spontaneous cell death lesions [22], it was tested if cosilencing of autophagy genes with *Adi3* would lead to a similar uncontrolled cell death phenotype. Tomato seedlings were infiltrated with *Agrobacterium* containing the VIGS constructs for *Adi3* [22], *Atg6*, *PI3K*, *Atg7*, *Atg3* [31] and *Atg8h*. Cell death symptoms were monitored and pictures taken at three and five weeks after *Agrobacterium* infiltration. As has been seen previously [22], *Adi3* silencing lead to cell death lesions (not shown) and an overall reduction in plant stature (Fig. 3B–F). Silencing of the autophagy genes by themselves, did not lead to an abnormal phenotype (Fig. 3B–F), as has been seen before [31]. However, cosilencing of *PI3K*, *Atg6*, *Atg7*, or *Atg3* with *Adi3* lead to severe plant stunting and near complete death of the plants at three weeks after *Agrobacterium* infiltration (Fig. 3B–E). After five weeks of silencing, these cosilenced plants were completely dead (Supplemental Fig. 2). In contrast, cosilencing of *Adi3* and *Atg8h* did not lead to increased plant stunting or cell death even five weeks after *Agrobacterium* infiltration (Fig. 3F). This is most likely due to the other *Atg8* genes being able to complement the loss of *Atg8h*. In fact, all *Arabidopsis Atg8* genes have identical expression patterns and are induced by nitrogen or sucrose starvation [30,42]. Silencing of *Atg8a* and *Atg8f* was not carried out due to an inability to generate VIGS clones for these genes.

These studies show that the cell death suppressor Adi3 interacts with the autophagy gene Atg8h with some specificity since Adi3 weakly interacts with two other Atg8 proteins (Fig. 2). Additionally, these data would suggest that there is similarity to the cell death controlled by Adi3 and that seen for the HR in that they both involve autophagy for controlling the spread of the cell death (Fig. 3). This may not be surprising given that Adi3 interacts with Pto and AvrPto [22,24] and autophagy is involved controlling HR cell death [27]. Future work will focus on determining the specific role for the Adi3/Atg8h interaction in controlling autophagy and/or cell death.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.08.031.

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AtAtg8a     MAKSSFKISNPLEARMSESSRIREKYPDRIPVIVEKAGQSDVPDIDKKKYLVPADLTVGQFV 62
SlAtg8a     MAKSSFKLEHPLERRQAEAARIREKYPDRIPVIVEKAERSDIPDIDKKKYLVPADLTVGQFV 62
AtAtg8f     MAKSSFKQEHDLEKRRAEAARIREKYPDRIPVIVEKAEKSDIPTIDKKKYLVPADLTVGQFV 62
SlAtg8f     MAKSSFKQEHDLEKRRAEAARIREKYADRIPVIVEKAEKSDIPNIDKKKYLVPADLTVGQFV 62
            ******* .: ** * :*::******.********** :**:* ******************

                                                    ↓
AtAtg8a     YVVRKRIKLGAEKAIFVFVKNTLPPTAALMSAIYEEHKDEDGFLYMTYSGENTFGSLTVA 122
SlAtg8a     YVVRKRIKLSAEKAIFIFVKNILPPTAAMMSAIYEEHKDEDGFLYMTYSGENTFGSF--- 119
AtAtg8f     YVIRKRIKLSAEKAIFIFVDNVLPPAGALMSSVYEEKKDDDGFLYVTYSGENTFGFGSP- 121
SlAtg8f     YVIRKRIKLSAEKAIFIFIDNVLPPTGAIMSAIYDEKKDDDGFLYVTYSGENTFGVLSEL 122
            **:******.******:*:.* ***:.*:**:*:*:**:*****:*********
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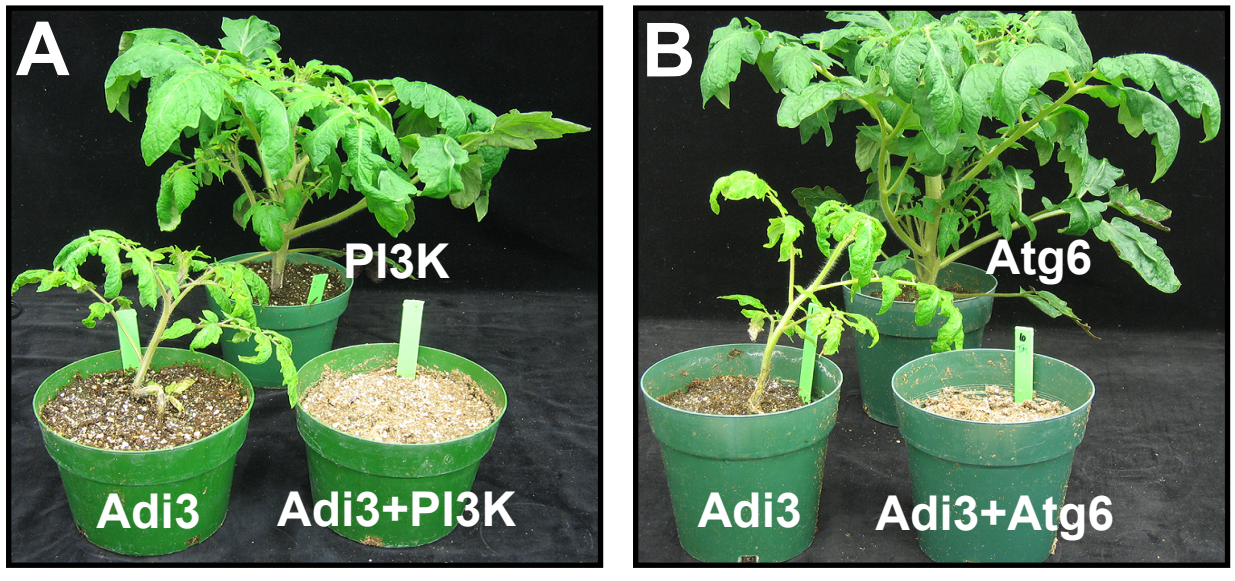
Supplemental Fig. 1. Alignment of Atg8a and Atg8f proteins from *Arabidopsis* (*At*Atg8a, At4g21980, accession #NP567642; *At*Atg8f, At4g16520, #NP849395) and tomato (*Solanum lycopersicum*; *Sl*Atg8a accession #JF304784; *Sl*Atg8f accession #JF304785). Identical amino acids are boxed in black. In the consensus line "*" = identical amino acids, ":" = conserved substitutions. "." = semi-conserved substitution. Arrow indicates the position of cleavage by Atg4 in *Arabidopsis* Atg8 proteins.



Supplemental Fig. 2. Cosilencing of *Adi3* with *PI3K* (A), *Atg6* (B), *Atg7* (C), and *Atg3* (D) after five weeks of silencing.