

1 **Title: Golgi anti-apoptotic proteins are evolutionarily conserved ion channels that regulate cell**  
2 **death in plants**

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32 **Highlight:**

33 Arabidopsis Golgi anti-apoptotic proteins (GAAPs) share functional conservation with their human  
34 and viral counterparts in cell death regulation and ion channel activity

35

36 **ABSTRACT**

37 Programmed cell death regulates developmental and stress responses in eukaryotes. Golgi anti-  
38 apoptotic proteins (GAAPs) are evolutionarily conserved cell death regulators. Human and viral  
39 GAAPs inhibit apoptosis and modulate intracellular Ca<sup>2+</sup> fluxes, and viral GAAPs form cation-  
40 selective channels. Although most mammalian cell death regulators are not conserved at the  
41 sequence level in plants, the GAAP gene family shows expansion, with five paralogues (*AtGAAP1-5*)  
42 in the Arabidopsis genome. We pursued molecular and physiological characterization of AtGAAPs  
43 making use of the advanced knowledge of their human and viral counterparts. Structural modeling  
44 of AtGAAPs predicted the presence of a channel-like pore, and electrophysiological recordings from  
45 purified AtGAAP3 reconstituted into lipid bilayers confirmed that plant GAAPs can function as ion  
46 channels. AtGAAP1 and AtGAAP4 localized exclusively to the Golgi within the plant cell, while  
47 AtGAAP2, AtGAAP3 and AtGAAP5 also showed tonoplastic localization. Gene expression analysis  
48 revealed differential spatial expression and abundance of transcript for *AtGAAP* paralogues in  
49 Arabidopsis tissues. We demonstrate that AtGAAP1-5 inhibit Bax-induced cell death in yeast.  
50 However, overexpression of AtGAAP1 induces cell death in *Nicotiana benthamiana* leaves and lesion  
51 mimic phenotype in Arabidopsis. We propose that AtGAAPs function as Golgi-localized ion channels  
52 that regulate cell death by affecting ionic homeostasis within the cell.

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55 **Key words:** apoptosis, Arabidopsis, Bax-induced cell death, Bax inhibitor-1, Ca<sup>2+</sup> signaling, Golgi,  
56 Golgi anti-apoptotic protein, ion channel, lesion mimic mutant, programmed cell death

57

58 **Abbreviations:** AtGAAP, *Arabidopsis thaliana* GAAP; BI-1, Bax inhibitor-1; CFP, cyan fluorescent  
59 protein; CMLV, camelpox virus; ER, Endoplasmic reticulum; GAAP, Golgi anti-apoptotic protein; GFP,  
60 green fluorescent protein; hGAAP, human GAAP; LFG, Lifeguard; LMM, lesion mimic mutant; PCD,  
61 programmed cell death; TMBIM, transmembrane Bax inhibitor-1 motif-containing; TMDs,  
62 transmembrane domains; vGAAP, viral GAAP; YFP, yellow fluorescent protein

## 63 INTRODUCTION

64 Programmed cell death (PCD) is a process that plays essential roles in the life cycle of plants and  
65 animals. In multicellular organisms, PCD is a key mechanism controlling developmental pattern  
66 formation, organ shape, and the removal of unwanted, damaged or infected cells. In plants,  
67 developmental PCD contributes to many processes, such as death of suspensor cells in the  
68 developing embryo (Bozhkov *et al.*, 2005), tracheary element differentiation (Fukuda, 2000) and  
69 organ senescence (Thomas, 2013). In addition to its role in development, PCD in plants can also be  
70 induced by abiotic and biotic cues. Rapid induction of PCD at the site of pathogen infection, the  
71 hypersensitive response (HR), is a powerful defense mechanism that limits the spread of biotrophic  
72 pathogens in plants (Coll *et al.*, 2011). A variety of abiotic stresses, including heat-shock (Vacca *et*  
73 *al.*, 2004; Watanabe and Lam, 2006), low temperature (Koukalova *et al.*, 1997), ultraviolet  
74 irradiation (Danon *et al.*, 2004) and ozone exposure (Overmyer *et al.*, 2005) also induce PCD.

75 Despite its central importance, the molecular mechanisms governing the initiation and  
76 execution of PCD in plants are largely unknown. This contrasts with animals, where apoptosis is a  
77 well characterized form of PCD (Dickman *et al.*, 2017). Mitochondrial pathway of apoptosis involves  
78 release of cytochrome c from mitochondria into the cytoplasm, which triggers a signaling cascade  
79 leading to activation of caspases, a family of cysteine proteases, and subsequently apoptosis. Bcl-2  
80 proteins are core regulators of apoptosis that can either inhibit (e.g. Bcl-2 and Bcl-XL) or promote  
81 (e.g. Bax and Bak) apoptosis by controlling the integrity of the mitochondrial outer membrane  
82 (Chipuk *et al.*, 2010).

83 PCD in animals and plants shares several morphological and biochemical features,  
84 including cell shrinkage, DNA fragmentation, Ca<sup>2+</sup> fluxes, and production of reactive oxygen species  
85 (ROS) (Dickman *et al.*, 2017). No direct homologues of mammalian caspases have been identified in  
86 plants, but caspase-like protease activities are associated with the activation of various types of PCD  
87 in plants (Coffeen and Wolpert, 2004; Hatsugai *et al.*, 2004; Kuroyanagi *et al.*, 2005; Hatsugai *et al.*,  
88 2006; Hatsugai *et al.*, 2009). Similarly, although plants have no apparent homologues of Bcl-2  
89 proteins, expression of mammalian Bax triggers PCD in Arabidopsis (*Arabidopsis thaliana*) and  
90 tobacco (Lacomme and Santa Cruz, 1999; Kawai-Yamada *et al.*, 2001). Although much of the core  
91 machinery of animal apoptosis is not conserved in plants at the sequence level, two related families  
92 of anti-apoptotic proteins are present in both animals and plants: Bax inhibitor-1 (BI-1) and Golgi  
93 anti-apoptotic protein (GAAP). BI-1 proteins were originally identified as inhibitors of Bax-induced  
94 cell death in yeast (Xu and Reed, 1998), and their roles in the regulation of cell death in both animal

95 and plant cells are now established. BI-1 inhibits cell death by controlling the Ca<sup>2+</sup> content of the  
96 endoplasmic reticulum (ER) and intracellular Ca<sup>2+</sup> fluxes (Ishikawa *et al.*, 2011; Robinson *et al.*, 2011).

97 GAAPs are an evolutionarily conserved group of anti-apoptotic proteins that were  
98 originally discovered in poxviruses (Gubser *et al.*, 2007; Carrara *et al.*, 2017). GAAP orthologues have  
99 been identified throughout eukaryotes, including animals, plants and fungi, as well as some  
100 prokaryotes (Gubser *et al.*, 2007; Carrara *et al.*, 2015; Carrara *et al.*, 2017). Over-expressing human  
101 (hGAAP) or viral (vGAAP) GAAP in human cells protects against both intrinsic (e.g. Bax) and extrinsic  
102 (e.g. Fas) pro-apoptotic stimuli. Silencing of hGAAP with small interfering RNA (siRNA) leads to cell  
103 death in human cells, suggesting that hGAAP is essential for cell viability (Gubser *et al.*, 2007). vGAAP  
104 can complement for the loss of hGAAP, indicating functional conservation (Gubser *et al.*, 2007). Cell  
105 adhesion, spread and migration are also regulated by hGAAP (Saraiva *et al.*, 2013a). In mammalian  
106 cells, over-expression of hGAAP or vGAAP reduces the Ca<sup>2+</sup> content of the Golgi and ER, while knock-  
107 down of hGAAP has the opposite effects (de Mattia *et al.*, 2009; Saraiva *et al.*, 2013a; Saraiva *et al.*,  
108 2013b). The increases in cytosolic and mitochondrial Ca<sup>2+</sup> concentration evoked by an apoptotic  
109 stimulus (staurosporine) and by IP<sub>3</sub>-evoked Ca<sup>2+</sup> release from intracellular stores are reduced by  
110 over-expression of hGAAP (de Mattia *et al.*, 2009). Purified vGAAP and human BI-1 form cation  
111 channels in lipid bilayers (Carrara *et al.*, 2015), suggesting that they may directly mediate a Ca<sup>2+</sup> leak  
112 from the Golgi and ER, thus reducing the amount of Ca<sup>2+</sup> available for release in response to  
113 apoptotic stimulation. Since intracellular Ca<sup>2+</sup> fluxes affect the sensitivity of cells to apoptosis (Giorgi  
114 *et al.*, 2012), hGAAP might suppress apoptosis by down-regulating cytosolic and mitochondrial Ca<sup>2+</sup>  
115 signals (de Mattia *et al.*, 2009).

116 The molecular and physiological functions of plant GAAPs remain largely  
117 uncharacterised. Roles for plant GAAPs have been proposed in plant-fungal interactions (GAAP  
118 referred to as Lifeguard (LFG); Weis *et al.*, 2013), ER stress responses (Wang *et al.*, 2019; Guo *et al.*,  
119 2018) and brassinosteroid signaling (Yamagami *et al.*, 2009). However, it remains unexplored  
120 whether plant GAAPs share functional conservation in cell death regulation and ion channel  
121 properties with their animal and viral counterparts. Here, we show that an expanded family of  
122 GAAPs exists in plants and undertake a thorough functional characterization of the five Arabidopsis  
123 GAAPs (AtGAAPs). Homology modeling was used to predict the structures of AtGAAPs and define a  
124 putative ion channel pore. Experimentation showed that AtGAAPs form ion channels in lipid bilayers  
125 and inhibit Bax-induced cell death in yeast. Fluorescently-tagged AtGAAPs displayed subtype-  
126 specific localization within the Golgi and tonoplast of cells *in planta*, and gene expression analyses

127 show that the five AtGAAP display different tissue distributions in Arabidopsis, suggesting subtype-  
128 specific functional roles of AtGAAPs. Arabidopsis strains with single, double and triple knockouts of  
129 AtGAAPs did not display obvious morphological phenotypes, which may suggest a high degree of  
130 redundancy in the function of AtGAAPs, or that AtGAAPs regulate responses to specific  
131 environmental stimuli or stresses. In contrast, fluorescently-tagged AtGAAP1 induced cell death  
132 when over-expressed *in planta*, demonstrating a role for AtGAAPs in the regulation of cell death in  
133 Arabidopsis.

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## 151 **MATERIALS AND METHODS**

### 152 Protein identification and phylogenetic analysis

153 Plant GAAP and BI-1 sequences were retrieved from the National Center for Biotechnology  
154 Information (NCBI; <https://www.ncbi.nlm.nih.gov/>) and Phytozome (<https://phytozome.jgi.doe.gov>)  
155 (Goodstein *et al.*, 2012) databases using hGAAP and hBI-1 sequences as initial queries, followed by  
156 queries using identified Arabidopsis sequences. Amino acid sequences were aligned using Pagan  
157 (Löytynoja *et al.*, 2012) and the Maximum-Likelihood tree was constructed in RAxML (Stamatakis,  
158 2014) with 1000 bootstrap replicates. Percentage identity and similarity of sequences were  
159 calculated using Basic Local Alignment Search Tool (BLAST) (Altschul and Lipman, 1990).

### 160 Sequence alignments and topology predictions

161 Sequence alignments were generated using Clustal Omega (European Molecular Biology  
162 Laboratory-European Bioinformatics Institute) (Sievers *et al.*, 2011). The GenBank accession  
163 numbers for the sequences used were: NP\_193209 (AtGAAP1); NP\_191890 (AtGAAP2); NP\_192178  
164 (AtGAAP3); NP\_567466 (AtGAAP4); NP\_171806 (AtGAAP5); O31539 (BsYetJ) and AAG37461  
165 (camelpox virus, CMLV, GAAP). TMDs were predicted using TOPCONS (Bernsel *et al.*, 2009). Asterisks  
166 indicate positions of fully conserved residue, while colons indicate residues with highly similar  
167 properties.

### 168 Structural modelling

169 Structural modelling was performed as described (Carrara *et al.*, 2015). I-TASSER (Zhang, 2008; Roy  
170 *et al.*, 2010; Roy *et al.*, 2012) was used to create homology models of AtGAAPs. The sequences of  
171 AtGAAPs were used in structure-based sequence alignments that search for suitable structures  
172 within the Protein Data Bank (PDB). The sequences used were: NP\_193209 (AtGAAP1); NP\_191890  
173 (AtGAAP2); NP\_192178 (AtGAAP3); NP\_567466 (AtGAAP4); and NP\_171806 (AtGAAP5). The  
174 searches showed that BsYetJ structures as templates gave the best models. The crystal structures  
175 of BsYetJ in closed (PDB: 4PGR) and open (PDB: 4PGS) states were used as templates for the models  
176 shown (Chang *et al.*, 2014). These models had confidence scores (C-scores) in the range -1.21 to  
177 +0.06, which are indicative of correct models (Roy *et al.*, 2010).

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179 Protein expression and purification in yeast

180 Protein expression and purification was carried out as reported (Carrara *et al.*, 2015). AtGAAP3 was  
181 expressed in *S. cerevisiae* strain FGY217 (Kota *et al.*, 2007), under control of the galactose promoter  
182 (Mumberg *et al.*, 1995). Recombinant AtGAAP3 was purified in 150 mM NaCl, 20 mM Tris-base, 5%  
183 glycerol and 0.06% lauryldimethylamine N-oxide (LDAO), pH 7.5 and analysed according to a  
184 protocol developed for transmembrane proteins (Drew *et al.*, 2008). Briefly, the GFP-8His tag was  
185 cleaved by adding 8His-tagged tobacco etch virus (TEV) protease to the purified GFP-8His-tagged  
186 AtGAAP3 at a molar ratio of 1:1, and digested overnight at 4°C. Cleaved GFP-8His and the His-tagged  
187 protease were removed using a HisTrap nickel column (GE Healthcare), and the untagged target  
188 AtGAAP3 was harvested from the flow-through. The purified protein was concentrated using an  
189 Amicon Ultra centrifugal filter with a molecular mass cut-off (MWCO) of 30 kDa (Millipore) and  
190 analysed on a Superdex 200 size-exclusion chromatography (SEC) column (GE Healthcare). Target  
191 protein fractions were collected and concentrated to 1.5 - 2 mg/ml.

192 Reconstitution of proteins into giant unilamellar vesicles (GUVs)

193 GUVs were produced as reported (Carrara *et al.*, 2015) by electroformation from a mixture of 1:10  
194 cholesterol (Sigma) to 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) (Avanti Polar Lipids)  
195 dissolved in chloroform (Carl Roth). The lipid mixture (20 µl) was spread on the conductive side of  
196 an indium tin oxide (ITO)-coated slide, and dried for 10 min. The dried lipid film was covered with 1  
197 M sorbitol (250 µl), enclosed within a greased O-ring and overlaid with another ITO-coated slide  
198 with its conductive side facing the lipids. The assembly was connected to a Vesicle Prep Pro (Nanion).  
199 Parameters of the electric field used for electroformation of GUVs were: 5 Hz, 3 V, for 128 min at  
200 20°C. GUVs were re-suspended from the slide within the sorbitol overlay, collected and stored for  
201 3-4 days at 4°C. Proteins were incorporated into GUVs as reported (Carrara *et al.*, 2015) by mixing  
202 purified protein (10 µl) with GUVs (90 µl) (0.2 mg/ml final protein concentration). Bio-Beads SM-2  
203 absorbents (152-8920, Bio-Rad) that had been washed in methanol (3 x 10 min), ethanol (3 x 10 min)  
204 and Milli-Q water (6 x 5 min) were added (40 mg/ml, 15 min), and then removed three times during  
205 the reconstitution procedure (45 min total incubation) to remove excess detergent micelles. GUVs  
206 containing protein were stored at 4°C and used within a few h for recordings.

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## 208 Electrophysiological recording

209 Single-channel recordings were performed as reported (Carrara *et al.*, 2015) with a Port-a-Patch  
210 system (Nanion) (Fertig *et al.*, 2002; Bruggemann *et al.*, 2003), using NPC-1 borosilicate glass chips  
211 (5-10 M $\Omega$  resistance). GUVs in 1 M sorbitol (5  $\mu$ l) were added to the *cis* side of the chip and planar  
212 lipid bilayers were formed across the aperture using suction (see **Figure 3D**). Bilayer formation  
213 generated seal resistances of 1-10 G $\Omega$ . Protein-reconstituted GUVs (5  $\mu$ l) (in 15 mM NaCl, 2 mM Tris-  
214 base, 0.5% glycerol, 0.006% LDAO and 0.9 M sorbitol, pH 7.25) were then added to allow  
215 incorporation of purified protein into the bilayer. The final composition of the medium in the *trans*  
216 chamber (5  $\mu$ l) was: 140 mM KCl, 200 nM free Ca<sup>2+</sup> (220  $\mu$ M CaCl<sub>2</sub> buffered with 0.5 mM BAPTA-  
217 Na<sub>4</sub>), 10 mM HEPES-free acid, adjusted to pH 7 with KOH. In the *cis* chamber (15  $\mu$ l, ground) the final  
218 composition of the medium was: 46.7 mM KCl, 200 nM free Ca<sup>2+</sup> (73  $\mu$ M CaCl<sub>2</sub> buffered with 0.17  
219 mM BAPTA-Na<sub>4</sub>), 5 mM NaCl, 3.33 mM HEPES-free acid, 0.67 M sorbitol, 0.67 mM Tris-base, pH 7  
220 (see **Figure 3D**). Recordings were acquired in the “on cell” mode using the PatchMaster software  
221 (Nanion) and an EPC 10 patch-clamp amplifier (HEKA). Voltages are expressed as the potential on  
222 the *cis* side relative to the *trans* side. Single-channel currents are shown such that downward  
223 deflections represent cations flowing from the *trans* to the *cis* side of the bilayer. Continuous current  
224 recordings were acquired by applying holding potentials for 1 min durations in increments of 20 mV,  
225 or until bursts of spontaneous channel activity appeared. Data were filtered at 2.9 kHz (Bessel filter,  
226 HEKA amplifier), digitized at 50 kHz and exported to Clampfit (Molecular Devices) via MatLab  
227 (MathWorks). Recordings were analysed with PatchMaster and Clampfit software.

## 228 Cloning and recombination procedure for generation of yeast expression constructs

229 The coding region of Bax from pBM272 Bax (Addgene) was amplified by PCR using forward (5'-  
230 caccaataatggatgggtccggggagcagc) and reverse (5'-tcagcccatcttctccagatggtgagc) primers, and  
231 inserted into pENTR/D-TOPO using a pENTR/D-TOPO Cloning Kit (Invitrogen). Gateway LR Clonase  
232 (Invitrogen) was then used to transfer the coding region of Bax into pAG303GAL-ccdb or  
233 pAG305GAL-ccdb vectors (Addgene) to give pAG303GAL-Bax and pAG305GAL-Bax. Green  
234 fluorescent protein (GFP) from the Gateway entry vector pENTR4-GFP-C1 (Addgene) was  
235 transferred to pYES-DEST52 and pAG426GAL-ccdb using Gateway LR Clonase, to create pYES-GFP  
236 and pAG426GAL-GFP. Yellow fluorescent protein (YFP) was transferred from a Gateway entry vector  
237 to pYES-DEST52 using Gateway LR Clonase, to form pYES-YFP. Gateway LR Clonase was used to

238 transfer AtGAAP, AtGAAP-GFP and AtGAAP-YFP inserts from Gateway entry vectors to pYES-DEST52  
239 (Invitrogen) and pAG426GAL-ccdb (Addgene). Sequences and orientations of all constructs were  
240 verified by sequencing (Source Bioscience).

#### 241 Yeast apoptosis assays

242 Strains of *S. cerevisiae* expressing two chromosomally integrated copies of either Bax (termed Bax<sub>2</sub>)  
243 or control GFP (termed GFP<sub>2</sub>) under control of the galactose-inducible GAL1 promoter were  
244 constructed as follows. The isogenic BY4742 strain of *S. cerevisiae* (MAT $\alpha$ , his3 $\Delta$ 1, leu2 $\Delta$ 0, lys2 $\Delta$ 0,  
245 ura3 $\Delta$ 0; Euroscarf) was transformed with pAG303GAL-Bax or pAG303GAL-GFP using a Frozen-EZ  
246 yeast transformation kit (Zymo Research). Transformants (termed Bax<sub>1</sub> and GFP<sub>1</sub>, respectively) were  
247 selected on agar containing synthetic defined (SD) medium with glucose and dropout supplement  
248 lacking histidine (SDglucose-His). Bax<sub>1</sub> and GFP<sub>1</sub> yeast were then cultured in SDglucose-His medium  
249 at 30°C and transformed with pAG305GAL-Bax or pAG305GAL-GFP, respectively. The resulting  
250 transformants (Bax<sub>2</sub> and GFP<sub>2</sub>, respectively) were isolated on agar containing SDglucose with  
251 dropout supplement lacking histidine and leucine (SDglucose-His-Leu). To assess the effects of  
252 untagged GAAPs on apoptosis, the Bax<sub>2</sub> strain was transformed with GFP, AtGAAPs or AtBI-1 in the  
253 vector pYES-DEST52, while the GFP<sub>2</sub> strain was transformed with GFP in pYES-DEST52. For assessing  
254 the effects of fluorescently-tagged GAAPs on apoptosis, the Bax<sub>2</sub> strain was transformed with  
255 pAG426GAL-GAAP-GFP, pAG426GAL-GAAP-YFP, pAG426GAL-GFP or pAG426GAL-YFP, while the  
256 GFP<sub>2</sub> strain was transformed with pAG426GAL-GFP. Transformants were selected on agar containing  
257 SDglucose with dropout supplement lacking histidine, leucine and uracil (SDglucose-His-Leu-Ura).  
258 Colonies were then grown overnight at 30°C in SDglucose-His-Leu-Ura. Cultures were adjusted to  
259 identical OD<sub>600</sub> and a 10-fold dilution series was spotted on either SDglucose-His-Leu-Ura or  
260 SDgalactose-His-Leu-Ura selective agar plates (7  $\mu$ l of each dilution). Growth at 30°C is shown after  
261 2 days (glucose), 6 days (galactose), or 10 days (galactose) for the slowest growing strains. Results  
262 are representative of experiments from three independent transformations. Growth of yeast was  
263 quantified by measuring the mean pixel intensity of spots (for the 10<sup>1</sup> dilution) after background  
264 subtraction (ImageJ) and normalizing to the mean pixel intensity of spots formed by the GFP<sub>2</sub>+GFP  
265 strain on SDgalactose-His-Leu-Ura agar (10<sup>1</sup> dilution). Media and dropout supplements for the  
266 growth of yeast were obtained from ForMedium.

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## 268 Plant materials and growth conditions

269 Both wild-type (Columbia-0 and Landsberg *erecta*) and mutant *Arabidopsis* plants were grown in a  
270 controlled growth room under 16 h light/ 8 h dark (referred to as long day) or 10 h light/ 14 h dark  
271 (referred to as short day) cycles, approximately 60% humidity, light intensity of  $130 \mu\text{E m}^{-2} \text{s}^{-1}$  and  
272 at a temperature of 23°C. *Nicotiana benthamiana* plants were grown under 16 h light/ 8 h dark  
273 cycles and under light intensity of  $200 \mu\text{E m}^{-2} \text{s}^{-1}$ , humidity and temperature as above. Soil-grown  
274 plants were sown on seed and modular compost plus sand (Levington) and germinated under short  
275 day conditions for two weeks after which plants were transferred to fresh soil. Plants were then  
276 placed under long day conditions to induce flowering when necessary.

## 277 Cloning and recombination procedure for generation of transgenic plants

278 PCR fragments were amplified by PCR using Phusion High-Fidelity DNA Polymerase (Finnzymes)  
279 using gene-specific primers. Col-0 genomic DNA was extracted using DNeasy plant mini kits (Qiagen)  
280 and used as template for the PCR. PCR products were cloned into the pENTR/D-TOPO vector  
281 following manufacturer's instructions (Thermo Fisher). Inserts were sequenced and recombined  
282 into Gateway destination vectors by LR recombination using Gateway LR ClonaseII Enzyme Mix  
283 (Thermo Fisher). The pGWB3 destination vector was used to create promoter-GUS fusions. The  
284 pGWB5 and pEG101 vectors were used for cauliflower mosaic virus 35S promoter-driven expression  
285 of AtGAAPs, with C-terminal GFP tags or YFP tags, respectively. The pGWB vector series was a kind  
286 gift from Dr Tsuyoshi Nakagawa (Research Institute of Molecular Genetics, Matsue, Japan)  
287 (Nakagawa *et al.*, 2009) and the pEarleyGate vectors were described (Earley *et al.*, 2006). The  
288 AtGAAP and empty vector constructs were transformed into *Agrobacterium tumefaciens* GV3101  
289 cells by electroporation. Electroporation was performed in a pre-chilled 2 mm cuvette, using a  
290 BioRad Gene Pulser according to the manufacturer's recommendations (capacitance 25  $\mu\text{F}$ ,  
291 resistance 200  $\Omega$ , voltage 1.8 kV). Cells were then transferred to 1 ml of SOB medium (2% Difco  
292 Bacto-tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM  $\text{MgCl}_2$ , and 10 mM  $\text{MgSO}_4$ ) and  
293 incubated for 40 min at 28°C incubator. The transformed cells were selected on LB plates  
294 supplemented with the following antibiotics: 50  $\mu\text{g/ml}$  rifampicin, 50  $\mu\text{g/ml}$  gentamycin, 50  $\mu\text{g/ml}$   
295 hygromycin and 50  $\mu\text{g/ml}$  kanamycin for pGBW vectors; 50  $\mu\text{g/ml}$  rifampicin, 50  $\mu\text{g/ml}$  gentamycin  
296 and 50  $\mu\text{g/ml}$  kanamycin for pEG vectors. Colony PCR was performed to confirm the presence of the

297 insert with gene-specific primers. Individual colonies were resuspended in 15 µl of sterile water and  
298 2 µl of this was used subsequently as a template in PCRs.

#### 299 Arabidopsis transformation and selection of transgenic plants

300 Binary vectors were transformed into Arabidopsis plants by floral dip (Clough and Bent, 1998). The  
301 pGWB3 and pGWB5 transgenic seeds were selected on 0.5 x Murashige and Skoog (MS) plates  
302 containing 50 µg/ml hygromycin B. The pEG101 seeds were grown on soil for seven days and  
303 screened for Basta (glufosinate-ammonium) resistance.

#### 304 Reverse transcriptase PCR (RT-PCR)

305 Total RNA was isolated using TRI REAGENT (Sigma) according to manufacturer's instructions and  
306 quantified using a spectrophotometer (BioPhotometer, Eppendorf). RNA (2 µg) and an anchored  
307 oligo(dT) primer were used for cDNA synthesis using Superscript<sup>TM</sup> III Reverse Transcriptase  
308 (Invitrogen) as instructed. cDNA was used as template for PCR with gene specific primers using  
309 Phusion High-Fidelity DNA Polymerase (Finnzymes) according to manufacturer's instructions.

#### 310 GUS staining

311 Plant tissue was immersed in GUS staining solution: 1 mM X-Gluc, 0.1% (w/v) Triton X-100, 0.5 mM  
312  $K_3Fe(CN)_6$  (ferricyanide), 0.5 mM  $K_4Fe(CN)_6 \cdot 3H_2O$  (ferrocyanide), 10 mM  $Na_2EDTA$ , 50 mM  $PO_4$   
313 buffer, pH 7.0. Tissue was infiltrated twice for two mins in a vacuum chamber before placing samples  
314 at 37°C for 16 h. GUS solution was then removed and the tissue was washed twice with 70% ethanol.

#### 315 Lactophenol trypan blue staining

316 Visualisation of dead and dying cells was performed as described (Koch and Slusarenko, 1990).  
317 Leaves or leaf discs were collected and immersed in staining solution (25% v/v of each  $H_2O$ , lactic  
318 acid, phenol and glycerol plus 0.025% w/v trypan blue) and boiled for 5 mins. Staining solution, once  
319 cooled down, was replaced by de-staining solution (250% w/v chloral hydrate). Samples were  
320 shaken for 7 days until leaves were cleared. Leaves were mounted in 60% glycerol and viewed with  
321 a Leica light microscope (MZ16F, Leica Microsystems).

322

323

### 324 Expression of fusion proteins in *Nicotiana benthamiana*

325 Fluorescent fusion proteins were transiently expressed in *Nicotiana benthamiana* leaf tissue using  
326 *Agrobacterium*-mediated infiltration. *Agrobacterium* cells were grown in LB media overnight.  
327 Cultures were diluted ten-fold into fresh LB and grown for another 24 hrs. Samples were centrifuged,  
328 the pellet was washed once in infiltration media (10 mM MES pH 5.6, 10 mM MgCl<sub>2</sub>, 200 μM  
329 acetosyringone) and resuspended to a final OD<sub>600</sub> of 0.5. Six-week old *N. benthamiana* plants were  
330 inoculated with a 1 ml needleless syringe. Leaf discs were viewed using a confocal microscope 1-4  
331 days after inoculation. AtGAAP-GFP/YFP fusion constructs were expressed alone, or co-expressed  
332 with organelle markers generated previously (Nelson *et al.*, 2007). For colocalisation studies,  
333 bacterial suspensions were mixed in a 1:1 ratio before infiltration. Organelle markers used in this  
334 study and the corresponding Arabidopsis Biological Resource Centre (ABRC) stock numbers are as  
335 follows: Golgi-CFP (CD3-962), Golgi-YFP (CD3-966).

### 336 Confocal microscopy

337 Leaf discs were mounted on glass slides with water. GFP, YFP, and CFP fluorescence was observed  
338 with a Leica DMIRE2 confocal microscope after excitation at 488 nm, 514 nm and 458 nm,  
339 respectively. Images were captured and analysed using Leica confocal software (Version 2.61; Leica  
340 Microsystems). For colocalization studies sequential scanning alternating between CFP and YFP/GFP  
341 channels was performed to avoid crosstalk between the fluorosphores.

### 342 Immunoblot analysis of *Nicotiana benthamiana* tissue

343 Ivoclar Vivadent Silamat S6 mixer was used to grind frozen leaf discs, followed by protein extraction  
344 in 2% SDS, 50 mM Tris-HCl pH 7.5 and protease inhibitors (Sigma P9599, 1:100) at 37°C for 20  
345 minutes. Total protein (50 μg) was run on a 15% SDS polyacrylamide gel, and electroblotted onto a  
346 polyvinylidene difluoride (PVDF) membrane. Immunological reactions were performed with anti-  
347 GFP mouse monoclonal antisera (1:1000) before detection with a horseradish peroxidase (HRP)-  
348 conjugated goat anti-mouse secondary antibody (1:10000).

### 349 Accession numbers

350 Sequence data can be found in the GenBank/EMBL libraries under the following accession numbers:  
351 At4g14730 (*AtGAAP1*), At3g63310 (*AtGAAP2*), At4g02690 (*AtGAAP3*), At4g15470 (*AtGAAP4*), and  
352 At1g03070 (*AtGAAP5*).

## 353 RESULTS

### 354 The GAAP gene family is expanded in plants

355 Both vGAAP and hGAAP belong to the Transmembrane Bax Inhibitor-1 Motif-containing (TMBIM) or  
356 Lifeguard (LFG) protein family (Gubser *et al.*, 2007; Carrara *et al.*, 2012; Saraiva *et al.*, 2013a; Saraiva  
357 *et al.*, 2013b; Carrara *et al.*, 2015). Mammals possess six TMBIM subtypes (TMBIM1-6) (Hu *et al.*,  
358 2009; Rojas-Rivera and Hetz, 2015). Only two subtypes, GAAP (LFG4/TMBIM4) and BI-1 (TMBIM6),  
359 are conserved in plants but several plant species show an intriguing expansion of the GAAP protein  
360 family (Gubser *et al.*, 2007; Gamboa-Tuz *et al.*, 2018). The Arabidopsis, rice (*Oryza sativa*) and the  
361 basal angiosperm *Amborella trichopoda* genomes contain five, seven and two putative GAAPs,  
362 respectively (**Figure 1**). Arabidopsis GAAPs were designated as AtGAAP1-5 (**Supplementary Table**  
363 **S1**; Guo *et al.*, 2018) after the founding members of this gene family (Gubser *et al.*, 2007).  
364 Arabidopsis GAAP paralogues show 48-82% amino acid sequence identity with each other  
365 (**Supplementary Table S2**). Based on phylogenetic analysis AtGAAP2, AtGAAP3 and AtGAAP5 form  
366 a distinct clade with high sequence similarity while AtGAAP4 and AtGAAP1 are distinct from both  
367 the AtGAAP2/3/5 group and each other (**Figure 1; Supplementary Table S2**). AtGAAP4 shares the  
368 highest level of identity with hGAAP (40%), followed by AtGAAP1 (37%), AtGAAP2 (36%), AtGAAP5  
369 and AtGAAP3 (34%; **Supplementary Table S2**). Protein length, transmembrane domain structure  
370 and hydrophobicity profiles are also evolutionarily well conserved between Arabidopsis, viral and  
371 human GAAPs (**Supplementary Table S1; Supplementary Figures S1A and S1B**; Gubser *et al.*, 2007).  
372 This includes as little as two to three amino acid difference in protein length between GAAP from  
373 camelpox virus (CMLV) and AtGAAP1 and AtGAAP2.

### 374 Structural models of AtGAAPs define a putative ion channel pore

375 The structure of a bacterial orthologue of GAAP and BI-1, BsYetJ, has been determined (Chang *et al.*,  
376 2014). This structure revealed seven transmembrane domains (TMDs) and a transmembrane pore  
377 capable of assuming open and closed conformations (Chang *et al.*, 2014). This supports the reported  
378 functions of GAAPs and BI-1 as ion channels or antiporters (Kim *et al.*, 2008; Ahn *et al.*, 2009; Lee *et al.*,  
379 2010; Bultynck *et al.*, 2014; Carrara *et al.*, 2015). Like BsYetJ, AtGAAPs were predicted to contain  
380 seven TMDs (**Supplementary Figure S1**). Sequence alignments of AtGAAPs and BsYetJ suggest a  
381 similar arrangement of predicted TMDs and substantial (25-32% overall) sequence identity, which  
382 was particularly high (37-57%) in the predicted TMD7 region (**Supplementary Figure S1**).



383 Using the crystal structures of BsYetJ (Chang *et al.*, 2014) as templates, we constructed  
384 homology models of AtGAAPs in putative closed (**Figure 2** and **Supplementary Figures S2-S5**, left  
385 columns) and open states (**Figure 2** and **Supplementary Figures S2-S5**, middle columns). The models  
386 of AtGAAPs contain seven TMDs, with TMD7 at the core of each structure similar to BsYetJ (Chang  
387 *et al.*, 2014), BI-1 and vGAAP (Carrara *et al.*, 2012; Carrara *et al.*, 2015). In BsYetJ salt bridges  
388 between two aspartates in the pore (D171 and D195), and between D171 and the basic residue R60  
389 in TMD2 stabilize the closed state (Chang *et al.*, 2014). Disruption of these salt bridges opens the  
390 pore by displacing TMD2 (Chang *et al.*, 2014). The AtGAAPs contain aspartate residues  
391 corresponding to D171 and D195 of BsYetJ (**Figure 2A**; **Supplementary Figures S1B**; **S2A-S5A**), and  
392 all AtGAAPs except AtGAAP1 have a positively charged histidine at the position corresponding to  
393 R60 of BsYetJ (**Supplementary Figure S1B**). The models suggest that the conserved aspartates are  
394 positioned towards the centre of the pore and that the equivalents of BsYetJ R60 are positioned  
395 near the cytosolic end of TMD2 (**Figure 2** and **Supplementary Figures S2-S5**), similar to their  
396 positions in BsYetJ. The closed-state models of AtGAAPs support the possibility that interactions  
397 between the aforementioned residues leads to blocking of a transmembrane pore (**Figure 2B** and  
398 **2C** and **Supplementary Figures S2B-S5B**; **S2C-S5C**, left and right columns) as in BsYetJ (Chang *et al.*,  
399 2014). In the open-state models of AtGAAPs, the residues are spaced further apart (**Figure 2B** and  
400 **2C** and **Supplementary Figures S2B-S5B**; **S2C-S5C**, middle columns) consistent with disruption of  
401 potential salt bridges. Surface models of AtGAAPs show a pronounced cavity extending along the  
402 axis of the pore (**Figure 2D** and **Supplementary Figure S6**). In AtGAAP2 and AtGAAP3, a continuous  
403 pore that fully traverses the membrane is observed in the open state (**Figure 2D** and **Supplementary**  
404 **Figure S6**, middle and right columns). These results suggest that AtGAAPs may be ion channels or  
405 exchangers.

#### 406 **AtGAAPs homo-oligomerise and form ion channels in lipid bilayers**

407 Since structural modelling of AtGAAPs predicted the presence of a channel-like pore, we  
408 investigated their ability to form ion channels in artificial lipid bilayers. AtGAAP3 was chosen for this  
409 analysis because after expression in *Saccharomyces cerevisiae*, detergent extraction and purification  
410 it showed a high degree of stability and remained in a non-aggregated state (**Figure 3A-C**).  
411 Recombinant AtGAAP3 was then used for electrophysiological recording after reconstitution into  
412 lipid bilayers. Size exclusion chromatography (SEC) (**Figure 3A**) and non-reducing SDS-PAGE (**Figure**  
413 **3B**) of purified AtGAAP3 revealed the expected presence of three or more oligomeric states, similar

414 to vGAAPs and hBI-1 (Saraiva *et al.*, 2013b; Carrara *et al.*, 2015). Purified AtGAAP3 separated into  
415 two oligomeric populations by SEC, with the smallest (monomers) eluting last (**Figure 3A**). This  
416 matches the profiles of the previously characterised vGAAP monomer (**Figure 3A**, dotted line) and  
417 a higher state oligomer (likely a dimer) (Carrara *et al.*, 2015). Previous work showed that monomeric  
418 vGAAP retained anti-apoptotic activity and reduced the Ca<sup>2+</sup> content of intracellular stores, while  
419 the functional contributions of oligomeric vGAAPs are unknown (Saraiva *et al.*, 2013b). Both  
420 monomeric and oligomeric populations of purified AtGAAP3 were pooled (**Figure 3A**, bracket) and  
421 used for functional reconstitution into bilayers, in order to optimise opportunities for detecting  
422 channel activity.

423           Incorporation of purified AtGAAP3 in giant unilamellar vesicles (GUVs) into artificial  
424 planar bilayers (**Figure 3D**) gave rise to spontaneous openings of single channels (**Figure 3E**) similar  
425 to those reported previously for vGAAP and BI-1 (Carrara *et al.*, 2015). These conductances were  
426 not observed in untreated lipid bilayers following the addition of GUVs reconstituted in the absence  
427 of protein or with 0.002% lauryldimethylamine N-oxide (LDAO) to mimic any possible detergent-  
428 induced artefacts (**Figure 3E**). These results provide the first direct evidence that AtGAAP3 forms a  
429 channel.

### 430 **AtGAAPs rescue yeast from apoptosis**

431 Since hGAAP, vGAAP and BI-1 inhibit apoptosis (Xu and Reed, 1998; Gubser *et al.*, 2007), we tested  
432 whether AtGAAPs also had anti-apoptotic activity. A strain of *S. cerevisiae* (termed Bax<sub>2</sub>) was  
433 generated that expressed the pro-apoptotic protein Bax (Oltvai *et al.*, 1993) under the control of a  
434 galactose-inducible promoter. This Bax<sub>2</sub> strain showed a dramatic reduction of growth on galactose-  
435 containing medium compared to a control strain (GFP<sub>2</sub>) (**Figure 4**), consistent with an induction of  
436 apoptosis in the Bax<sub>2</sub> strain. Expression of AtGAAPs or AtBI-1 in the Bax<sub>2</sub> strain markedly enhanced  
437 growth after induction of Bax expression, relative to the control strain expressing GFP (**Figure 4**).  
438 The enhanced growth was greater for AtGAAP1-4 than for AtGAAP5, the latter only showing  
439 significantly enhanced growth after longer periods (10 days) of growth (**Figure 4**). These results  
440 demonstrate that AtGAAPs exert an anti-apoptotic effect in yeast, similar to BI-1 (Xu and Reed,  
441 1998).

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444 **AtGAAPs localize mainly to the Golgi in planta**

445 Human and viral GAAPs localize mainly to the Golgi (Gubser *et al.*, 2007). To study the subcellular  
446 localization of Arabidopsis GAAPs *in planta*, AtGAAPs tagged with fluorescent proteins (GFP or YFP)  
447 were transiently expressed in *Nicotiana benthamiana* leaves and analysed by confocal microscopy.  
448 Expression of the fluorescently-tagged AtGAAPs in the Bax<sub>2</sub> yeast strain confirmed that they retained  
449 their anti-apoptotic effects (**Supplementary Figure S7**), consistent with previous studies using  
450 tagged BI-1 (Xu and Reed, 1998) and GAAPs (Gubser *et al.*, 2007; de Mattia *et al.*, 2009; Saraiva *et*  
451 *al.*, 2013a; Saraiva *et al.*, 2013b; Carrara *et al.*, 2015). At the modest expression levels achieved  
452 within one to two days after transformation of *N. benthamiana*, fluorescence localized to small,  
453 punctate structures distributed throughout epidermal cells, and colocalized with a Golgi-CFP marker  
454 (**Figure 5A**) (Nelson *et al.*, 2007).

455 At high expression levels, human and viral GAAPs are also expressed in the ER (Gubser  
456 *et al.*, 2007). At the higher expression levels achieved three to four days after transformation,  
457 AtGAAP1-YFP and AtGAAP4-GFP colocalised exclusively with the Golgi-CFP marker. However, the  
458 distributions of YFP-tagged AtGAAP2/3/5 were similar to that of tonoplast proteins (Saito *et al.*,  
459 2002; Nelson *et al.*, 2007), with a clearly visible cell wall space between two neighbouring cells  
460 (**Figure 5B**, arrows) and ring-like structures (Saito *et al.*, 2002). Some colocalisation with the Golgi  
461 stacks was also maintained at these later time points for YFP-tagged AtGAAP2/3/5 (**Figure 5B**). We  
462 conclude that like hGAAP and vGAAP, AtGAAPs are expressed primarily in the Golgi at low levels of  
463 expression. At higher expression levels, AtGAAP1 and AtGAAP4 remain within the Golgi, whereas  
464 AtGAAP2/3/5 are expressed mainly at the tonoplast.

465 **AtGAAPs show differential spatial expression and abundance of transcript**

466 To gain further insight into the possible functions of the *AtGAAP* family, we fused the *AtGAAP*  
467 promoters (*proAtGAAP*) to the  $\beta$ -glucuronidase (GUS) reporter gene (*uidA*) and generated  
468 transgenic lines. Distinct expression patterns for *AtGAAP1-5* could be observed both spatially and in  
469 transcript abundance based on the pattern and intensity of GUS staining between the transgenic  
470 lines (**Figure 6A; Supplementary Figure S8**). *AtGAAP4* showed the strongest and most uniform  
471 expression throughout the inflorescence and leaf tissues. *AtGAAP2* expression was also  
472 comparatively high in both tissue types while *AtGAAP1* and *AtGAAP5* expression was markedly

473 lower. No GUS staining was observed in plants containing the *proAtGAAP3::uidA* transgene (**Figure**  
474 **6A; Supplementary Figure S8**).

475 *AtGAAP4* was expressed widely in the reproductive tissues, including the pistil, stamen,  
476 sepals, petals, siliques and inflorescence stems (**Figure 6A; Supplementary Figure S8**). *AtGAAP2* and  
477 *AtGAAP5* expression was elevated particularly in the anther, including the pollen grains, and the tip  
478 of the style and stigma. Unlike the other *AtGAAP* paralogues, *AtGAAP1* was expressed distinctly in  
479 the ovules within the pistil and also highly expressed in the petal and sepal abscission zones. In the  
480 vegetative tissues, *AtGAAP2* and *AtGAAP4* were highly expressed throughout the rosette with  
481 particularly intense GUS staining observed in the leaf vasculature and the hydathodes (**Figure 6A**).  
482 Similar spatial pattern of expression was observed for *AtGAAP5* although at much reduced amounts  
483 while *AtGAAP1* showed hydathode-specific expression in the leaves.

484 To complement the histological analysis of *AtGAAP* promoter activity by GUS staining,  
485 the abundance of *AtGAAP* transcripts in leaf and inflorescence tissues was analyzed using semi-  
486 quantitative RT-PCR. *AtGAAP2* and *AtGAAP4* showed high transcript abundance in leaves and  
487 flowers (**Figure 6B**). Transcript levels of *AtGAAP1*, *AtGAAP3* and *AtGAAP5* were substantially lower  
488 compared to *AtGAAP2* and *AtGAAP4*. The abundance of *AtGAAP1*, 3 and 5 transcripts was higher in  
489 inflorescence tissue compared to leaves (**Figure 6B**). Analysis of publicly available expression data  
490 in Genevestigator (Hruz *et al.*, 2008; Zimmermann *et al.*, 2004) supported the results from both  
491 *ProAtGAAP::uidA* and RT-PCR analyses (**Supplementary Fig. S9**). We conclude that *AtGAAPs* show  
492 widespread expression in Arabidopsis, and that relative transcript abundance and spatial expression  
493 pattern in plant tissues differs between the *AtGAAP* paralogues.

#### 494 **atgaap single, double and triple mutants show no obvious growth defects**

495 Gene expression analysis indicated that all *AtGAAPs* are expressed in Arabidopsis. In order to  
496 investigate the physiological functions of individual *AtGAAPs* we isolated homozygous T-DNA  
497 insertion mutants in the Columbia-0 (Col-0) background for all *AtGAAPs*. Absence of *AtGAAP1*,  
498 *AtGAAP3*, *AtGAAP4* and *AtGAAP5* transcripts suggested that the plants were loss-of-function  
499 mutants (*atgaap1* - SALK\_46652, *atgaap3* - GABI186E10, *atgaap4* - SAIL\_151\_F11, *atgaap5* -  
500 SALK\_066103; **Supplementary Figure S10A-B**). The only available T-DNA line for *AtGAAP2* in the Col-  
501 0 background, SALK\_52507, showed *AtGAAP2* expression levels similar to wild-type (**Supplementary**  
502 **Figure S10A-B**), but no *AtGAAP2* transcript was detected in a transposon-tagged line in the

503 Landsberg *erecta* (*Ler*) background, *atgaap2* - GT\_93791. Homozygous mutant plants showed no  
504 obvious growth phenotypes and were indistinguishable from wild-type plants when grown under  
505 either 10- or 16-hour day regimes (**Supplementary Figure S11**). The mutants were fertile and  
506 produced seeds that germinated and developed normally.

507 Since the lack of phenotypes in *atgaap* single mutants could be the result of genetic  
508 redundancy between AtGAAP subtypes, *atgaap* double and triple mutants were generated. We  
509 were able to isolate all *atgaap* double mutant combinations except *atgaap1atgaap4*. The short  
510 genetic distance between *AtGAAP1* and *AtGAAP4* is the likely reason for not obtaining a double  
511 mutant for these genes. Furthermore, we generated *atgaap2atgaap3atgaap4*,  
512 *atgaap2atgaap3atgaap5*, and *atgaap2atgaap4atgaap5*. Intriguingly, no obvious developmental  
513 defects were observed in any of the double or triple mutants (**Supplementary Figure S11**). As  
514 expected, *atgaap2* double and triple mutants, with mixed Col-0 and *Ler* background, displayed  
515 varying characteristics of parental ecotypes, similarly to offspring of crosses between the wild-type  
516 plants. We conclude from our results that AtGAAP subtypes may display a high degree of functional  
517 redundancy in Arabidopsis under normal growth conditions, or that AtGAAPs regulate responses to  
518 specific environmental stimuli or stresses.

### 519 **Over-expression of AtGAAP1-YFP induces cell death of *Nicotiana benthamiana* leaves and severe** 520 **growth defects in Arabidopsis**

521 As loss-of-function mutants did not provide insights into AtGAAP function, we studied the effects of  
522 transient and stable over-expression of AtGAAPs in *N. benthamiana* and Arabidopsis, respectively.  
523 Transient expression in *N. benthamiana* leaf tissue has been employed successfully to study the role  
524 of proteins in the induction and inhibition of cell death (Yoshioka *et al.*, 2006; Urquhart *et al.*, 2007;  
525 Yang *et al.*, 2007; Baxter *et al.*, 2008; Chin *et al.*, 2010; Xu *et al.*, 2017). Transient over-expression of  
526 AtGAAP1-YFP in *N. benthamiana* led to visible symptoms of cell death 2-3 days after inoculation  
527 (**Figure 7A-B**). Conversely, over-expression of fluorescently-tagged AtGAAP2-5, Golgi-YFP marker  
528 (Nelson *et al.*, 2007) or the fluorescent tags alone did not induce cell death (**Figure 7A-B**).  
529 Immunoblot analysis confirmed transgene expression with YFP-tagged AtGAAP2/3/5, the Golgi-YFP  
530 marker and YFP showing the greatest abundance and AtGAAP1-YFP present at lower levels (**Figure**  
531 **7C**).

532           In order to address whether the induction of cell death by AtGAAP1 was also apparent  
533 in Arabidopsis, transgenic plants stably expressing AtGAAP1-YFP driven by the strong constitutive  
534 promoter 35S in the *atgaap1* mutant background were generated. Plant morphology was  
535 dramatically altered in the plants over-expressing AtGAAP1-YFP (**Figure 8**). Rosettes were small in  
536 size compared to *atgaap1* plants, and over-expression plants displaying a range of sizes could be  
537 identified. A representative panel of 5-week old plants displaying varying degrees of the  
538 developmental phenotype is shown in **Figure 8A**. Lower rosette leaves of plants over-expressing  
539 AtGAAP1-YFP exhibited early senescence compared to *atgaap1* plants (**Figure 8B-C**). In plants over-  
540 expressing AtGAAP1-YFP, the oldest rosette leaves that lacked visible signs of yellowing, as indicated  
541 by arrows in **Figure 8C**, had brown lesions (**Figure 8D**). These lesions were clearly identified as  
542 regions of dead cells with trypan blue staining (**Figure 8E**). While trypan blue staining for dead cells  
543 was most pronounced around the site of the lesions, staining was also observed throughout these  
544 leaves. No cell death was observed in *atgaap1* leaves of similar age (**Figure 8F**). The distinct punctate  
545 pattern of YFP fluorescence typical of the Golgi was observed in plants over-expressing AtGAAP1-  
546 YFP, indicating that the observed phenotypes were likely due to accumulation of the recombinant  
547 protein in its native location (**Supplementary Figure S12**).

548           In summary, these data show that ectopic over-expression of AtGAAP1-YFP, but not  
549 AtGAAP2-5, induces cell death in *N. benthamiana* leaf tissue. Over-expression of AtGAAP1-YFP in  
550 Arabidopsis leads to dwarfism, early senescence and ectopic cell death. This supports a role for plant  
551 GAAPs in the control of cell death *in planta*.

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560 **DISCUSSION**

561 **Arabidopsis GAAPs are channel proteins that localize to the Golgi and tonoplast**

562 The homology models based on the structure of BsYetJ suggested that AtGAAPs form  
563 transmembrane pores that function as ion channels or exchangers. This is similar to previous  
564 proposals that BI-1 (Bultynck *et al.*, 2014), vGAAPs (Carrara *et al.*, 2015) and BsYetJ (Chang *et al.*,  
565 2014) form ion channels or exchangers that mediate Ca<sup>2+</sup> flux across membranes. The aspartate pair  
566 (Asp171-Asp195) in BsYetJ is involved in hydrogen bonding responsible for the reversible, pH-  
567 dependent transitions between open and closed conformation of BsYetJ that are associated with  
568 Ca<sup>2+</sup> leak across the membrane (Chang *et al.*, 2014). Importantly, the conservation of this di-aspartyl  
569 pH sensor in AtGAAPs as well as viruses and vertebrates (Carrara *et al.*, 2015) suggests a similar  
570 regulatory mechanism for GAAPs from bacteria, animals, viruses and plants. Mutagenesis, in  
571 conjunction with functional assays, will be required to verify the importance of the residues  
572 concerned.

573 vGAAP and hBI-1 form ion channels and vGAAP is selective for cations (Carrara *et al.*,  
574 2015). In this study, we demonstrate that purified AtGAAP3 exhibits ion channel activity in planar  
575 lipid bilayers. To our knowledge this is the first direct evidence that a plant TMBIM family member  
576 exhibits ion channel functions. The relative selectivity of the AtGAAPs for cations remains to be  
577 determined. Although it has been suggested that plant BI-1 may be directly involved in Ca<sup>2+</sup>  
578 transport across the ER membrane (Ishikawa *et al.*, 2011), experimental evidence is still lacking. In  
579 contrast, Ca<sup>2+</sup> leak properties reported for the C terminus of BI-1 were conserved in animal but not  
580 plant orthologues, suggesting that the Ca<sup>2+</sup> channel-like activity of BI-1 may have been acquired  
581 specifically in the animal lineage (Bultynck *et al.*, 2012). Residues that are important for ion  
582 conductance (Asp219 and Glu207 of vGAAP) and ionic selectivity (Glu207) of vGAAP (Carrara *et al.*,  
583 2015) are conserved in AtGAAPs (**Supplementary Figure S1**), further implying functional  
584 conservation.

585 The possible role of AtGAAPs as a novel class of Ca<sup>2+</sup>-conducting channels in plants is  
586 interesting in light of the evidence showing that plants generally show a loss of diversity in the  
587 mechanisms for Ca<sup>2+</sup> influx from the extracellular space and intracellular stores into the cytoplasm  
588 compared to animals (Wheeler and Brownlee, 2008; Verret *et al.*, 2010; Edel and Kudla, 2015;  
589 Marchadier *et al.*, 2016; Edel *et al.*, 2017); many of the classic animal Ca<sup>2+</sup> channels like the inositol

590 1,4,5-trisphosphate receptors (IP<sub>3</sub>Rs) are not present in plant genomes (Wheeler and Brownlee,  
591 2008; Verret *et al.*, 2010; Edel and Kudla, 2015). Thus far, only five families of Ca<sup>2+</sup>-permeable influx  
592 channels have been shown to function in plants: cyclic nucleotide-gated channels (CNGCs)(Zelman  
593 *et al.*, 2012), glutamate-like receptors (GLRs)(Price *et al.*, 2012), two-pore channels (TPCs) (Morgan  
594 and Galione, 2014), mechanosensitive channels (MCAs) (Kurusu *et al.*, 2013) and reduced  
595 hyperosmolality-induced Ca<sup>2+</sup> increase channels (OSCs) (Yuan *et al.*, 2014). Reduced diversity of  
596 mechanisms for Ca<sup>2+</sup> influx in plants compared to animals is associated with amplification of specific  
597 mechanisms, as many of these gene families show expansion in plants (Edel *et al.*, 2017) as is also  
598 demonstrated in the case of plant GAAPs (**Figure 1**).

599 Like hGAAP and vGAAP (Gubser *et al.*, 2007), AtGAAPs localize to Golgi membranes,  
600 suggesting a conserved function as Golgi-localized ion channels. The role of the Golgi as an  
601 important Ca<sup>2+</sup> store that contributes to Ca<sup>2+</sup> signaling in animal cells is established (Pizzo *et al.*,  
602 2011). Although information on Ca<sup>2+</sup> handling by the plant Golgi is scarce (Costa *et al.*, 2018), it has  
603 been shown that free Ca<sup>2+</sup> concentration is higher in the Golgi than in the cytosol of plant cells, and  
604 abiotic cues can affect luminal Ca<sup>2+</sup> dynamics (Ordenes *et al.*, 2012). The nature and identity of Ca<sup>2+</sup>  
605 channels and transporters of the plant Golgi awaits clarification, although a role for P2A-type ATPase  
606 AtECA3 in the transport of Ca<sup>2+</sup> and Mn<sup>2+</sup> into the Golgi has been proposed (Mills *et al.*, 2008).  
607 AtGAAP2/3/5, but not AtGAAP1/4, also localized in the tonoplast (**Figure 5**). The vacuole is the main  
608 storage compartment of Ca<sup>2+</sup> in plant cells (Peiter, 2011; Costa *et al.*, 2018) and many tonoplast Ca<sup>2+</sup>  
609 transporters have been identified (Martinoia *et al.*, 2012; Neuhaus and Trentmann, 2014; Costa *et*  
610 *al.*, 2018). Ca<sup>2+</sup> release from the vacuole likely contributes to signaling in plants and here evidence  
611 for a role for TPC1 as a tonoplast channel critical for many physiological processes is accumulating  
612 (Peiter *et al.*, 2005; Carpaneto and Gradogna, 2018). Localization of AtGAAPs to these organelles  
613 makes them ideally placed to act as functional Ca<sup>2+</sup> release channels.

#### 614 **Arabidopsis GAAPs regulate cell death in yeast cells and *in planta***

615 Similar to hGAAP and vGAAP (Gubser *et al.*, 2007), all five AtGAAPs suppressed cell death induced  
616 by Bax-expression in yeast (**Figure 4 and Supplementary Figure S7**). This suggests that the anti-  
617 apoptotic effects of GAAPs are conserved between plants, viruses and vertebrates. Various studies  
618 in mammals indicate that other members of the TMBIM family also display anti-apoptotic activity  
619 (Rojas-Rivera and Hetz, 2015). BI-1 suppresses Bax-induced cell death in yeast (Xu and Reed, 1998),



620 and Bax-induced PCD is suppressed by BI-1 proteins in Arabidopsis, rice, rapeseed and tobacco  
621 (Kawai *et al.*, 1999; Sanchez *et al.*, 2000; Kawai-Yamada *et al.*, 2001; Bolduc *et al.*, 2003). This  
622 conservation of function within the TMBIM family suggests that these proteins target one of the  
623 core molecular mechanisms of PCD that is conserved across kingdoms.

624 Expression of AtGAAP1-YFP in yeast inhibited Bax-induced cell death, whereas  
625 expression of this fusion protein in *N. benthamiana* and Arabidopsis induced cell death,  
626 demonstrating that AtGAAP1 can function as a positive or negative regulator of cell death depending  
627 on the context. Opposite effects of BI-1 in regulating cell death have also been demonstrated.  
628 Mammalian and plant BI-1 have been mainly associated with cytoprotective functions (Xu and Reed,  
629 1998; Kawai *et al.*, 1999; Sanchez *et al.*, 2000; Kawai-Yamada *et al.*, 2001; Bolduc *et al.*, 2003;  
630 Robinson *et al.*, 2011). However, AtBI-1 has been reported to induce cell death in mammalian cells  
631 (Yu *et al.*, 2002) and upon over-expression in *N. benthamiana* (Xu *et al.*, 2017). The former effect  
632 was speculated to be due to AtBI-1 functioning as a dominant inhibitor of the endogenous  
633 mammalian BI-1, while the latter was shown to be dependent on certain autophagy-related proteins.  
634 A cell death-promoting phenotype for mammalian BI-1 at low pH has also been reported (Kim *et al.*,  
635 2008; Lee *et al.*, 2011); over-expression of BI-1 leads to increased Ca<sup>2+</sup> release from the ER and  
636 promotion of cell death in acidic conditions (Kim *et al.*, 2008), while BI-1 knock-down had the  
637 opposite effects (Lee *et al.*, 2011). These studies and the results reported here demonstrate dual  
638 and contrasting roles for TMBIM proteins in regulating cell death, depending on the context. The  
639 molecular mechanisms underlying these observed effects remain to be characterized in detail but  
640 may be related to the function of these proteins as cation or Ca<sup>2+</sup> channels.

641 Stable over-expression of AtGAAP1-YFP fusion protein in Arabidopsis led to a severe  
642 dwarf phenotype, enhanced leaf senescence and development of spontaneous lesions in leaves  
643 (**Figure 8**). This phenotype resembles that of lesion mimic mutants (LMM), which display  
644 spontaneous PCD and have been widely used as models for unraveling cell death signaling pathways  
645 (Lorrain *et al.*, 2003; Bruggeman *et al.*, 2015). Some previously characterized LMMs contain  
646 mutations in genes encoding ion channels. *Constitutive expresser of PR genes 22 (cpr22)* encodes  
647 the chimeric cyclic nucleotide-gated ion channel CNGC11/CNGC12, which is a constitutively active  
648 form of the putative Ca<sup>2+</sup> channels responsible for the lesions (Yoshioka *et al.*, 2001; Yoshioka *et al.*,  
649 2006; Urquhart *et al.*, 2007; Chin *et al.*, 2010). In contrast, mutations disrupting CNGC2 and CNGC4  
650 in *defence no death1 (dnd1)* and *dnd2*, respectively, lead to LMM phenotypes (Yu *et al.*, 1998;

651 Clough *et al.*, 2000; Balague *et al.*, 2003; Jurkowski *et al.*, 2004; Ahn, 2007; Ali *et al.*, 2007). Given  
652 the critical role of ion fluxes in the regulation of PCD, and the LMM phenotypes previously recorded  
653 for plant ion channel mutants, it is tempting to speculate that the LMM phenotype induced by  
654 AtGAAP1-YFP over-expression could be due to unbalanced ionic homeostasis caused by the over-  
655 expression of a member of this novel family of ion conducting channels.

#### 656 **Further physiological roles of AtGAAPs**

657 Our gene expression analysis provided clear evidence for expression of all five *AtGAAP* paralogues.  
658 *AtGAAP2* and *AtGAAP4* showed the highest and most uniform abundance of transcript throughout  
659 plant tissues. Expression of *AtGAAP1* and *AtGAAP5* was much lower, with increased expression in  
660 the inflorescence tissue compared to rosettes. *AtGAAP3* was the least abundant *AtGAAP* isoform  
661 according to all methods used. It is noteworthy that in the sexual organs, *AtGAAP2*, *AtGAAP4* and  
662 *AtGAAP5* showed prominent expression in the pollen whereas *AtGAAP1* expression was most  
663 prominently elevated in the ovary. Taken together, these data suggest that AtGAAPs may fulfil  
664 subtype-specific roles in different tissues. The tissue expression analysis will facilitate predictions of  
665 possible redundancy between individual AtGAAP subtypes and the processes that they regulate. For  
666 example, the abundance of *AtGAAP2*, *AtGAAP4* and *AtGAAP5* transcripts in pollen could indicate  
667 the involvement of these proteins in the tapetal PCD that occurs during pollen development (Kurusu  
668 and Kuchitsu, 2017), a process in which AtBI-1 may play an inhibitory role (Kawanabe *et al.*, 2006).

669 In order to obtain further insights into the *in vivo* functions of AtGAAPs we analyzed  
670 single, double and triple mutants. The lack of developmental phenotypes in the mutants suggested  
671 that AtGAAPs may display a high degree of functional redundancy in Arabidopsis, or that AtGAAPs  
672 regulate responses to specific environmental stimuli or stresses. Recently, a redundant role for  
673 AtGAAP1 to AtGAAP3 in the unfolded protein response and the onset of cell death in response to  
674 ER stress was reported (Wang *et al.*, 2019; Guo *et al.*, 2018). A role for barley GAAP in plant-fungal  
675 interaction has also been demonstrated (Weis *et al.*, 2013) and this function is conserved in barley  
676 BI-1 (Huckelhoven *et al.*, 2003; Eichmann *et al.*, 2006; Babaeizad *et al.*, 2009). Knockdown of  
677 AtGAAP1 (AtLFG1) or AtGAAP2 (AtLFG2) also delayed fungal development on Arabidopsis (Weis *et al.*,  
678 *et al.*, 2013). Intriguingly, AtGAAP2 has also been linked to brassinosteroid signaling (Yamagami *et al.*,  
679 2009). Further studies are required to uncover the molecular mechanisms underlying the reported  
680 phenotypes and additional phenotypes.



681                    In summary we describe and characterize a family of five GAAPs in Arabidopsis. We  
682 demonstrate that they form ion channels and share an evolutionarily conserved function in cell  
683 death regulation with GAAPs from animals and viruses. We propose that the physiological role of  
684 AtGAAPs in cell death regulation is based on the ability of these Golgi- and tonoplast-localized ion  
685 channels to modify the finely balanced ionic homeostasis within the cell. Differential expression of  
686 AtGAAP subtypes in different plant tissues suggests that this family of proteins may play roles in a  
687 variety of physiological processes.

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700 **SUPPLEMENTARY DATA**

- 701 Supplementary Figure S1. Sequence conservation between GAAPs and BsYetJ.
- 702 Supplementary Figure S2. Structural models of AtGAAP1 define a putative ion channel pore.
- 703 Supplementary Figure S3. Structural models of AtGAAP2 define a putative ion channel pore.
- 704 Supplementary Figure S4. Structural models of AtGAAP4 define a putative ion channel pore.
- 705 Supplementary Figure S5. Structural models of AtGAAP5 define a putative ion channel pore.
- 706 Supplementary Figure S6. Structural models of AtGAAPs define a putative ion channel pore.
- 707 Supplementary Figure S7. Fluorescently tagged AtGAAPs rescue yeast from Bax-mediated apoptosis.
- 708 Supplementary Figure S8. Histochemical analysis of GUS expression in *proAtGAAP1-5::uidA*
- 709 transgenic plants.
- 710 Supplementary Figure S9. *AtGAAP* gene expression analysis.
- 711 Supplementary Figure S10. RT-PCR analysis of *AtGAAP1-5* gene expression in the wild-type and
- 712 *atgaap* mutant plants.
- 713 Supplementary Figure S11. Phenotypes of *atgaap* single, double and triple mutants.
- 714 Supplementary Figure S12. Subcellular localisation of AtGAAP1-YFP fusion protein in transgenic
- 715 Arabidopsis lines.
- 716 Supplementary Table S1. Designation of Arabidopsis GAAPs.
- 717 Supplementary Table S2. Comparison of deduced amino acid sequences of human GAAP, viral GAAP
- 718 and Arabidopsis GAAPs.

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## FIGURE LEGENDS

**Figure 1. Phylogenetic relationship of GAAP and BI-1 proteins.** Maximum-Likelihood tree of GAAP and BI-1 sequences from *Homo sapiens*, camelpox virus, *Arabidopsis thaliana*, *Oryza sativa* and *Amborella trichopoda*. Numbers next to the nodes represent bootstrap values from 1000 replicates. hGAAP, human GAAP; hBI-1, human BI-1; vGAAP, viral GAAP: AtGAAP1-5, Arabidopsis GAAP1-5; AtBI-1, Arabidopsis BI-1; other sequences are labeled according to the gene identifiers.

**Figure 2. Structural models of AtGAAP3 define a putative ion channel pore.** Homology models of AtGAAP3 based on the crystal structures of BsYetJ. **(A)** A sequence alignment of BsYetJ with AtGAAP3. Residues discussed in the text are labeled and predicted transmembrane domains (TMDs) are underlined. **(B-D)** Models of AtGAAP3 in the closed (left column) and open (middle column) states, viewed from the membrane **(B)** or from the lumen of the Golgi apparatus/vacuole **(C-D)**. The side-chains of residues discussed in the text are coloured magenta (equivalents of BsYetJ-D171/D195) and cyan (equivalents of BsYetJ-R60). Helices are coloured for clarity: TMD1 (dark blue), TMD2 (light blue), TMD3 (green), TMD4 (yellow), TMD5 (light orange), TMD6 (dark orange) and TMD7 (red). The insets (right column) show enlarged regions of the closed-state models **(A-B)** or the open-state model **(D)**. Residues discussed in the text are labeled and the TMDs are numbered for clarity.

**Figure 3. Purified AtGAAP3 exhibits ion channel activity in planar lipid bilayers. (A-C)** Biochemical analyses of purified AtGAAP3, carried out as described for other GAAPs (Carrara *et al.*, 2015). **(A)** Ultraviolet (UV) absorbance profile of purified AtGAAP3 during size exclusion chromatography (SEC) overlapped with previously characterised purified camelpox virus GAAP (CMLV GAAP) (Carrara *et al.* 2015) for comparison. \*Corresponds to the protein aggregation peak and the ramp indicates the directional increase in size of eluting GAAP oligomers. Fractions corresponding to monomeric and oligomeric populations of AtGAAP3 (indicated in bracket) were pooled, concentrated, and **(B)** their contents were analysed by non-reducing SDS-PAGE and Coomassie staining. The expected positions of the monomeric (x1) and oligomeric proteins (x2, x3 and x4) are shown. **(C)** The stability and aggregation of the purified AtGAAP3 was assessed over a period of 12 days at 4°C and 20°C and proteins were visualised by Coomassie stain. **(D-E)** Electrophysiological analyses of AtGAAP3 function, were carried out as described previously for other GAAPs (Carrara *et al.*, 2015). **(D)** Conditions of the bilayer chamber used. A planar lipid bilayer is formed across a  $\mu\text{m}$ -sized aperture within the chip. Purified protein is incorporated into the bilayer by adding protein reconstituted GUVs to the *cis* chamber (ground). The KCl concentration is greater in the *trans* chamber relative to the *cis* chamber. **(E)** Electrophysiological recordings from artificial lipid bilayers reconstituted with purified AtGAAP3 show spontaneous channel openings. Representative current traces were recorded at the indicated holding potentials, which are expressed as the potential on the *cis* side relative to the *trans* side (n = 2 independent experiments). Downward deflections represent positive ions flowing from the *trans* to the *cis* side of the bilayer. The lipid bilayer alone (n = 35) or after addition of GUVs reconstituted in the presence of LDAO (n = 10) were used as negative controls. The dotted line indicates the closed state.



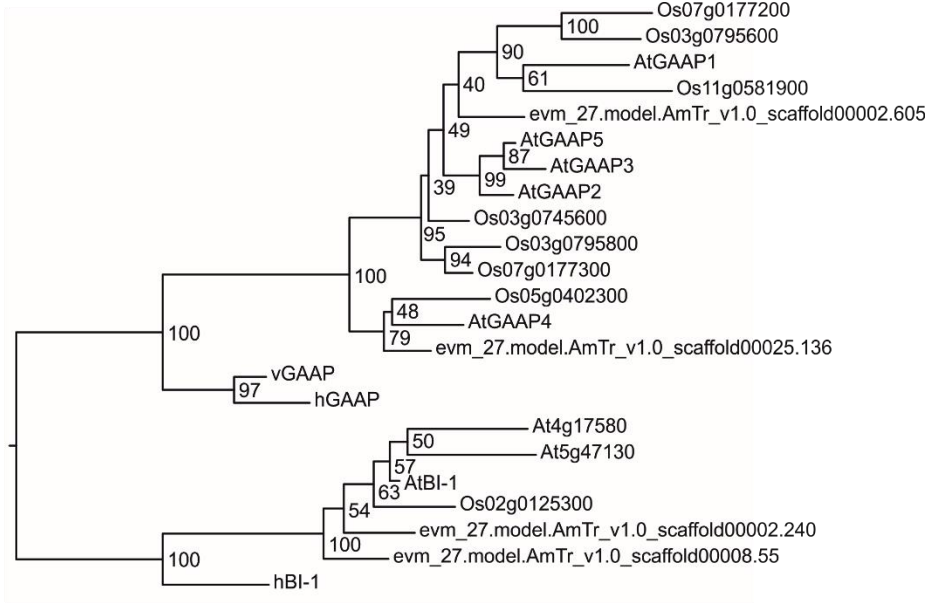
**Figure 4. AtGAAPs rescue yeast from Bax-mediated apoptosis.** (A) Representative growth is shown after 2 days (glucose) or 5 days (galactose), and the inset shows growth after 10 days (galactose) for the slowest growing transformants. (B) Mean data from independent transformations (n=3), showing normalized growth on SDgalactose-His-Leu-Ura. Data are represented as mean  $\pm$  SEM and statistical significance is indicated at \* $p$ <0.05 and \*\* $p$ <0.01 levels, using one-way ANOVA and Dunnett's multiple comparison test.

**Figure 5. Subcellular localisation of AtGAAP1-5:GFP/YFP fusion proteins in *Nicotiana benthamiana* leaf epidermal cells.** Leaves were co-infiltrated with *Agrobacterium* carrying AtGAAP1-5:GFP/YFP and Golgi-CFP marker constructs and imaged using a confocal laser scanning microscope two days **(A)** and three days **(B)** after inoculation. YFP/GFP (left column) and CFP (middle column) signals and merged images (right column) are shown. Scale bars are as indicated. Arrows denote cell wall space between two neighbouring cells. Experiment was repeated at least three times and representative images are shown.

**Figure 6. *AtGAAP* gene expression analysis. (A)** Histochemical analysis of GUS expression in *proAtGAAP1-5::uidA* transgenic plants. Tissue of six-week old Arabidopsis plants expressing *AtGAAP* promoter-GUS fusions was subjected to histochemical staining for GUS activity. 1800 bp, 1800 bp, 1694 bp, 1794 bp and 1803 bp of promoter region was used for *AtGAAP1* to *AtGAAP5*, respectively. **(a)** Inflorescence tissue containing flowers and young siliques, **(b)** individual flower, **(c)** silique, **(d)** rosette leaf, **(e)** magnification of rosette leaf showing staining in veins and hydathodes. Hydathodes are indicated with arrowheads. The expression of *uidA* is driven by *AtGAAP* promoters as indicated. 12 transgenic lines were analysed for each construct and images of representative lines are shown. **(B)** RT-PCR analysis of *AtGAAP* expression. RT-PCR analysis of *AtGAAP1*, *AtGAAP2*, *AtGAAP3*, *AtGAAP4* and *AtGAAP5* expression in five-week old wild type Col-0 inflorescence and leaf tissue. *ACTIN8* (*ACT8*) expression provided a control for RT-PCR. <sup>a</sup> Primer pairs used were specific for a particular *AtGAAP* paralogue, or *ACT8* as indicated. <sup>b</sup> Total RNA was extracted from either inflorescence (I) or rosette leaf (L) tissue of wild type Col-0 plants as indicated.

**Figure 7. Over-expression of AtGAAP1-YFP induces cell death upon transient expression in *Nicotiana benthamiana* leaf tissue. (A)** Leaf areas marked with circles were infiltrated with *Agrobacterium* carrying AtGAAP1/2/3/5-YFP, AtGAAP4-GFP, Golgi-YFP marker, YFP and GFP constructs or infiltration media (Inf.) as indicated. Shown are representative leaves at three days post inoculation (dpi). **(B)** Inoculated leaf areas were excised from the leaves and subjected to trypan blue staining for visualization of cell death at three dpi. **(C)** Immunoblots showing expression of fusion proteins in inoculated leaf areas at three dpi. Experiments were repeated at least three times, representative images are shown.

**Figure 8. Over-expression of AtGAAP1-YFP leads to dwarfism, enhanced senescence and development of spontaneous lesions on the rosette leaves in Arabidopsis. (A)** An *atgaap1* plant and a panel of *atgaap1* plants expressing AtGAAP1-YFP (*atgaap1::AtGAAP1-YFP*) as indicated photographed at five weeks. Expression of AtGAAP1-YFP led to a dwarf phenotype and induced senescence of lower rosette leaves. Two to five transgenic lines deriving from three independent transformations were analysed. All displayed similar phenotypes. **(B-C)** Rosette leaves of a five-week old *atgaap1* plant **(B)** and an *atgaap1* plant expressing AtGAAP1-YFP **(C)**. **(D)** The oldest rosette leaves of plants expressing AtGAAP1-YFP without signs of yellowing (indicated by arrowheads in panel C) developed brown lesions (arrows) **(E)** The leaf in **(D)** after trypan blue staining for cell death. Brown lesions in **(D)** are clearly visible as regions of dead cells in **(E)**. **(F)** Trypan blue stained leaf of an *atgaap1* plant. No cell death was visible. Leaves of the same age were used for **(D-F)**.



**Figure 1.**

	<b>R60</b>	<b>TMD 3</b>	<b>TMD 4</b>	
<b>BsYetJ</b>	WMRRRKA	VGYAFVYTF	AFVSGITLFP	IVSHYAS
<b>AtGAAP3</b>	YYHQKHPV	NYLLLGIF	TALALAFV	----VGLTCA
	<b>H99</b>			<b>TMD 5</b>
<b>BsYetJ</b>	GAKMKK	DLSFLWS	FLLVAVL	LALAVVG
<b>AtGAAP3</b>	AARKGY	DFNFLG	PFLFGAL	TVLIF
				<b>TMD 6</b>
<b>BsYetJ</b>				FDLNQ
<b>AtGAAP3</b>				DTDN
				<b>D171</b>
				<b>D209</b>
<b>BsYetJ</b>	IKHRHIT	EDLIPV	MALSLYL	DFINLF
<b>AtGAAP3</b>	LIKRH	TYDEYI	-WAAV	SLYLDI
				<b>D195</b>
				<b>D232</b>
				<b>TMD 7</b>
				INLLRFFG
				ILSSDD
				214
				INLLRFFG
				ILSSDD
				214
				INLLRFFG
				ILSSDD
				214

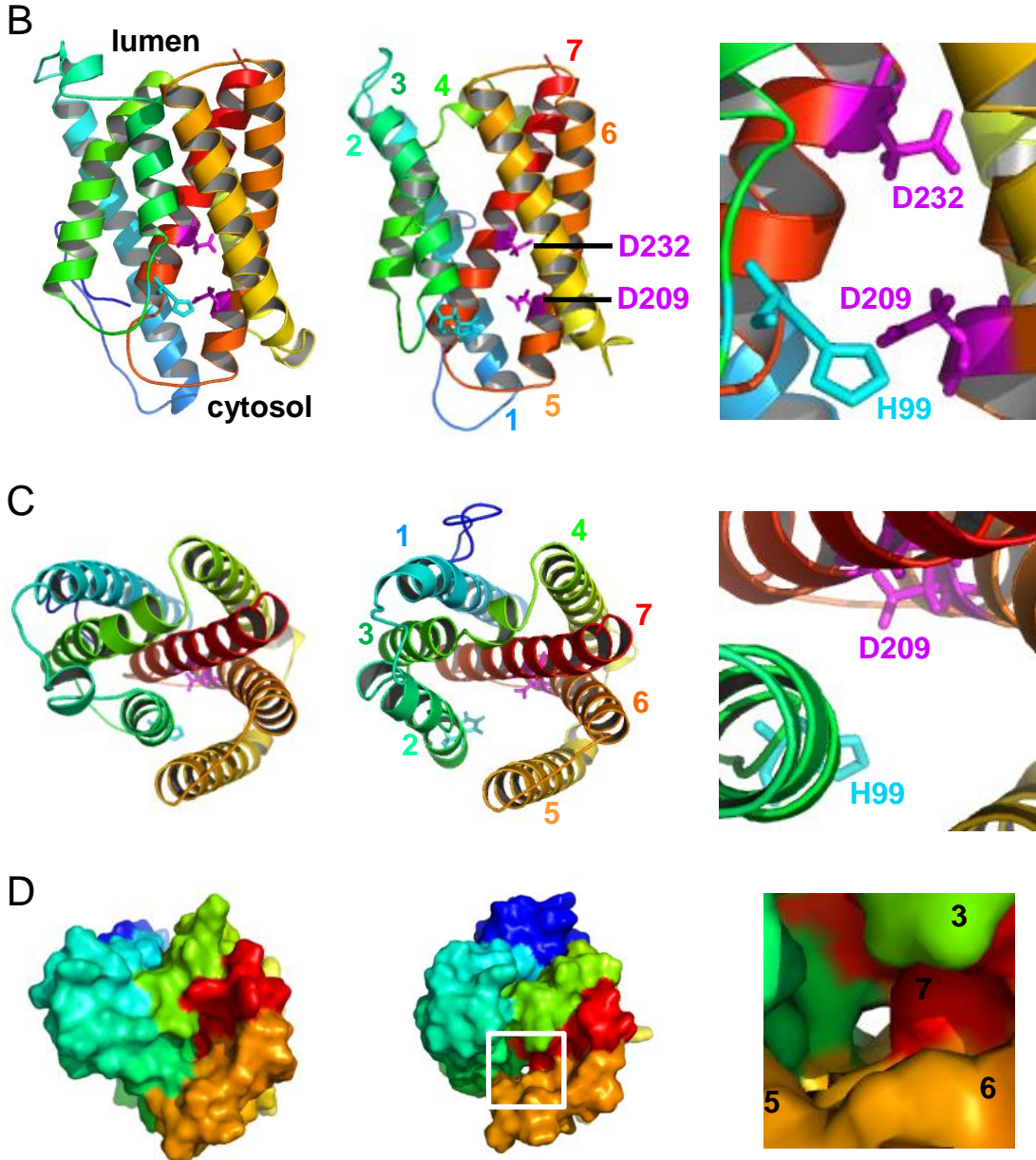
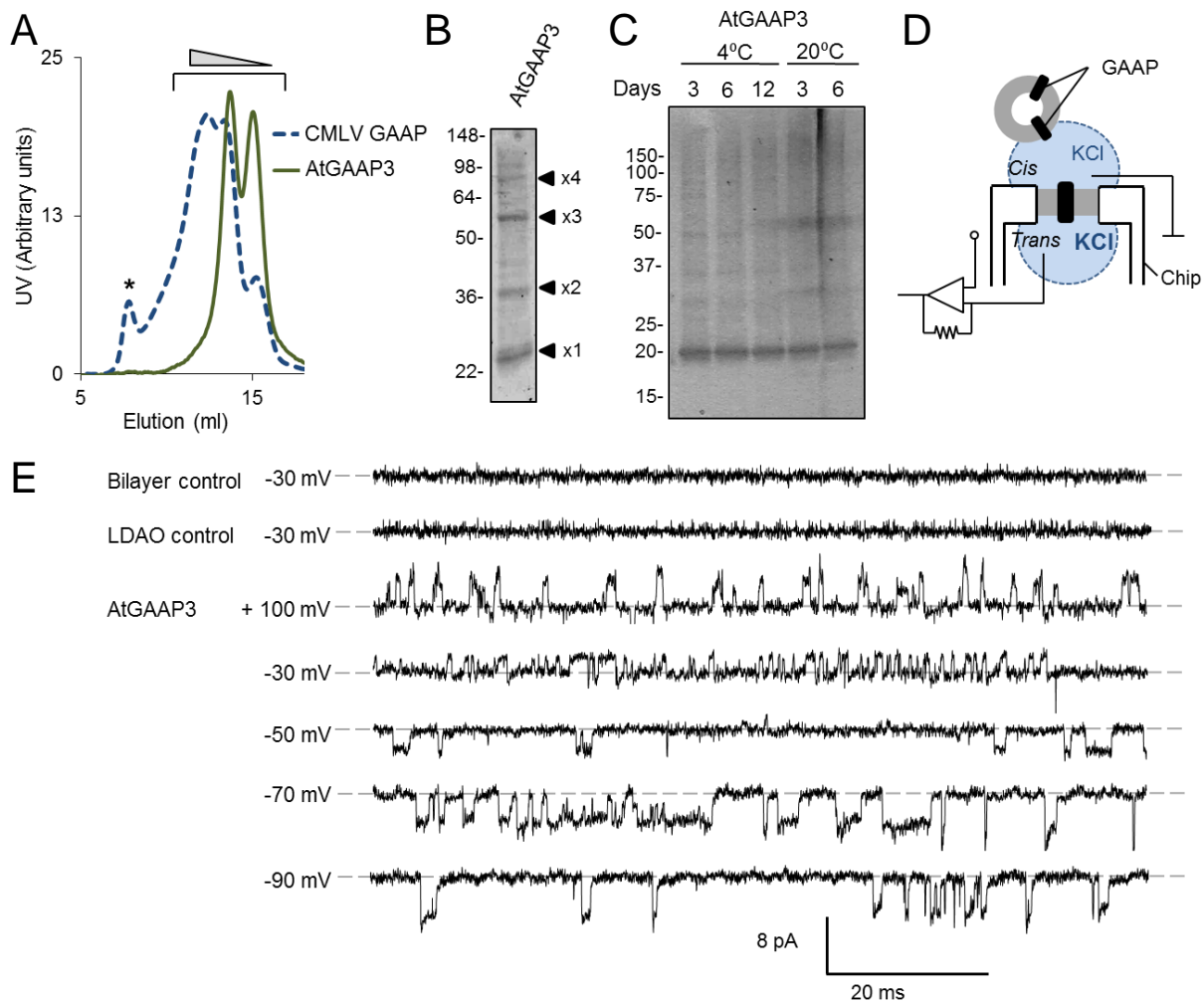
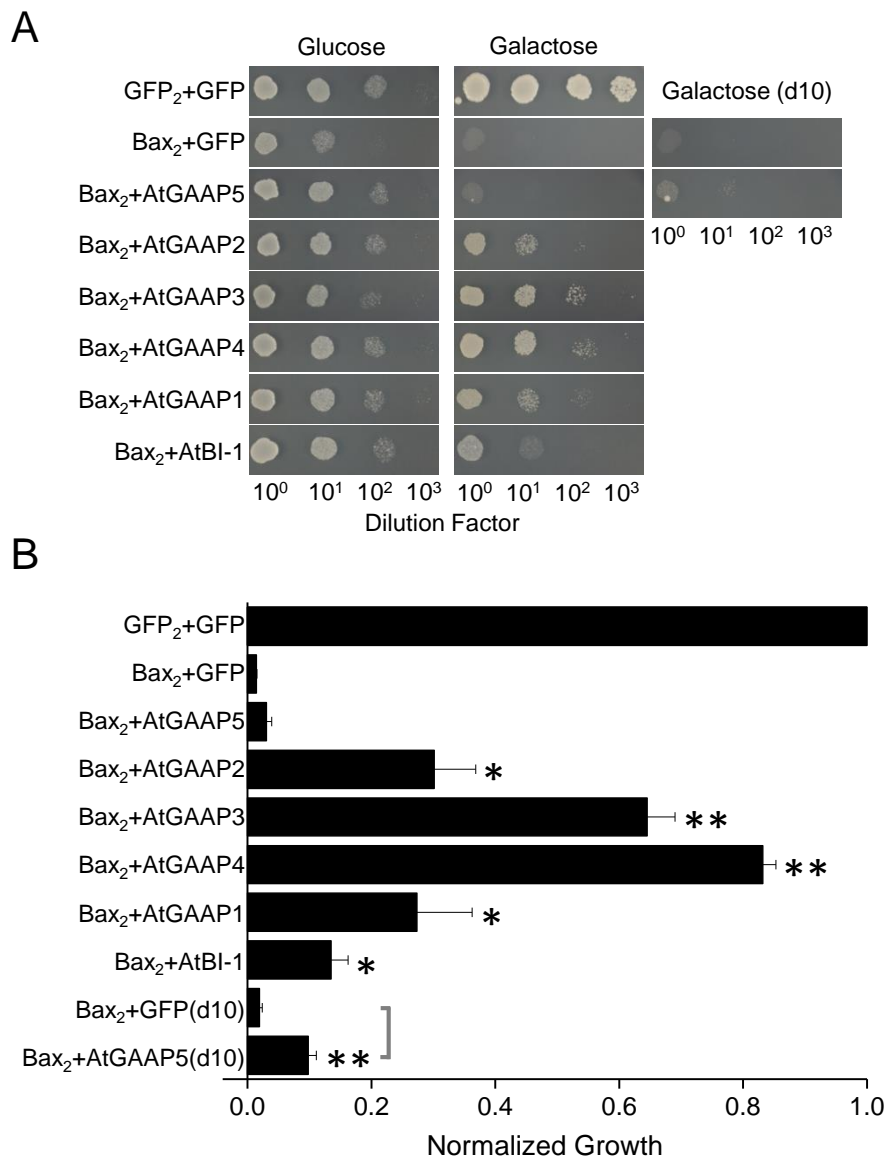


Figure 2.

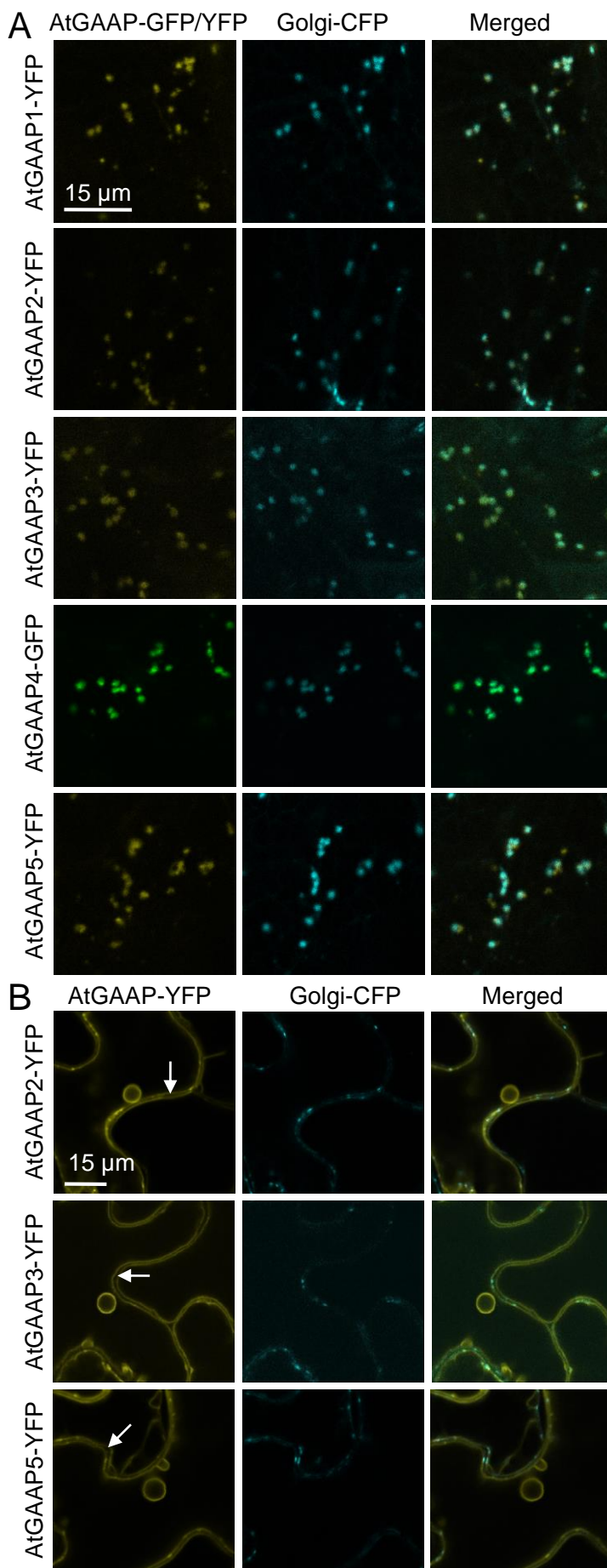


**Figure 3.**

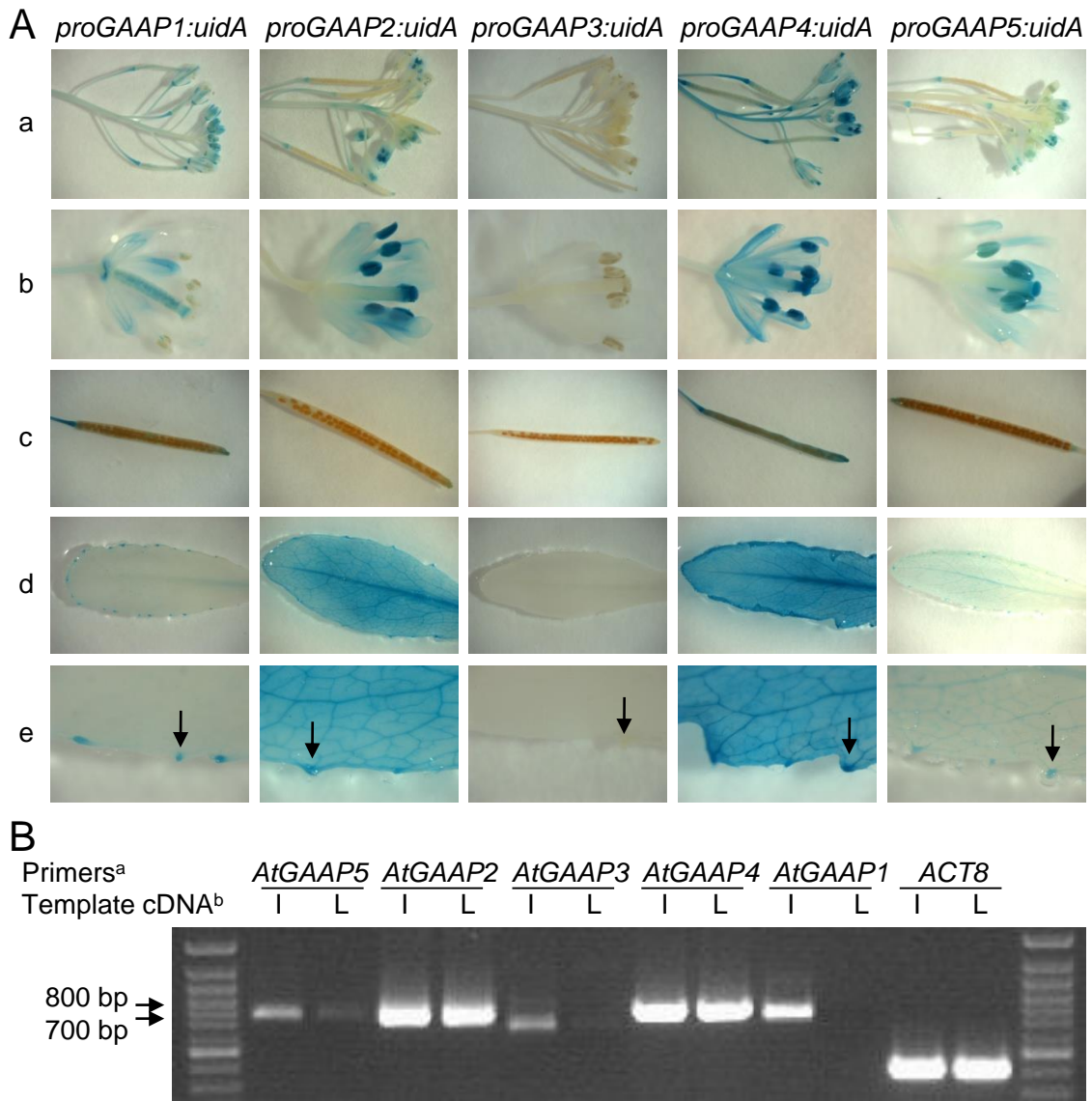




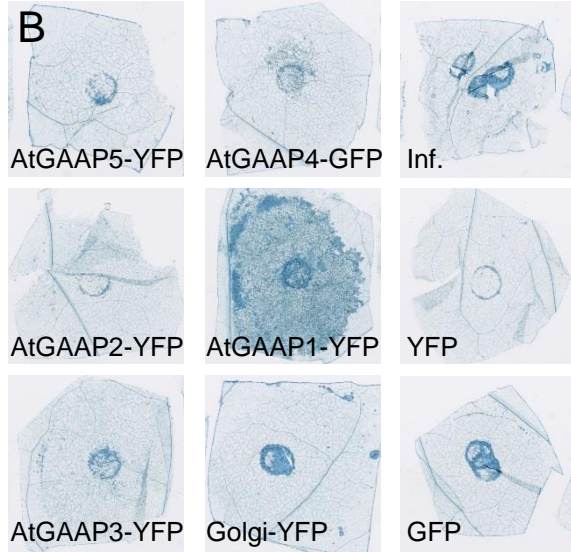
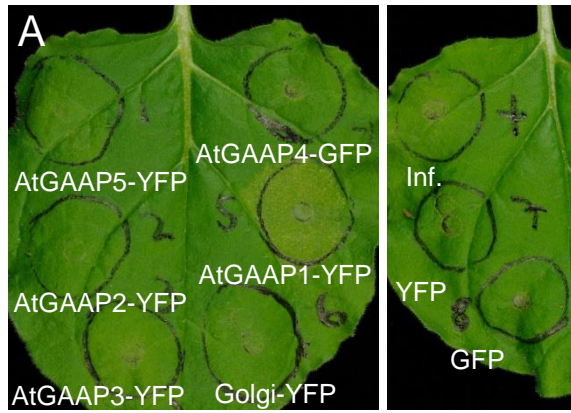
**Figure 4.**



**Figure 5.**



**Figure 6.**



**Figure 7.**

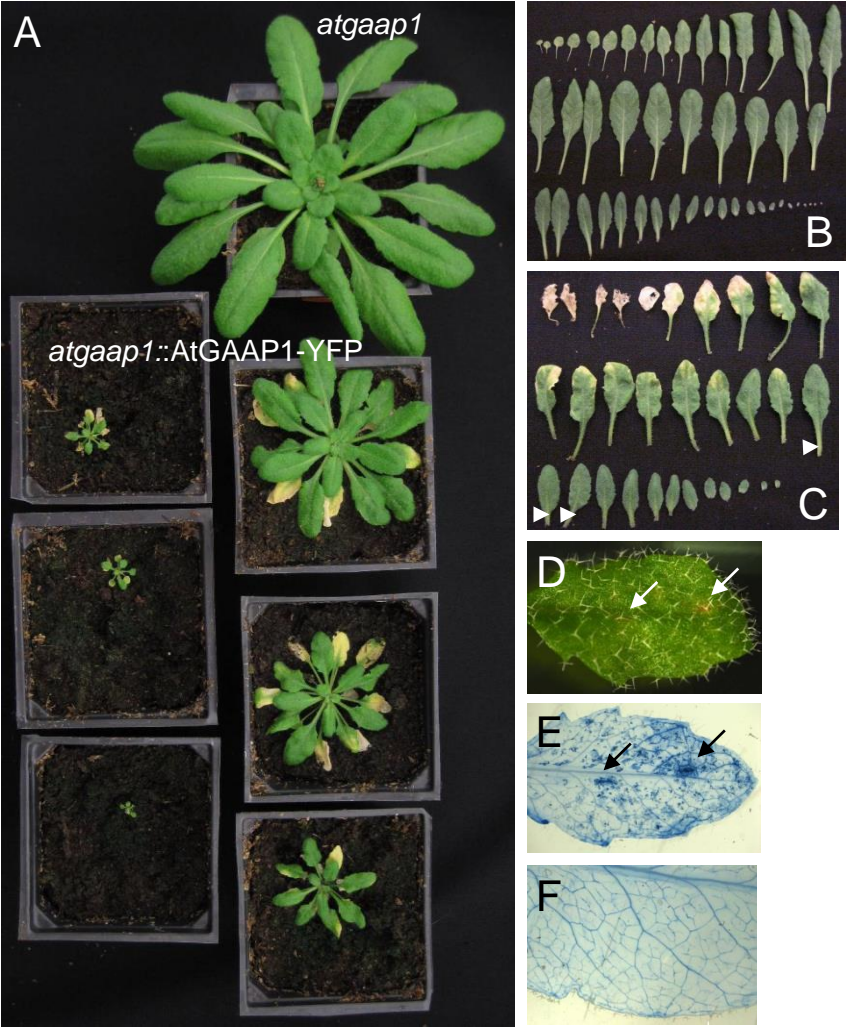


Figure 8.