Characterisation of the GAAP (Golgi anti-apoptotic protein) gene family in Arabidopsis thaliana

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A thesis submitted for the degree of Doctor of Philosophy and the Diploma of Membership of Imperial College
Statement of Originality

The material in this thesis has not been previously submitted for a degree in any university, and to the best of my knowledge contains no material previously published or written by another person except where due acknowledgement is made in the thesis itself.
Äidille ja isälle
ABSTRACT

Programmed cell death (PCD) plays an essential role in eukaryotes during growth and development and in response to stress signals. GAAPs (Golgi anti-apoptotic protein) are a novel, evolutionarily conserved group of anti-apoptotic proteins. Human and viral GAAPs have been shown to inhibit apoptosis and modulate intracellular calcium fluxes. There is an apparent expansion of the GAAP gene family in plants, with five paralogous genes present in the Arabidopsis thaliana genome (AtGAAP1-5). AtGAAPs share the UPF0005 signature motif with animal and plant proteins that have been shown to function as inhibitors of cell death, including Bax inhibitor-1 and Lifeguard. AtGAAP genes show distinct expression patterns with AtGAAP4 and AtGAAP2 showing the highest overall transcript abundance based on publicly available microarray data and RT-PCR analysis. AtGAAP gene expression analysis using promoter-GUS fusions revealed overlapping expression patterns for AtGAAP1, AtGAAP2 and AtGAAP4 in floral organs, with AtGAAP2 and AtGAAP4 also highly expressed in leaf tissue. AtGAAP5 however showed floral-specific expression that was mostly distinct from the expression pattern of AtGAAP1, AtGAAP2 and AtGAAP4 in the flowers. AtGAAP3 expression was undetectable by GUS staining. Intracellular localisation of fluorescent protein-tagged AtGAAPs was studied using stable or transient expression in Arabidopsis and Nicotiana benthamiana, respectively. All AtGAAPs were confirmed to localise to the Golgi at low expression levels and AtGAAP1 and AtGAAP2 additionally localised to the tonoplast at higher expression levels. Analysis of single knock-out mutants of AtGAAPs revealed no obvious developmental or PCD-related phenotypes. Measurement of cytosolic Ca^{2+} rises following H_{2}O_{2} or mannitol treatment in atgaap null mutants, transgenically expressing proaequorin, indicated a potential role for AtGAAPs in Ca^{2+} signalling, however, these data are preliminary. Several double and triple atgaap mutants have been generated, all of which display a wild-type growth habit suggesting either redundancy within the AtGAAP gene family or the existence of a subtle phenotype that is not apparent under the conditions used. Phenotypes have however been uncovered in plants overexpressing AtGAAP-YFP fusion proteins. AtGAAP1 overexpressors display a slight dwarf phenotype whereas AtGAAP2 overexpressors show severely twisted branches. AtGAAP5 overexpressors display a severe dwarf phenotype, enhanced senescence and development of spontaneous lesions in both
rosette and cauline leaves. Moderate to high expression of AtGAAP5 presumably leads to lethality, as no transgenic plants that express AtGAAP5-YFP at these levels have been recovered.
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List of abbreviations

**AtGAAP**, *Arabidopsis thaliana* GAAP
**Bax**, Bcl-2-associated X protein
**Bcl-2**, B-cell lymphoma 2
**BI-1**, Bax inhibitor-1
**BR**, brassinosteroid

$[Ca^{2+}]_{cyt}$, cytosolic calcium concentration
**CFP**, cyan fluorescent protein
**CNGC**, cyclic-nucleotide-gated ion channel
**CPA**, cyclopiazonic acid
**ER**, endoplasmic reticulum
**GAAP**, Golgi anti-apoptotic protein
**GFP**, green fluorescent protein
**h-GAAP**, human GAAP
**HR**, hypersensitive response
**IP3**, inositol 1,4,5-trisphosphate
**KO**, knock-out
**LFG**, lifeguard
**LMM**, lesion mimic mutant
**MOM**, mitochondrial outer membrane
**MOMP**, mitochondrial outer membrane permeabilisation
**PCD**, programmed cell death
**PM**, plasma membrane

**Pst**, *Pseudomonas syringae pv tomato* DC3000
**TMV**, Tobacco mosaic virus
**v-GAAP**, viral GAAP
**VPE**, vacuolar processing enzyme
**WT**, wild-type
**YFP**, yellow fluorescent protein
Chapter 1: General introduction

1.1 Programmed cell death in plants

Programmed cell death (PCD) is a genetically controlled process that plays an essential role in the life cycle of plants and animals. PCD is one of the key mechanisms controlling generation of developmental patterns and specific organ shapes and the removal of unwanted, damaged or infected cells in multicellular organisms. In plants, developmental PCD is manifested in a plethora of processes from seed germination to seed production. A few well known examples include the death of the suspensor cells in the developing embryo (Bozhkov et al., 2005) and differentiation of tracheary elements, specialized cells in the xylem of vascular plants that are critical for transport of solutes and water and for mechanical support (Fukuda, 2000). Leaf senescence is also a type of developmental PCD and involves orderly changes in cellular functions that allow efficient recycling of nutrients that are released during senescence (Lim et al., 2003). A variety of abiotic stresses, such as heat shock (Vacca et al., 2004; Watanabe & Lam, 2006), low temperature (Koukalova et al., 1997), UV irradiation (Danon et al., 2004) and ozone exposure (Overmyer et al., 2005) are also known to induce PCD in plants.

Despite the studies of PCD during developmental processes and abiotic stress adaptation, the best known examples of PCD in plants occur in response to pathogens. Inhibition of growth of avirulent pathogens during incompatible plant-pathogen interaction involves the rapid induction of PCD at the site of infection. This localized response, termed hypersensitive response (HR), limits the spread of the pathogen playing an important role in the plant’s defence response. HR occurs during race-specific disease resistance mediated by the host disease resistance (R) gene and the corresponding pathogen avirulence (avr) gene in an allele specific manner (Hofius et al., 2007). It can also be triggered by host receptor-mediated perception of pathogen non-race specific elicitors (Zipfel & Felix, 2005). Virulent pathogens can also induce cell death with apoptotic features during compatible plant-pathogen interactions that lead to disease development (Greenberg & Yao, 2004).
1.2 Apoptosis – an overview

Apoptosis in animals is a form of programmed cell death that has been well characterised. Apoptosis is an integral part of normal development. However, a major interest in this field stems from the fact that defective apoptotic processes have been linked to disease development. AIDS and neurodegenerative diseases such as Parkinson’s and Alzheimer’s disease involve excessive apoptosis whereas decreased levels of apoptosis can lead to cancer and autoimmune diseases (Casella & Finkel, 1997; Hanahan & Weinberg, 2000; Prasad & Prabhakar, 2003; Rohn et al., 2001).

Apoptosis is associated with a distinct set of morphological and biochemical changes in the cell (Kerr et al., 1972). A family of cysteine proteases termed caspases play a central role in the orchestration and execution of apoptosis; cleavage of numerous cellular substrates by caspases eventually leads to apoptosis (Adrain and Martin, 2001). Caspases specifically cleave after the aspartate residue of many target proteins. Caspases exist in healthy tissues as inactive pro-enzymes and can be broadly grouped into initiator and executioner/effector caspases. Apoptotic signals first activate initiator caspases, including caspase -2, -8, -9 and -10, which then proteolytically activate the effector caspases, primarily caspases -3,-6 and -7. Cleavage of essential cellular structures by effector caspases leads to apoptotic morphotype (Adrain & Martin, 2001; Lawen, 2003). For example, activated effector caspase-3 is able to cleave inhibitor of caspase activated DNAse (ICAD), leading to activation and nuclear localization of caspase activated DNAse (CAD) and DNA fragmentation (Enari et al., 1998).

Apoptosis can be triggered by two major mechanisms: extrinsic/death receptor pathway or intrinsic/mitochondrial pathway. A number of intra- and extracellular stresses including DNA damage, endoplasmic reticulum (ER) stress, oxidative stress and treatment with cytotoxic drugs can activate the intrinsic apoptotic pathway. Mitochondrial outer membrane permeabilisation (MOMP) is the critical step governing the intrinsic pathway, leading to release of cytochrome c and other proapoptotic molecules from the mitochondrial intermembrane space into the cytosol. Once in the cytosol, cytochrome c drives the assembly of apoptosome, a multiprotein
complex containing adapter molecule apoptosis protease activating factor 1 (APAF-1), (d)ATP, and initiator caspase-9. Apoptosome assembly activates caspase-9, which can then activate effector caspases, which in turn cleave cellular substrates leading to apoptotic outcome (Adrain & Martin, 2001; Adrain & Martin, 2009).

Another set of core regulators of apoptotic pathways is the Bcl-2 (B-cell lymphoma 2) family of proteins, members of which either inhibit or promote apoptosis. Bcl-2 family members contain one to four Bcl-2 homology regions (BH1-4) and can be subdivided into three groups based on domain structure and function of the proteins: 1) Antiapoptotic multidomain proteins, which possess all four BH domains (BH1-4) (eg. Bcl-2 and Bcl-XL), 2) Proapoptotic multidomain proteins, with three BH domains (BH123) (eg. Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak)), and 3) BH3-only proteins (eg. Bid, Bim, Bad). Bcl-2 family members control apoptosis through regulation of mitochondrial integrity. Bax and Bak are responsible for permeabilisation of the mitochondrial outer membrane (MOM) and release of proapoptotic factors including cytochrome c from the mitochondria into the cytosol upon apoptotic stimuli. It is yet unclear whether Bax and Bak form a pore in the outer mitochondrial membrane or release proapoptotic factors by other means (Kroemer et al., 2007). Bax and Bak reside in the cytoplasm or mitochondrial outer membrane, respectively, as inactive monomers in healthy tissues. Following an apoptotic stimulus, certain members of the BH3 group, including tBid (truncated Bid) and Bim directly bind to Bax and Bak, and trigger formation of active homo-oligomers of Bax and Bak at the MOM, thereby inducing MOMP (Kim et al., 2009). Antiapoptotic Bcl-2 and Bcl-XL inhibit MOMP induction by keeping BH3 proteins in inactive complexes, thus preventing Bax/Bak activation. Proapoptotic activity of certain members of the BH3 group (eg Bad) does not occur through direct activation of Bax/Bak, but instead through inactivation of Bcl-2 and Bcl-XL (Kim et al., 2009; Yao & Marassi, 2009).

Activation of the extracellular pathway occurs upon binding of extracellular ligands to plasma membrane localised death receptors. Typical death receptors include members of the tumor necrosis factor (TNF) receptor (TNFR) family, including TNFR1 and Fas. Upon ligand binding, the activated death receptors recruit adaptor protein FADD (Fas-associated death domain-containing protein), which then promotes the assembly
of multiprotein signalling complex (death-inducing signalling complex (DISC)) on the cytoplasmic side of the plasma membrane. DISC assembly leads to the recruitment of initiator caspases -8 and -10 to the complex, causing their activation and release into the cytoplasm. These initiator caspases then cleave and activate effector caspases -3, -6, and -7, which cleave cellular substrates leading to an apoptotic phenotype. The extrinsic pathway can also lead to the activation of the mitochondrial pathway. Once activated, initiator caspase-8 can cleave BH3 protein Bid, leading to activation of Bak/Bax and induction of MOMP (Kroemer et al., 2007; Russo et al., 2010).

1.3 PCD and apoptosis have similarities

In contrast to mammalian apoptosis, mechanisms driving and controlling PCD in plants still remain rather obscure. Plant cells undergoing PCD display several characteristic features of apoptosis and plant homologues of certain regulators of apoptosis have been identified and characterised. However, some obvious differences exist between apoptosis and programmed cell death in plants. Final stages of apoptosis are characterised by the formation of apoptotic bodies, fragments of nucleus and cell material encapsulated within plasma membrane fragments (Kerr et al., 1972). Apoptotic bodies are engulfed by specialised macrophages or cells in the direct vicinity of the apoptotic bodies. Phagocytosis is not found in plant tissues and thus true apoptosis apparently does not occur in plants (van Doorn & Woltering, 2005). Instead, the dying cells themselves are responsible for degrading the cell material. Release of hydrolytic enzymes from the vacuole has been implicated in this process and tonoplast rupture is a feature of certain PCD processes in plants [eg. Tracheary element formation (Fukuda, 2000)], but not in others [eg. Victorin-induced PCD in oats (Curtis & Wolpert, 2004)]. Also, no obvious homologues of caspases or Bcl-2 family members have been identified in plants. Despite these differences, recent evidence suggests conservation of a number of features between apoptosis and PCD in plants. Some of these similarities are highlighted in the following paragraphs.
1.3.1 Apoptotic-like morphology in plant cells undergoing PCD

Morphological features associated with apoptosis were first described in a seminal paper by Kerr et al. (1972), clearly distinguishing this controlled form of cell death from necrosis. Apoptosis is associated with a distinct set of cytological changes, including cell shrinkage and chromatin condensation, nuclear fragmentation and formation of apoptotic bodies (fragments of nucleus and cell material encapsulated within plasma membrane fragments) which are phagocytosed by other cells (Kerr et al., 1972). Several morphological and biochemical features characteristic of animal apoptosis appear to be conserved in plant cells undergoing programmed cell death. McCabe et al. (1997) used carrot cell suspension culture to study effect of heat shock on cell death morphology. Heat shock with temperatures below 55°C resulted in apoptotic features, including cell condensation and shrinkage and fragmentation of DNA. Importantly, exposure of cells to temperatures above 75°C resulted in necrotic phenotypes; cell swelling and lysis, demonstrating that condensation of the protoplast away from the cell wall was an active result of PCD (McCabe et al., 1997). Similar systems whereby cells undergoing PCD or necrosis can be distinguished based on morphology of the dying cells upon heat shock have now also been established in Arabidopsis and tobacco cell culture (Burbridge et al., 2007; McCabe & Leaver, 2000). Cytoplasmic shrinkage, along with chromatin condensation and DNA laddering was observed by Vacca et al. (2004) during heat-shock induced PCD in tobacco Bright Yellow 2 (TBY-2) cell culture. Cell shrinkage, chromatin condensation and DNA fragmentation have also been observed in response to a number of abiotic stresses, including H$_2$O$_2$ and ceramides (Houot et al., 2001; Townley et al., 2005), and biotic stresses, including host-selective toxin, victorin, in oats, and fungal elicitors in tobacco (Curtis & Wolpert, 2004; Yano et al., 1998).

Apoptotic morphology has also been observed in a number of developmental processes. Death of the tapetum in developing anthers is essential for pollen development. Cell condensation and shrinkage, together with chromatin condensation and DNA fragmentation are associated with death of the tapetum in a number of plant species (Balk & Leaver, 2001; Papini et al., 1999; Wang et al., 1999). Similar morphologies have also been observed in root tips of Arabidopsis during root cap formation (Zhu & Rost, 2000).
1.3.2 DNA laddering

A well characterised feature of cells undergoing apoptosis is the cleavage of genomic DNA by endonucleases. Internucleosomal cleavage occurs at specific sites, leading to fragments that are multiples of 180bp (Wyllie, 1980). This so called DNA laddering, detected by in situ labelling of 3’hydroxyl termini (TUNEL; (Gavrieli et al., 1992)) or by visualising fragments on an agarose gel, has been extensively used as an indication of plant cells undergoing PCD (Danon et al., 2000). In suspension cultured carrot cells, internucleosomal cleavage of DNA was detected 5 hours after heat shock treatment at 55°C. Upon heat treatment at 85°C, degradation of DNA was detected immediately after the stimuli, and appeared as a smear on the agarose gel indicating random breakage of the DNA due to heat damage (McCabe et al., 1997). The authors were therefore able to distinguish whether cells died in a programmed or necrotic manner. The time delay in DNA cleavage observed upon PCD-inducing stimuli is presumably due to the time nucleases require to be activated and cleave DNA at specific sites (McCabe et al., 1997; Reape & McCabe, 2008; Reape et al., 2008). DNA laddering has since been observed in response to several biotic and abiotic stress treatments that induce cell death, including cold (Koukalova et al., 1997), UV radiation (Danon et al., 2004) and pathogens (Greenberg & Yao, 2004; Hofius et al., 2007). DNA laddering has also been associated with developmental cell death during endosperm development (Young et al., 1997), a range of senescing plant tissues including petal, carpel tissue and leaves (Orzaez & Granell, 1997a; Orzaez & Granell, 1997b; Yen & Yang, 1998), and during tapetal cell death (Balk & Leaver, 2001). During apoptosis, cleavage of DNA into large 50 kb fragments usually precedes DNA laddering (Susin et al., 1999). The presence of 50 kb fragments during PCD has also been observed in plants, for example during embryogenesis (Filonova et al., 2000), and in response to pathogens, H₂O₂ and mitochondrial extracts (Balk et al., 2003; Levine et al., 1996; Mittler et al., 1997). Although nuclease activity has been detected during PCD, the enzymes responsible for internucleosomal cleavage in plants remain poorly characterised (Reape & McCabe, 2008; Sugiyama et al., 2000).
1.3.3 Release of cytochrome c upon PCD induction

The release of cytochrome c from the mitochondrion, followed by formation of the apoptosome and activation of a caspase cascade leading to cleavage of cellular targets is one of the determining features of apoptosis in animals. The release of cytochrome c has been detected in different plant systems, in which PCD was induced. Release of cytochrome c in Arabidopsis plants and maize cells treated with mannose precedes the appearance of PCD symptoms (Stein & Hansen, 1999) and induction of PCD in Arabidopsis cell cultures by ceramide, protoporphyrin IX and an elicitor of HR (avrRpt2) leads to cytochrome c release (Yao et al., 2004). Also, heat-induced programmed cell death in tobacco BY-2 cells and menadione induced death in tobacco protoplasts is associated with cytochrome c release (Sun et al., 1999; Vacca et al., 2006). However, the possible function of cytochrome c as a cell death signalling molecule in plants is yet to be established. Some evidence suggests that unlike in animal cells, cytochrome c is not sufficient in inducing cell death in plant cells. Balk et al. (2003) developed a cell-free system containing purified Arabidopsis nuclei to study whether mitochondrial extracts can induce hallmarks of PCD, including chromatin condensation and DNA laddering. These hallmarks of PCD were induced upon addition of broken mitochondria to Arabidopsis nuclei, but were absent when purified cytochrome c was applied. This indicated that factors capable of inducing PCD reside in the plant mitochondria, but cytochrome c is either not necessary, or not sufficient for such induction (Balk et al., 2003).

1.3.4 Caspases, caspase-like proteases and metacaspases

1.3.4.1 Caspase-like activities associated with PCD in plants

A class of cysteine proteases called caspases play a central role during programmed cell death in animals. Some caspases function as signalling molecules in the apoptotic pathway, others mediate cleavage of a variety of cellular targets ultimately leading to cell death (Lawen, 2003). All caspases show specificity for cleavage next to an aspartate residue. No direct homologues of mammalian caspases have been identified in the available plant genome sequences (Hofius et al., 2007; Watanabe & Lam, 2004). However, caspase inhibitor studies suggest the involvement of caspase-like
proteases during the activation of various types of PCD in plants. Synthetic peptide caspase inhibitors and p35 protein from baculovirus can inhibit caspase activation and subsequently apoptosis in animals. Infiltration of tobacco leaves with synthetic caspase inhibitors abolished the HR induced by Pseudomonas syringae pv. phaseolicola (del Pozo & Lam, 1998). Transgenic tobacco plants expressing p35 showed partial inhibition of nonhost HR caused by P. syringae pv. phaseolicola, and gene-for-gene interaction mediated HR in response to Tobacco Mosaic Virus (TMV) infection. Importantly, mutant forms of p35 that do not suppress caspase activity in animal systems were also unable to suppress HR cell death in transgenic tobacco plants (del Pozo & Lam, 2003). Expression of p35 protein in transgenic tomato plants blocked AAL-toxin induced cell death and protected the plants against infection by Alternaria alternata f. sp. Lycopersici. Also, p35 mutant that does not function against animal caspases is also unable to inhibit AAL-toxin induced cell death (Lincoln et al., 2002). These results provide evidence for the participation of caspase-like proteases during pathogen infection and the HR in plants.

Involvement of caspase-like activities in PCD in plants has also been detected in a number of studies using synthetic caspase substrates (Piszczek & Gutman, 2007). For example, PCD induced by ultraviolet-C irradiation was associated with cleavage of a specific caspase substrate. Furthermore, this protease activity was insensitive to broad-range cysteine protease inhibitors that are unable to inhibit animal caspases (Vacca et al., 2004). Also, caspase-like proteolytic activity has been detected in tobacco plants during the N-gene mediated hypersensitive response against Tobacco Mosaic Virus (TMV) infection (Chichkova et al., 2004; del Pozo & Lam, 1998).

1.3.4.2 Plant proteases with caspase-like activities

1.3.4.2.1 Vacuolar processing enzyme

Vacuolar processing enzyme (VPE) has been identified as a caspase-like protease involved in PCD processes in plant cells (Hatsugai et al., 2004). Although overall sequence similarity with caspases is lacking, VPE and caspase-1 share several structural and enzymatic properties. Like caspases, VPE is a cysteine protease that cleaves adjacent to an aspartate residue, although VPE can additionally cleave
adjacent to an asparagine residue. VPE is able to cleave caspase-1 substrate (VDAC), but not caspase-3 substrate (DEVD). VPE and caspase-1 have similar substrate pockets and active sites and like caspase-1, VPE is synthesised as an inactive proenzyme that becomes active through self-catalytic cleavage (Hatsugai et al., 2006).

VPE has been shown to play a role in virus-induced PCD in Arabidopsis and tobacco cells. Hatsugai et al. (2004) studied the involvement of VPE in tobacco N-gene mediated resistance response to TMV. The authors found that VPE expression was rapidly upregulated in response to TMV infection. The HR lesion formation was inhibited by application of caspase-1 inhibitor and VPE inhibitor. Furthermore, virus-induced gene silencing of VPE in *Nicotiana benthamiana* resulted in abolition of caspase-1 activity, VPE activity and HR lesion formation. Disintegration of the tonoplast and DNA degradation accompanying the HR were also abolished in VPE silenced plants. Kuroyanagi et al. (2005) demonstrated that VPE also regulates fungal toxin (fumonicin B1 (FB1)) –induced PCD in Arabidopsis plants. Unlike wild-type plants, a VPE-null mutant did not develop lesions in response to FB1-infiltration. Tonoplast rupture associated with FB-1-induced cell death was also abolished in VPE-null mutants. Application of VPE and caspase-1 inhibitors also abolished lesion development. FB-1-inoculated wild-type leaves displayed VPE and caspase-1 activity and these activities were not detected in the presence of caspase-1 inhibitor. These activities were also absent in FB1-treated leaves of VPE-null mutant. Tonoplast rupture is a feature of both FB-1-induced cell death in Arabidopsis and TMV-induced HR in *N. benthamiana*. Tonoplast rupture did not occur in VPE-deficient plants (Hatsugai et al., 2004; Kuroyanagi et al., 2005). Authors propose that VPE is a key molecule in plant PCD pathways that are associated with vacuolar collapse, a phenomenon not associated with apoptosis in animals. VPE-mediated activation of target proteins may lead to vacuolar collapse thereby releasing hydrolytic enzymes from the vacuole to the cytosol. These enzymes degrade cellular components and prevent viral proliferation (Hatsugai et al., 2004; Hatsugai et al., 2006; Kuroyanagi et al., 2005).
1.3.4.2.2 Saspase

Coffeen and Wolpert (2004) purified and characterised two proteases involved in the victorin-induced PCD of oat (Avena sativa). These proteases show specificity for aspartate residue but unlike caspases contain an active site serine residue, and were hence termed saspases. Saspase showed proteolytic activity towards a range of caspase substrates and was inhibited by caspase inhibitors. Serine proteases which show caspase-like activity, granzyme B and the proteasome, have previously been identified from mammals (Kisselev et al., 2003; Thornberry et al., 1997). Victorin induces cleavage of Rubisco during PCD in oats, however, purified saspases were unable to cleave Rubisco. Authors hypothesised that saspases are part of a proteolytic cascade during the victorin-induced PCD-response that ultimately leads to cleavage of Rubisco. Unlike caspases, the active saspases are constitutively present in the cell. They appear to function in the extracellular space as they are released there upon victorin-induction. Saspase sequences show homology to subtilisin-like serine proteases that exist as multigene families in plants (Meichtry et al., 1999).

1.3.4.2.3 The plant proteasome exhibits caspase-like activity

Hatsugai et al. (2009) reported a novel mechanism whereby plants defend themselves against avirulent bacterial pathogens. Fusion between vacuolar membrane (tonoplast) and plasma membrane was observed in Arabidopsis plants inoculated with an avirulent Pseudomonas syringae strain. Membrane fusion released antibacterial proteins from the vacuole into the apoplast where bacterial pathogens proliferate. Furthermore, the authors linked the cell death response and observed membrane fusion to the function of proteasome and caspase-3-like activity exhibited by the proteasome. Hypersensitive response was abolished by application of inhibitors for caspase-3 and a general proteasome inhibitor. The proteasome consists of three subunits, β1, β2, and β5, encoded by PBA1, PBB and PBE, respectively. Mammalian and yeast proteasomes have previously been shown to exhibit caspase-like activity, cleaving substrates adjacent to an aspartate residue (Kisselev et al., 2003). Application of an inhibitor of the β1 subunit also abolished the HR. Arabidopsis leaves inoculated with bacteria showed activity towards a caspase-3 substrate.
(DEVD) and this activity was abolished by the proteasome inhibitors. PBA1 activity in inoculated leaves was inhibited by a caspase-3 inhibitor. Silencing of PBA1 by RNAi resulted in decreased activity against caspase-3 substrate and reduced PBA1 activity. The authors showed that the β1 subunit of the proteasome is the target of the caspase-3 inhibitor by performing immunoprecipitation with an irreversible caspase-3 inhibitor. Furthermore, silencing of PBA1 suppressed membrane fusion and HR in response to infection by avirulent bacteria suggesting that the proteasome is required for gene-for-gene mediated cell death and membrane fusion.

1.3.4.3 Metacaspases

Structural element-based iterative searches using sequences distantly related to caspases identified a family of plant caspase-like proteases called metacaspases (Aravind et al., 1999; Uren et al., 2000). The catalytic cysteine and histidine diad required for catalysis by cysteine proteases is conserved within the caspase-like proteolytic domain in metacaspases. Caspases usually contain an N-terminal prodomain encoding interaction modules. The plant metacaspases can be divided into two subclasses based on the predicted domain structure and sequence similarity within the caspase-like regions. Type I metacaspases have proline-rich prodomains at the N-terminus. Prodomains of this class also contain a zinc finger motif that is also found in LSD1, a negative regulator of HR cell death conserved in plants. Type II metacaspases do not possess a prodomain. Metacaspases, like caspases, possess a small subunit at the C-terminus. In type II metacaspases this subunit is separated from the large proteolytic subunit by a conserved linker region of approximately 180 amino acids (Uren et al., 2000).

In the Arabidopsis genome, there are 3 predicted type I metacaspases (AtMC1-3) and 6 predicted type II metacaspases (AtMC4-9) (Vercammen et al., 2004; Watanabe & Lam, 2004). Rapid upregulation of AtMCP1-5 gene expression was observed in response to infection of leaves with bacterial pathogens (Watanabe & Lam, 2005). Similarly, transcript levels of LeMCA1, a Lycopersicon esculentum type II metacaspase, were rapidly induced in tomato leaves in response to the fungal
necrotroph *Botrytis cinerea* (Hoeberichts et al., 2003). These results suggest metacaspase participation in the process of PCD.

More direct evidence for a role of metacaspases in plant PCD has recently emerged. Van baarlen et al. (2007) studied the role of Arabidopsis metacaspases during *Botrytis* infection using metacaspase knock-out mutants. KO of type II metacaspases rendered plants less susceptible to *Botrytis* infection, suggesting that type II metacaspases have a stimulatory role in cell death. Conversely, KO of type I metacaspases leads to increased susceptibility to *Botrytis* compared to wild-type, as well as yellowing at the whole plant level and leaf discoloration, indicative of accelerated senescence, and development of spontaneous lesions (lesion mimic phenotype). The observed phenotypes suggest that type 1 metacaspases may function as inhibitors of cell death. Alterations in sensitivity to *Botrytis* was slight but significant in the individual MCA KO mutants. The authors postulate that this could be due to functional redundancy (van Baarlen et al., 2007). He et al. (2008) showed that Arabidopsis type 2 metacaspase (AtMC8) is involved in PCD triggered by oxidative stress. Transcript levels of *AtMC8* increased upon treatment of plants with UVC, H$_2$O$_2$, and the herbicide methyl violagen. These treatments have previously been shown to induce apoptotic-like PCD in plants. Overexpression of *AtMC8* enhanced H$_2$O$_2$ and UVC-induced PCD in transgenic plants and protoplasts. Furthermore, mutation of *AtMC8* resulted in reduction of H$_2$O$_2$ and UVC induced cell death.

Although the above results provide strong evidence for involvement of metacaspases in the regulation of PCD in plants, metacaspases might not be responsible for caspase-like activities observed in plants. All plant metacaspases tested so far cleave adjacent to an arginine, instead of aspartate, as caspases do. Metacaspases have no activity towards caspase substrates and are not inhibited by caspase inhibitors (Bonneau et al., 2008; Bozhkov et al., 2005; He et al., 2008; Vercammen et al., 2004; Watanabe & Lam, 2005).
1.3.5 Calcium fluxes and cell death

Ca$^{2+}$ is a ubiquitous secondary messenger in eukaryotic organisms and plays a role in a multitude of signalling cascades involved in development and stress responses. Cytosolic calcium concentrations [Ca$^{2+}$]$_{cyt}$ are tightly regulated and maintained at low concentrations (~0.1-0.3 μM) (Mcainsh et al., 1992). Ca$^{2+}$-induced signalling is triggered by rises in cytoplasmic calcium concentration. These rises result from flux of calcium into the cytosol across the plasma membrane or from subcellular compartments in which the concentration of calcium is high compared to the cytosol. The ER and Golgi have been implicated as the most important Ca$^{2+}$ storage compartments in animal cells (Hajnoczky et al., 2003; Pinton et al., 1998). The ER is also an internal source of calcium in plant cells and the Golgi is thought to function as a mobile Ca$^{2+}$ store contributing to Ca$^{2+}$ signalling (Ma & Berkowitz, 2007; Menteyne et al., 2006). The vacuole in plant cells has been shown to constitute an additional pool of calcium ions contributing to cytosolic Ca$^{2+}$ changes (Lecourieux et al., 2006; Ma & Berkowitz, 2007). In animal cells, calcium release occurs mainly through inositol-1,4,5-trisphosphate (IP3) receptors (IP3Rs), Ca$^{2+}$ channels located on the ER and Golgi membrane. These channels open to release Ca$^{2+}$ upon binding of inositol-1,4,5-trisphosphate (Rizzuto et al., 2009). No homologues of animal IP3Rs have been identified in higher plants. However, release of Ca$^{2+}$ from the vacuole leading to an increase in cytosolic calcium levels has been observed in response to IP3 (Gilroy et al., 1990; MacRobbie, 2000). Identification and molecular characterisation of plant Ca$^{2+}$ channels is an area of intense research in the plant field (Dodd et al., 2010; Kudla et al., 2010; McAinsh & Pittman, 2009; Ward et al., 2009).

Ca$^{2+}$ is a key molecule controlling physiological functions and cell survival in animal cells. However, Ca$^{2+}$ also plays a role during apoptotic responses. Calcium signals induced by a number of stress agents trigger mitochondrial membrane permeabilization and apoptosis (Demaurex & Distelhorst, 2003; Pinton et al., 2001; Szalai et al., 1999). Calcium release from storage organelles are thought to play a role in the apoptotic changes of mitochondria (Pinton & Rizzuto, 2006). Organelle Ca$^{2+}$ loading can determine the sensitivity of cells to apoptotic stress; Ca$^{2+}$ overload in the storage organelles can increase apoptosis. Lowering Ca$^{2+}$ levels in the stores protects cells from apoptosis, and increasing Ca$^{2+}$ levels in the stores sensitises cells to
apoptosis. This is thought to involve reduced or increased levels of Ca^{2+} signalling from the stores to the mitochondria, respectively (Ma \textit{et al.}, 1999; Nakamura \textit{et al.}, 2000; Pinton \textit{et al.}, 2001; Pinton & Rizzuto, 2006; Rizzuto \textit{et al.}, 2009). Ca^{2+} cross-talk between stores and mitochondria is thought to involve some members of the Bcl-2 family of apoptotic regulators. Bcl-2 has been shown to localise to the ER in addition to mitochondria. Overexpression of Bcl-2 lowers the level of free Ca^{2+} in ER and Golgi, thereby reducing Ca^{2+} signalling to the mitochondria leading to protection from apoptotic challenges (Pinton \textit{et al.}, 2000).

As with animal cells, rapid rises in cytosolic calcium concentration have been detected in response to numerous stress stimuli. Abiotic stress stimuli that induce [Ca^{2+}]_{cyt} rises include cold (Knight \textit{et al.}, 1996; Knight \textit{et al.}, 1991), heat shock (Gong \textit{et al.}, 1998), oxidative stress (Evans \textit{et al.}, 2005) and osmotic, salt and drought signals (Kiegle \textit{et al.}, 2000; Knight \textit{et al.}, 1997). Changes in [Ca^{2+}]_{cyt} have also been reported in response to a number of pathogens, pathogen elicitors and phytotoxins (Lecourieux \textit{et al.}, 2006).

Whether these Ca^{2+} fluxes play a role in PCD in plants has remained rather elusive. However, it is becoming evident that Ca^{2+} is an important molecule in cell death signalling in plant cells. A number of studies using plasma membrane calcium channel blockers or calcium chelators indicate an essential role for Ca^{2+} in PCD control. HR triggered in Arabidopsis by the avrRpm1 avirulence protein has been associated with a sustained increase in cytosolic Ca^{2+}. Application of the calcium channel blocker lanthanum abolished the Ca^{2+} increase and blocked HR development (Grant \textit{et al.}, 2000). Sasabe \textit{et al.} (2000) reported that HR of tobacco BY-2 cells resulting from application of INF1, an oomycete elicitor, was significantly reduced after application of a general chelator of cations, a specific chelator of Ca^{2+}, and by lanthanum. Levine \textit{et al.} (1996) studied PCD triggered by H_{2}O_{2} and an avirulent \textit{Pseudomonas syringae} strain in soybean cells. Application of lanthanum to soybean cells almost completely abolished the PCD response. These studies suggest that cytosolic Ca^{2+} increases are necessary for HR cell death in plants. Lanthanum has been used to demonstrate the necessity of Ca^{2+} fluxes for PCD in response to a number of PCD triggers. McCabe \textit{et al.} (1997) observed that application of lanthanum to carrot suspension cultures treated with various PCD inducers, including H_{2}O_{2} and
heat stress, abolished cell condensation and shrinkage associated with PCD. Townley et al. (2005) showed that C2-ceramide, a well-established inducer of apoptosis in animals, also triggers PCD with characteristic hallmarks of apoptosis in Arabidopsis suspension cultures. Furthermore, preventing Ca$^{2+}$ signalling by lanthanum inhibited ceramide-induced cell death. Lanthanum has also been used to show that Ca$^{2+}$ influx is necessary for bacterial toxin induced PCD in Arabidopsis cell cultures, and camptothecin-induced PCD in suspension-cultured tomato cells (Errakhi et al., 2008; Hoeberichts et al., 2001).

Additional data suggesting a role for calcium as a mediator of PCD has come from molecular characterisation of putative calcium channels in plant cells. Kurusu et al. (2005) studied the role of a putative voltage-gated Ca$^{2+}$ channel, rice two-pore channel (OsTPC1), in fungal-elicitor induced defence responses, including PCD, in rice cells. They showed that overexpression of this channel accelerated the PCD induced by the elicitor and KO of the channel suppressed the PCD, suggesting a role for this putative Ca$^{2+}$ channel in PCD. The Role of calcium-permeable cyclic-nucleotide-gated ion channels (CNGC) in cell death has also been demonstrated. The Arabidopsis dnd1 mutant was originally isolated from a mutant screen for plants with altered HR against Pseudomonas syringae carrying the avrRpt2 avirulence gene (Yu et al., 1998). dnd1 displays a dwarf phenotype. dnd1 fails to produce an HR in response to pathogen infection although it retains resistance against this pathogen. DND1 was subsequently shown to encode AtCNGC2, a plasma membrane localised channel capable of conducting Ca$^{2+}$ into cells (Ali et al., 2007; Clough et al., 2000) demonstrating that inhibition of HR can result from loss of Ca$^{2+}$ conducting CNGC channel.

Characterisation of Arabidopsis lesion mimic mutants (LMM) has also shed light on a possible link between PCD and Ca$^{2+}$ in plant cells. Lesion mimic mutants develop spontaneous HR-like lesions in the absence of a pathogen (Lorrain et al., 2003). The Arabidopsis mutant constitutive expressor of PR genes 22 (cpr22) exhibits stunted growth with curly leaves and constitutively activated defence responses, including HR-like cell death (Yoshioka et al., 2001). This mutant contains a novel chimeric CNGC that consists of a fusion between the AtCNGC11 and AtCNGC12 channel proteins (Yoshioka et al., 2006). The AtCNGC11/12 chimeric protein was shown to
complement a $\text{Ca}^{2+}$ uptake deficient yeast mutant, thereby confirming its function as a calcium channel (Urquhart et al., 2007). AtCNGC11/12 localises to the plasma membrane and overexpression of AtCNGC11/12 in *Nicotiana benthamiana* induces PCD with characteristic hallmarks of apoptosis, including plasma membrane shrinkage and DNA fragmentation. Interestingly, this PCD was suppressed by caspase-1 inhibitors and calcium channel blockers. Furthermore, silencing of the caspase-like protease, vacuolar processing enzyme (VPE) also reduced the severity of PCD (Urquhart et al., 2007).

The data presented above clearly indicates that calcium signalling is an important mediator of PCD in plants, as well as in animal cells. However, the exact mechanisms underlying this role are largely obscure, especially in plant cells. Most studies demonstrate the importance of influx of calcium across the plasma membrane in PCD in plant cells, much less is known about the importance of intracellular calcium stores in PCD. In contrast, in animal cells the link between intracellular calcium stores and mitochondria in apoptosis has been clearly documented. Studies on a conserved regulator of apoptosis, Bax inhibitor-1, has demonstrated the importance of calcium release from the ER in both animal and plant cells in cell death. This will be discussed below.

### 1.3.6 Function of core regulators of animal apoptosis is conserved in plants

Bcl-2 (B-cell lymphoma 2) family of proteins form a set of core regulators of apoptotic pathways in animals, members of which either inhibit (eg. Bcl-2 and Bcl-XL) or promote (eg. Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak)) apoptosis (Lawen, 2003). Examination of the completed genome sequence of *Arabidopsis*, as well as other plant genomic databases has not revealed any obvious homologues to these core apoptosis regulators (Watanabe & Lam, 2006). However, function of the members of the Bcl-2 family of proteins seems to be conserved when expressed in plants from a transgene. For example, constitutive or transient expression of Bax in Arabidopsis or tobacco plants, respectively, triggers PCD (Kawai-Yamada et al., 2001; Lacomme & Cruz, 1999). Furthermore, transient expression of Bax correlated with expression of PR1 (pathogenesis-related gene 1), a
protein commonly induced during pathogen responses in plants, suggesting Bax activated an endogenous cell-death program in plants (Lacomme & Cruz, 1999). The expression of antia apoptotic genes Bcl-2 and Bcl-XL in tobacco plants suppressed the extensive cell death caused by necrotrophic fungal pathogens and also enhanced resistance to some abiotic stresses such as wounding, salt, cold, UV-B, and paraquat treatment (Dickman et al., 2001; Mitsuhara et al., 1999; Qiao et al., 2002). Transgenic tomato plants expressing bcl-XL have been shown to exhibit enhanced tolerance to viral-induced necrosis and low-temperature-associated necrotic lesion formation and leaf senescence (Xu et al., 2004).

As discussed earlier, Bcl-2 family membranes control apoptosis through regulation of mitochondrial membrane permeabilisation (MMP) in mammalian cells. Although the function of Bcl-2 proteins is conserved in plants, there is, to our knowledge, no detail on how these proteins regulate MMP in plants.

1.3.7 Conserved regulators

Although none of the core machinery members of animal apoptosis have been identified in plants, the identification of two classes of proteins, Bax inhibitor-1 (BI-1) and Golgi anti-apoptotic protein (GAAP), that function as cell death modulators and which are widely conserved in eukaryotic organisms including plants, provides further evidence supporting the existence for a common mechanism for animal and plant cell death pathways.

1.3.7.1 Bax inhibitor-1

Bax inhibitor-1 is a small protein of 25-37 kDa with 6 or 7 predicted transmembrane domains. BI-1 localises predominantly to the endoplasmic reticulum (ER) (Huckelhoven, 2004; Watanabe & Lam, 2004). Bax-inhibitor-1 was first isolated from a human cDNA library screen as a suppressor of Bax-induced cell death in yeast (Xu & Reed, 1998). This function was found to be conserved in mammalian cells; overexpression of BI-1 in human cells inhibited cell death induced by Bax and other pro-apoptotic stimuli whereas knockout of BI-1 by antisense constructs induced
apoptosis (Xu & Reed, 1998). Since then, BI-1 has been shown to be widely conserved in animals and plants and plant BI-1 homologues have been isolated from various species including Arabidopsis (Kawai et al., 1999; Sanchez et al., 2000), Oryza sativa (Kawai et al., 1999), Hordeum vulgare (Huckelhoven, 2004), Brassica napus and Nicotiana tabacum (Bolduc et al., 2003). Interestingly, Arabidopsis BI-1 (AtBI-1) and Oryza sativa BI-1 (OsBI-1) are able to inhibit cell death induced by Bax expression in yeast (Kawai-Yamada et al., 2001; Kawai et al., 1999; Sanchez et al., 2000) and transient co-transfection of human cells with Bax and B. napus or N. tabacum BI-1 revealed that these proteins can significantly inhibit apoptosis induced by Bax overexpression (Bolduc et al., 2003). Overexpression of AtBI-1 from a transgene has also been shown to suppress Bax-induced lethality in planta (Kawai-Yamada et al., 2001).

Arabidopsis Bax inhibitor-1 regulates cell death triggered by both biotic and abiotic inducers of cell death. BI-1 expression has been shown to be upregulated in response to pathogen inoculation in barley (Huckelhoven, 2004), and Arabidopsis (Sanchez et al., 2000; Watanabe & Lam, 2006). In Arabidopsis, AtBI-1 expression was upregulated during both compatible and incompatible interactions with Pseudomonas syringae pv. tomato DC3000 and also in response to non-pathogenic bacteria (Sanchez et al., 2000). Ablastic stress treatments, heat shock and wounding, also lead to dramatic upregulation of AtBI-1 transcript levels (Sanchez et al., 2000; Watanabe & Lam, 2006). Direct evidence for the role of AtBI-1 in PCD in plants is described in a study by Watanabe and Lam (2006), where two Arabidopsis mutants with a T-DNA insertion in the AtBI-1 gene (atbi1-1 and atbi1-2) were characterized. These knockout mutants showed no obvious developmental defects under normal growth conditions, but exhibited accelerated cell death upon exposure to biotic and abiotic stimuli. Infiltration of leaf tissue with programmed cell death inducing fungal toxin fumonisin B1 (FB1) led to an increase in the rate of FB1-induced cell death in the mutant plants compared to wild-type plants. Also, mutant plants displayed substantially increased levels of heat shock-induced cell death compared to control plants. Overexpression of AtBI-1 transgene in the mutant backgrounds rescued the mutant phenotypes but did not result in further delay of cell death induced by either FB1 or heat shock treatments (Watanabe & Lam, 2006). Over-expression of AtBI-1 has been shown to overcome various stress-induced defects in other experimental
systems. For example, overexpression of AtBI-1 in rice cell culture suppressed cell death caused by treatment with fungal (*Magnaporthe grisea*) elicitor (Matsumura *et al.*, 2003) and overexpression of tomato BI-1 in tobacco leaves protected the leaves against heat and cold induced chlorosis (Chae *et al.*, 2003). Also, induction of PCD by H₂O₂ was suppressed in tobacco suspension cultured cells overexpressing AtBI-1, indicating a role for AtBI-1 in the oxidative stress response (Kawai-Yamada *et al.*, 2004). Plant Bax inhibitor clearly suppresses PCD triggered by a number of stress stimuli.

1.3.7.1.1 AtBI-1 and calcium

Studies with both mammalian and Arabidopsis BI-1 indicate that it may be involved in Ca²⁺ homeostasis at the ER. Cytoprotective activity of BI-1 has been correlated with free Ca²⁺ concentration in the ER of animal cells. Overexpression of BI-1 results in reduced levels of releasable luminal Ca²⁺ (Chae *et al.*, 2004; Kim *et al.*, 2008; Westphalen *et al.*, 2005; Xu *et al.*, 2008). Kim *et al.* (2008) suggested that BI-1 acts as a pH-dependent Ca²⁺ channel in the ER membrane. Furthermore, human BI-1 has been shown to have Ca²⁺/H⁺ antiporter-like activity in reconstituted liposomes (Ahn *et al.*, 2009).

Kawai-Yamada and co-workers have demonstrated that AtBI-1 may also be involved in controlling Ca²⁺ homeostasis at the ER. Ihara-Ohori *et al.* (2007) demonstrated that AtBI-1 can interact with Arabidopsis Calmodulin-7 (AtCaM7), a Ca²⁺-binding protein, in yeast and *in planta*. Kawai-Yamada *et al.* (2009) demonstrated that the C-terminal 6 amino acids of AtBI-1 are necessary for this interaction using a genomic mutant (AtBI-CM) in which the C-terminus of BI-1 protein is replaced by a T-DNA insertion. Arabidopsis mutants carrying this insertion showed accelerated HR in response to *Pst* carrying the *avrRpt2* avirulence gene. Also, this mutant failed to suppress Bax-induced lethality in yeast cells. These results indicated that CaM binding may be necessary for the function of AtBI-1 as a cell death suppressor (Kawai-Yamada *et al.*, 2009). To unravel the possible relationship between ER Ca²⁺ homeostasis and AtBI-1, Ihara-Ohori *et al.* (2007) used the ER-resident calcium pump inhibitor cyclopiazonic acid (CPA). Arabidopsis plants overexpressing AtBI-1 were
less sensitive to CPA than WT plants. Also, overexpression of AtBI-1 in tobacco BY-2 cells repressed the cell death induced by CPA treatment. CPA treatment of BY-2 cells was associated with a rapid cytosolic calcium elevation, which in turn was suppressed by overexpression of AtBI-1 (Ihara-Ohori et al., 2007). In the same study the authors tested whether proteins involved in calcium transport across membranes are necessary for the ability of AtBI-1 to rescue yeast cells from Bax-induced cell death. Using a panel of yeast mutants the authors showed that BI-1 was unable to suppress Bax-induced cell death in yeast mutants lacking Ca$^{2+}$ ATPase (Ca$^{2+}$ pump) located on the ER and Golgi membranes. However, BI-1 was able to rescue mutants deficient in calcium transport in the PM and vacuole from Bax-induced cell death. These results suggest that controlling calcium flux at the Golgi and ER may be necessary for cell death suppression by AtBI-1 in yeast.

1.3.7.1.2 BI-1 and ER stress

Certain stress stimuli lead to the accumulation of unfolded or misfolded proteins in the ER, leading to manifestation of “ER stress”. If ER stress cannot be relieved, apoptosis is induced in animal cells. ER stress–induced apoptosis is thought to be mediated through both mitochondria-dependent and –independent pathways in animal cells (Boyce & Yuan, 2006). PCD induction in plant cells also occurs upon ER stress (Watanabe & Lam, 2008a; Watanabe & Lam, 2008b; Watanabe & Lam, 2009; Zuppini et al., 2004). Studies from both animals and plants indicate that BI-1 plays a protective role in ER-stress induced cell death pathways. Cells isolated from BI-1 KO mice are hypersensitive to apoptosis induced by pharmacological agents that trigger ER stress, such as tunicamycin, an inhibitor of N-linked glycosylation that triggers ER stress and apoptosis through accumulation of unfolded proteins in the ER. Overexpression of BI-1 in vitro protects cells against these stimuli (Bailly-Maitre et al., 2006; Chae et al., 2004). Watanabe et al. (2008b) reported that tunicamycin can trigger ER stress in Arabidopsis seedlings. Roots of the treated seedlings exhibited hallmarks of apoptosis such as nuclear condensation and DNA laddering. Tunicamycin treatment led to strong upregulation of AtBI-1 transcript. Furthermore, AtBI-1 KO mutants exhibited accelerated PCD in response to tunicamycin. Conversely, overexpression of AtBI-1 protected cells against tunicamycin-induced
PCD. These data indicate a conserved role for BI-1 as a cell death suppressor against ER stress.

1.3.7.2 Golgi anti-apoptotic proteins

GAAPs (Golgi anti-apoptotic proteins) are an evolutionarily conserved group of anti-apoptotic proteins (Gubser et al., 2007). This protein was originally discovered from poxviruses, and subsequently orthologues were identified from mammals, insects, amphibia, and plants, including Arabidopsis (Gubser et al., 2007). Human (h-GAAP) and viral (v-GAAP) GAAPs share 73% identity at the amino acid level, and are thus far the only functionally characterized members of this gene family. Human and viral GAAPs have been shown to have anti-apoptotic properties against a variety of pro-apoptotic stimuli upon overexpression in human cell culture. The stimuli tested include inducers that are known to execute apoptosis through both the intrinsic (e.g. Bax and staurosporine) and extrinsic (e.g. Fas and tumor necrosis factor α) pathways. v-GAAP is not essential for the replication of the virus, but has been shown to have a role in the virulence of the virus in a murine infection model. h-GAAP, on the other hand, is essential for cell survival: knocking down the expression of h-GAAP using siRNA led to induction of cell death within 56 hours after transfection. Gubser et al. (2007) showed that v-GAAP can complement for the loss of h-GAAP, indicating a clear conservation of function within the human and viral proteins. h-GAAP is expressed throughout the human tissues tested. h- and v-GAAP proteins have been shown to localise mainly to the Golgi in stable human cell lines, and transient transfection assays. However, the protein also localised to the ER when expressed at higher levels. In stably transformed cell lines the protein can be detected throughout the membrane stacks of the Golgi complex (Gubser et al., 2007).

In order to unravel the mechanisms by which h-GAAP inhibits apoptosis, de Mattia et al. (2009) studied whether h-GAAP can affect Ca^{2+} signalling. Gubser et al., (2007) had previously shown that h-GAAP can inhibit apoptosis induced by staurosporine (STS), an agent that induces apoptosis through stimulation of Ca^{2+} influx across the PM. de Mattia et al. (2009) reported that both mitochondrial and cytosolic calcium concentrations increased in response to STS treatment. STS-induced increase in Ca2+
concentrations in both cytosol and mitochondria was reduced in cells that overexpressed h-GAAP. Knock-down of GAAP expression by RNAi led to much higher Ca\textsuperscript{2+} concentrations in both cellular locations upon STS-stimuli. In order to test whether GAAP also has an effect on release of Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} stores, the authors used histamine, an IP3 generating hormone that induces Ca\textsuperscript{2+} release from intracellular stores through IP3Rs. Overexpression of h-GAAP significantly reduced the histamine-induced rise in cytosolic and mitochondrial Ca\textsuperscript{2+} concentration whereas knock down of h-GAAP had the opposite effect. In order to establish whether the observed effects were due to free releasable Ca\textsuperscript{2+} concentrations in the intracellular stores, the amount of stored Ca\textsuperscript{2+} was studied in intact cells. It was shown that Ca2+ content in intracellular Ca\textsuperscript{2+} stores (both ER and Golgi) was reduced in cells overexpressing h-GAAP, and increased in h-GAAP downregulated cells (de Mattia et al., 2009).

As histamine-induced release of Ca\textsuperscript{2+} from the intracellular stores is largely governed by IP3Rs, de Mattia et al. (2009) postulated that h-GAAP might affect the function of these receptors. Coimmunoprecipitation experiments demonstrated that h-GAAP interacts with IP3Rs. However, whether this interaction is direct or indirect is yet to be determined. The Authors established the maximum level of Ca\textsuperscript{2+} that could be released from the Ca\textsuperscript{2+} stores upon IP3 stimulation (as opposed to the total amount releasable from the stores). They subsequently showed that the amount of Ca\textsuperscript{2+} released upon maximum IP3 stimulation was significantly reduced in h-GAAP overexpressing cells. The authors speculated that overexpression of h-GAAP could lead to reduction in the IP3-sensitive part of the releasable Ca\textsuperscript{2+} store. Alternatively, h-GAAP could decrease the ability of IP3Rs to release Ca\textsuperscript{2+} from the stores (de Mattia et al., 2009).

De Mattia et al.(2009) highlighted the role h-GAAP plays in regulating Ca\textsuperscript{2+} signalling between the intracellular Ca\textsuperscript{2+} stores, the cytosol and the mitochondria. The authors proposed that h-GAAP may exert its anti-apoptotic activity through regulation of intracellular Ca\textsuperscript{2+} fluxes.
1.4 Aims of the research

The aim of this research was to functionally characterize the GAAP protein family in Arabidopsis, consisting of five highly conserved paralogues, using a number of well established experimental approaches. This involved the generation, genetic and phenotypic analysis of single, double and higher order mutant combinations. Particular emphasis was placed on the analysis of processes that are known to involve forms of programmed cell death. Further approaches included gene expression analysis using available microarray data, RT-PCR and GAAP promoter-GUS reporter lines. Finally, subcellular localization of GAAPs in Arabidopsis and Nicotiana benthamiana was studied using confocal microscopy and GAAP-YFP/GFP fusion proteins.
Chapter 2: Materials and methods

2.1 Bioinformatics

Basic Local Alignment Search Tool (BLAST) searches were carried out to identify plant GAAP orthologues (Altschul & Lipman, 1990). Non-redundant protein sequence (nr) database was searched with protein-protein BLAST (blastp) using h-GAAP sequence as the query sequence. Following identification of plant GAAP sequences, a subset of these were subsequently used as query sequences in blastp searches. Amino acid sequences were aligned using ClustalW2 (Chenna et al., 2003) program with default settings (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Percentage identity and similarity of sequences were calculated using bl2seq BLAST program (Altschul & Lipman, 1990). Pfam (Finn et al., 2008) and Interpro (Quevillon et al., 2005) databases were used to determine putative conserved domains of GAAP proteins. The TMHMM Server (http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used to predict transmembrane topology. Subcellular localisation was predicted using WoLF PSORT (Horton et al., 2007), Subcellular Localisation using Local Alignments (SLP-L) (Matsuda et al., 2005) and ChloroP1.1 programs (Emanuelsson et al., 1999). Publicly available microarray gene expression sets were analysed using Genevestigator (Hruz et al., 2008).

2.2 General molecular techniques

2.2.1 Plant genomic DNA extraction

Genomic DNA was extracted by grinding Arabidopsis flowerheads with 600 μl Proteinase K Extraction Buffer (100 mM Tris HCl pH 8.0, 50 mM EDTA pH 8.0, 100 mM NaCl, 2% SDS; proteinase K added to final concentration of 0.1 mg/ml just before use) using an eppendorf pestle. Samples were shaken in a rotary shaker at 28°C for 30 min. Phenol Chloroform pH 8.0 (400 μl) was added, the sample was vortexed and subsequently shaken in a rotary shaker at 37°C for 10 min. The sample was spun for 5 min and the upper aqueous layer was removed to a clean microfuge tube. Five hundred μl of isopropanol and 50 μl 3M NaAc pH 5.2 was added and the sample was
mixed by inversion, followed by spin at 13,000 rpm for 2 min and resuspension of pellet in 500 μl TE pH 8.0 (10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8). Five hundred μl of ice cold ethanol and 50 μl 3M NaAc pH 5.2 was added, the sample was mixed by inversion and spun at 4°C, 13,000 rpm for 5 min. The supernatant was discarded, pellet was dried for 10 minutes and finally resuspended in 65μl TE pH 8.0. A dilution of 1/15 of this DNA stock was used as template for PCR.

2.2.2 Polymerase chain reaction (PCR)

PCR reactions were performed in 20 μl reaction using 2 μl (approximately 40 ng/μl) of plant genomic DNA as template, 0.2 μM forward and reverse primers, 200 μM dNTPs, 2.5 units of Taq DNA polymerase and 1x PCR buffer (50mM KCl, 10mM Tris-HCl, pH 8.0, 1.5 mM MgCl2, 0.01% (v/v) Triton X-100, 2 % (w/v) sucrose, 0.3 mM cresol red). PCR was performed in a PCT-200 thermocycler (MJ research) according to following conditions: 95°C for 5 minutes, 35 cycles (95°C for 30 sec, 56°C for 45 sec, 72°C, specific extension time (1 minute per kb)), 72°C for 5 minutes, 10°C forever.

2.2.3 Reverse transcriptase PCR (RT-PCR)

Total RNA was isolated using TRI REAGENT™ (Sigma) according to manufacturer’s instructions. RNA was quantified using a spectrophotometer (BioPhotometer, Eppendorf) to read the OD260 of a 1/100 dilution (1 OD260 = 40 μg/ml RNA). Two μg of RNA and an anchored oligo(dT) primer (Table 2-1) were used for cDNA synthesis using Superscript™ III Reverse Transcriptase (Invitrogen) as instructed. cDNA was used as template for PCR with gene specific primers (Table2-1). PCR (25 or 35 cycles) was carried out using Phusion™ High-Fidelity DNA Polymerase (Finnzymes) according to manufacturer’s instructions.
2.2.4 Agarose gel electrophoresis

Agarose gels (1% (w/v)) containing Sybr® Safe DNA gel stain (1 μl/50 ml of 10000x concentrate) were run at 100V for 1 hour in TBE buffer (Tris-Borate 45 mM, EDTA 1 mM pH 8.0). GeneRuler™ 1 Kb+ or 100 bp DNA ladders (Fermentas) were used to gauge molecular weight and concentration of samples. DNA was visualised on a Gel Doc-2000 (BioRad™) gel imager using a Safe Imager™ (Invitrogen) Blue light unit. Images were captured using PD Quest software (The Discovery Series, Version 7.31, Biorad Laboratories, Inc).

2.2.5 DNA sequencing

Sequencing reactions were performed in a 10 μl reaction containing 200 ng of DNA and 300 nM of primer using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Thermal cycling conditions as follows: 96 °C for 1 minute, 25 cycles (96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 min), 4 °C for 10 minutes. Samples were sequenced using an AbiPrism3730 capillary sequencer (John Innes Genome Laboratory).

2.3 Plant materials and growth conditions

Both wild-type (Columbia-0 and Landsberg erecta) and mutant Arabidopsis thaliana plants were grown in a controlled growth room under 16 h light/ 8 h dark (referred to as long day) or 10 h light/ 14 h dark (referred to as short day) cycle, approximately 60% humidity, light intensity of 130 μE m⁻² s⁻¹ and at a temperature of 23°C. Nicotiana benthamiana plants were grown under 16 h light/ 8 h dark cycle and under light intensity of 200 μE m⁻² s⁻¹, humidity and temperature as above. Soil-grown plants were sown on seed and modular compost plus sand (Levington) and germinated under short day conditions for two weeks after which plants were transferred to fresh soil. Plants were then placed under long day conditions to induce flowering when necessary.
2.4 Isolation of atgaap mutant plants

T-DNA or transposon-tagged insertion lines for AtGAAPs were identified using T-DNA Express database (www.signal.salk.edu). T3 segregating seeds of insertion lines were obtained from the Nottingham Arabidopsis Stock Centre (NASC; http://arabidopsis.info/). Homozygous plants were selected using PCR-based markers that can discriminate between the wild-type and mutant alleles using primer sets described in Table 2-1. Genomic DNA was used as template for the PCRs.

2.5 Seed sterilisation

Seeds were washed with 70% Ethanol for five minutes, 10% bleach/1%SDS for one minute, followed by seven one-minute washes with sterile water. Seeds were resuspended in 0.2% agarose and spread on ½ x Murashige and Skoog (MS) plates.

2.6 Generation of transgenic plants

2.6.1 Cloning and recombination procedure

PCR fragments were amplified by PCR using Phusion™ High-Fidelity DNA Polymerase (Finnzymes) using gene specific primers as shown in Table 2.1. Col-0 genomic DNA was extracted using DNeasy plant mini kit (Qiagen) and used as template for the PCR. PCR products were cloned into the pENTR™/D-TOPO® vector following manufacturer’s instructions (Invitrogen). Inserts were sequenced (see section 2.2.5) and recombined into Gateway destination vectors by LR recombination reaction using Gateway® LR Clonase™II Enzyme Mix (Invitrogen). pGWB3 destination vector was used to create promoter-GUS fusions. pGWB5 and pEG101 were used for cauliflower mosaic virus 35S promoter driven expression of AtGAAP, with C-terminal GFP or YFP-tag, respectively. pGWB vector series was a kind gift from Dr Tsuyoshi Nakagawa of Research Institute of Molecular Genetics (Matsue, Japan) (Nakagawa et al., 2009) and pEarleyGate vectors have been published by Earley et al. (2006). AtGAAP and empty vector constructs were transformed into Agrobacterium tumefaciens GV3101 cells by electroporation. Electroporation was
performed using a BIORAD Gene Pulser in a pre-chilled 2 mm cuvette according to conditions recommended by the manufacturer (Capacitance: 25 μFD, Resistance: 200 Ω, Voltage: 1.8 KV). Cells where then transferred to 1 ml of SOB media (2% Difco Bacto-tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM MgCl$_2$ and 10 mM MgSO$_4$) and incubated for 40 minutes at 28°C incubator. The transformed cells were selected on Luria Bertani plates supplemented with the following antibiotics: 50 μg/ml rifampicin, 50 μg/ml gentamycin, 50 μg/ml hygromycin and 50 μg/ml kanamycin for pGBW vectors; 50 μg/ml rifampicin, 50 μg/ml gentamycin, 50 μg/ml kanamycin for pEG vectors. Colony PCR was performed to confirm the presence of the insert with gene specific primers. Individual colonies were resuspended in 15 μl of sterile water and 2 μl of this was subsequently used as template in PCR reactions.

Table 2-1: Primers used in this study

<table>
<thead>
<tr>
<th>Amplified product</th>
<th>Forward primer (5' to 3')</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Detection of wild type and T-DNA mutant alleles for <em>AtGAAP</em>-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>AtGAAP1</em> wild type allele</td>
<td>CACCATGTATAAGTGGAATCTTCATTAC</td>
<td>GAGAACAATTTTGTCAAATATCTTG</td>
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<tr>
<td><em>AtGAAP2</em> wild type allele</td>
<td>GAC GAA AAA TGT GAA AAA AGT TGT TG</td>
<td>GAAGCAAGAAATTTTCATGCTCAAAAT</td>
</tr>
<tr>
<td><em>AtGAAP3</em> wild type allele</td>
<td>TTTCTTTGACTACATGAAAGTTGTCCT</td>
<td>GAATTGCAGCCTCCACAGAAAT</td>
</tr>
<tr>
<td><em>AtGAAP4</em> wild type allele</td>
<td>AGAAATGTCGACGATGATTG</td>
<td>GAAGAGAAGAATTTTCATGCTCAAAAT</td>
</tr>
<tr>
<td><em>AtGAAP5</em> wild type allele</td>
<td>GGGCGACAGGATGAAAATGGAATTG</td>
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</tr>
<tr>
<td><em>atgaap1</em> allele</td>
<td>TGGTTCACGTAGTGGGCCATCG</td>
<td>GAGAACAATTTTGTCAAATATCTTG</td>
</tr>
<tr>
<td><em>atgaap2</em> allele</td>
<td>GAC GAA AAA TGT GAA AAA AGT TGT TG</td>
<td>GAAGCAAGAAATTTTCATGCTCAAAAT</td>
</tr>
<tr>
<td><em>atgaap3</em> allele</td>
<td>GGGCGACAGGATGAAAATGGAATTG</td>
<td>GAATTGCAGCCTCCACAGAAAT</td>
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<td><em>atgaap4</em> allele</td>
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<td><em>atgaap5</em> allele</td>
<td>GGGCGACAGGATGAAAATGGAATTG</td>
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RT-PCR amplification of *AtGAAP*-5 cDNA

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<th>Reverse primer (5' to 3')</th>
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<tr>
<td><em>AtGAAP1</em> cDNA</td>
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<td><em>AtGAAP2</em> cDNA</td>
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<td><em>AtGAAP3</em> cDNA</td>
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<td><em>AtGAAP4</em> cDNA</td>
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<td><em>AtGAAP5</em> cDNA</td>
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<td><em>Actin8</em> cDNA</td>
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<td>GAGAACAATTTTGTCAAATATCTTG</td>
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</table>

1st strand cDNA synthesis (V represents mixture of C, G and A)

| Any mRNA with poly(A) tail | TTTTTTTTTTTTTTTTTTTTTTTTTTT |

Amplification of *AtGAAP*-5 genomic DNA excluding STOP codon

<table>
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<td><em>AtGAAP1</em> gDNA</td>
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<tr>
<td><em>AtGAAP2</em> gDNA</td>
<td>CACCATGTATAAGTGGAATCTTCATTAC</td>
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<td>CTCGGGACCTGCTGGAAATGGA</td>
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<tr>
<td><em>AtGAAP5</em> gDNA</td>
<td>CACCATGTATAAGTGGAATCTTCATTAC</td>
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Amplification of *AtGAAP*-5 and *BNF1* promoter regions

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<tr>
<td><em>AtGAAP2</em> promoter</td>
<td>CACCGGTACCTCGTAAGAAAATGGAATTG</td>
<td>CTCGGGACCTGCTGGAAATGGA</td>
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<tr>
<td><em>AtGAAP3</em> promoter</td>
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<td>CTCGGGACCTGCTGGAAATGGA</td>
</tr>
<tr>
<td><em>AtGAAP4</em> promoter</td>
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<td><em>AtGAAP5</em> promoter</td>
<td>CACCGGTACCTCGTAAGAAAATGGAATTG</td>
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</table>

*BFN1* promoter

<table>
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<tr>
<td><em>BFN1</em> promoter</td>
<td>CACCGGTACCTCGTAAGAAAATGGAATTG</td>
<td>CTCGGGACCTGCTGGAAATGGA</td>
</tr>
</tbody>
</table>
2.6.2 Agrobacterium competent cells

Agrobacterium GV3101 strain was streaked on an L (1% Difco Bacto-tryptone, 0.5% Yeast Extract, 0.5% NaCl, 0.1% D-glucose, 1.5% bacto-agar) plate supplemented with 50 μg/ml Rifampicin and 50 μg/ml gentamycin and grown two days at 28°C. Two single colonies were picked and grown overnight in 5 ml L medium antibiotics at 28°C shaking at 300 rpm. This starter culture was diluted 1:1000 into 5 x 100 ml L with tetracycline in baffled 250 ml Erlenmeyer flasks. The cultures were grown overnight at 28°C while shaking at 300 rpm until the OD600 of 0.5-0.7 was reached, and then cooled on ice. All subsequent steps were performed on ice using pre-chilled equipment and reagents. The cells were pelleted in 10 x 50 ml sterile cold conical centrifuge tubes (BD biosciences) for 20 minutes at 4000 rpm in a Mistral centrifuge (Sigma/Qiagen) at 4°C. The supernatant was discarded, and the pellet was resuspended fully in 10 ml ice-cold sterile 10% (v/v) glycerol (C$_3$H$_8$O$_3$). The volume was then increased to 50 ml (Vtot = 500 ml) and spun as previously apart from the reduced centrifugation time of 10 minutes. Wash step was repeated twice, but the pellets were pooled into five centrifuge tubes and washed in a total volume of 250 ml. For a final wash, the pellets were pooled into two centrifuge tubes and washed in a total volume of 100 ml. The cells were pelleted by centrifugation and the pellet was resuspended in approximately 2 ml ice-cold 10% (v/v) glycerol to yield a final OD600 of 70. Cells were kept on ice and used immediately for transformation or dispensed into 20 μl aliquots and snap frozen in liquid nitrogen. The aliquots were stored in -80°C freezer.

2.6.3 Arabidopsis transformation and selection of transgenic lines

Binary vectors were transformed into Arabidopsis plants by floral dip (Clough and Bent, 1998). pGWB3 and pGWB5 transgenic seeds were selected on ½ x Murashige and Skoog (MS) plates containing 50 μg/ml hygromycin B. pEG101 seeds were grown on soil for seven days and screened for Basta (glufosinate-ammonium) resistance. Agrobacterium GV3101-PM90RK with pGIF2(HindIII)-35S-Aq construct carrying the apoaequorin gene was a kind gift from Prof Marc Knight (Durham...
University, Durham, UK). pGIF-aequorin transgenic seeds were selected on $\frac{1}{2}$ x Murashige and Skoog (MS) plates containing 9 μg/ml Hygromycin B.

2.7 **GUS staining**

Plant tissue was immersed in GUS staining solution: 1 mM X-Gluc, 0.1% (w/v) Triton X-100, 0.5 mM K$_3$Fe(CN)$_6$ (ferricyanide), 0.5 mM K$_4$Fe(CN)$_6$·3H$_2$O (ferrocyanide), 10 mM Na$_2$EDTA, 50 mM PO$_4$ buffer pH 7.0. Tissue was infiltrated twice for two minutes in a vacuum chamber before placing samples in 37°C for 16 hours. GUS solution was then removed and tissue was washed twice with 70% Ethanol.

2.8 **Plant stress treatments and analysis of phenotypes**

2.8.1 *Pseudomonas syringae pv tomato DC3000 (avrRpm1) infection*

*Pseudomonas syringae pv tomato DC3000* expressing avirulence gene avrRpm1 was grown overnight at 28°C in 5ml of Luria Bertani (LB) media containing 40 μg/ml Rifampicin. Bacterial culture was spun down and resuspended in 10 mM MgCl$_2$. Final cell density was adjusted to 1x10$^7$ cfu/ml (equivalent of OD$_{600}$ = 0.02). Five-week old plants were syringe inoculated on the abaxial side of the leaf using a needleless 1 ml syringe. Control plants were inoculated with 10 mM MgCl$_2$. Progression of HR was monitored at 12 h intervals for 48 hours by quantification of electrolyte leakage and by staining leaves with lactophenol trypan blue at 12 and 24 hours as detailed below.

2.8.1.1 **Electrolyte leakage**

Electrolyte leakage from dead and dying cells was measured as described (Kwon et al., 2003). After inoculation plants were left to take up the inoculum for two hours. Six 0.5 cm$^2$ leaf discs per plant (2 leaf discs per leaf) were cut using a cork borer. Disks were placed in 90 mm Petri dishes containing 25 ml of purified water and shaken for
two hours to wash off excess inoculum. Leaf disks were transferred to 50 mm Petri dishes containing 6 ml of fresh purified water. Conductivity of the solution was measured using inoLab (Wissenschaftlich-Technische Werkstätten GmbH) conductivity meter. In the end of the experiment the solution and leaf discs were transferred to 15 mL Falcon tubes and boiled for 15 minutes. After cooling, the conductivity of samples was measured. This value represents 100 % electrolyte leakage and percentage of electrolyte leakage for each time point was determined using this value.

### 2.8.1.2 Lactophenol trypan blue staining

Visualisation of dead and dying cells was performed as described (Koch and Slusarenko, 1990). Leaves were collected and immersed in staining solution (25% v/v of each H$_2$O, lactic acid, phenol and glycerol plus 0.025% w/v trypan blue) and boiled for 5 minutes. Saining solution, once cooled down, was replaced by destaining solution (250% w/v chloral hydrate). Samples were shaken for 7 days until leaves were cleared. Leaves were mounted in 60% glycerol and viewed with Leica light microscope (MZ16F, Leica Microsystems).

### 2.8.2 Heat stress

Heat shock treatment was performed as described by Watanabe and Lam (2006) with slight modifications. Four-week old soil-grown plants were incubated in a hybridisation oven at 65°C for 20 minutes. Plants were returned to standard growth chamber. Progression of cell death was followed by eye and images were taken three days post-treatment.

### 2.8.3 Dark-induced senescence

Rosette leaves 5, 6, 7 and 8 were detached from 4-week old soil-grown plants and placed in Petri dish containing moist filter paper (Sartorius). Petri dishes were wrapped in foil for dark treatment or left unwrapped for control treatment and placed
in standard plant growth room. Development of senescence was analysed by observation of visible yellowing of the leaves. Images were captured 4 and 7 days after treatment.

2.8.4 Drought stress

Plants were grown on soil with sufficient water for seven weeks and were then subjected to water withholding for 10 days. Plants were observed on a daily basis and images were captured after 10 days of drought treatment.

2.9 Calcium measurements

All calcium measurements were carried out by the author in the laboratory of Professor Marc Knight at Durham University.

2.9.1 In vitro reconstitution of aequorin

Seeds were surface sterilised and grown on 1 x MS media. Six 7-day old seedlings were placed in 1.5 ml microfuge tubes and snap frozen in liquid nitrogen. Tissue was ground to powder using an eppendorf pestle. One hundred μl of freshly prepared chilled medium (0.5 M NaCl, 5 mM EDTA, 10 mM Tris-HCl pH 7.4, 5 mM mercaptoethanol, 0.1% (w/v) gelatin) was added. Tissue was homogenised further using a motorised micropestle. Further 0.4 ml of medium was added, samples were mixed and subsequently spun at 13 000 g for 10 min. One hundred μl of supernatant was placed in a fresh microfuge tube to which 1 μl of 100 μM coelenterazine (ProLume) was added. Samples were mixed and incubated in the dark at room temperature overnight. Samples were diluted 10-fold with 0.5 ml Tris-EDTA buffer (200 mM Tris-HCl, 0.5 mM EDTA pH 7.0) in a plastic luminometer cuvette (Sarstedt). The cuvette was placed into the luminometer chamber and luminescence counts were recorded to obtain sample background. Half a ml of 50 mM CaCl₂ was added from a syringe through a light-tight luminometer port and measurements were
continued for a further 10 seconds. Background was subtracted to give luminescence counts for each sample.

2.9.2 In vivo reconstitution of Aequorin

Eight- to ten–day old seedlings were floated in 3ml of water (approximately 25/well in 12-well plates). Coelenterazine was added to give a final concentration of 2.5 μM and samples were swirled gently and placed in the dark in standard growth chamber overnight.

2.9.3 Experimental measurements

Room lights were dimmed for the duration of the experiment. Freshly reconstituted individual seedling was gently placed in the bottom of a luminometer cuvette containing water. Seedling was allowed to settle for approximately 5 minutes in the dark. The cuvette was placed in the luminometer and luminescence counts were started. Readings were taken every second. After 10 seconds of counting, the desired solution was injected into the cuvette using a syringe with a long needle. Luminescence counts were continued for a further 3 minutes. Remaining aequorin was discharged with an equal volume of 2M CaCl₂, 20% ethanol at the end of the experiment in order to calibrate the results.

Seedlings used for H₂O₂, cyclopiazonic acid (CPA) and histamine treatments were placed in luminometer cuvettes containing 0.5 ml water. Subsequently, 0.5 ml of double strength solution was added to get the following final concentrations: 3mM for H₂O₂, 10 μM for CPA and 100 μm for histamine. Seedlings used for mannitol treatment were floated in 0.25 ml of water before injecting 0.5 ml of 0.666M mannitol to get a final concentration of 0.444M.
2.9.4 Luminometry and calibration of calcium measurements

\( \text{Ca}^{2+} \) measurements by luminometry were performed using a digital chemiluminimeter with discriminator (Electron Tubes), fitted with a cooling unit for the photomultiplier tube. Luminescence counts were measured at 1 sec intervals. The luminescence counts obtained were calibrated by applying the following equation:

\[
p\text{Ca}=0.332588(-\log k)+5.5593, \quad \text{where} \quad k = \frac{\text{luminescence counts s}^{-1}}{\text{total luminescence counts remaining}}.
\]

This equation takes into account the double logarithmic relationship between the concentration of free calcium present in the cells containing reconstituted aequorin and the remaining aequorin discharged at any point in time (Fricker, 1999). The equation has been empirically determined (Knight et al., 1996).

2.10 Subcellular localisation

2.10.1 Expression of fusion proteins in *Nicotiana benthamiana*

Fluorescent fusion proteins were transiently expressed in *Nicotiana benthamina* leaf tissue using Agrobacterium-mediated infiltration. Agrobacterium cells were grown in LB media overnight. Cultures were diluted ten-fold into fresh LB and grown for another 24 hours. Samples were centrifuged, pellet was washed once in infiltration media (10 mM MES pH 5.6, 10 mM MgCl\(_2\), 200 \(\mu\)M acetosyringone) and resuspended to a final OD600 of 0.5. Six-week old *N. benthamiana* plants were inoculated with a 1 ml needleless syringe. Leaf discs were viewed under confocal microscope 1-4 days post-inoculation.

AtGAAP-GFP/YFP fusion constructs were expressed alone, or coexpressed with organelle markers generated by Nelson et al (2007). For co-localisation studies bacterial suspensions were mixed in 1:1 ratio before infiltration. Organelle markers used in this study and the corresponding Arabidopsis Biological Resource Centre (ABRC) stock numbers as follows: Golgi-CFP (CD3-962), Golgi-YFP (CD3-966), ER-YFP (CD3-958), Tonoplast-CFP (CD3-970), Tonoplast-YFP (CD3-974), peroxisome-CFP (CD3-978), Mitochondria-CFP (CD3-986), Plastid-YFP (CD3-994), Plasma membrane-YFP (CD3-1006). Markers were ordered from ABRC (http://abrc.osu.edu/).
2.10.2 Expression of fusion proteins in transgenic Arabidopsis lines

Transgenic lines were generated as detailed in section 2.6.1. Expression levels of AtGAAP-YFP fusion proteins were analysed by viewing detached rosette leaves under UV with YFP filter set (Leica10447225) using a stereofluorescence microscope (Leica MZ16F). Images were captured with Leica camera (DFC300FX) and Leica application suite (Version2.7.1 R1) software using identical settings for each sample. Fluorescence levels were quantified using Image Processing and Analysis in Java software (ImageJ; http://rsb.info.nih.gov/ij/).

2.10.3 Confocal microscopy

Leaf discs were mounted on glass slides with water. GFP, YFP, and CFP fluorescence was observed with Leica DMIRE2 confocal microscope after excitation at 488nm, 514nm and 458nm, respectively. Pictures were captured and analysed using Leica confocal software (Version 2.61; Leica Microsystems). All co-localisation images were taken with sequencial scanning mode.
Chapter 3: Phylogenetic and gene expression analysis of AtGAAP gene family

3.1 INTRODUCTION

GAAPs are novel anti-apoptotic proteins, first described by Gubser et al. (2007). GAAPs represent a family of genes that are evolutionarily well conserved. Orthologues sequences have been identified from viruses, mammals, plants, insects and amphibians. Human and viral GAAPs (h- and v-GAAP) are thus far the only characterised members of this family. They exert a cytoprotective role against a number of apoptotic stimuli. GAAP proteins are well conserved in length and sequence identity, with human and viral GAAPs sharing 73% amino acid sequence identity. These proteins are predicted to have 6 or 7 transmembrane domains and have been experimentally shown to localise to the Golgi. Human GAAP is expressed in most human tissues (Gubser et al. (2007). Human and viral GAAPs belong to the uncharacterised protein family UPF0005. GAAPs share sequence similarity with two characterised members of this family: Bax-inhibitor-1 (BI-1), and lifeguard (LFG), both of which protect cells against apoptotic stimuli. BI-1 inhibits apoptosis triggered primarily by ER stress and LFG protects cells against Fas-receptor mediated apoptosis (Chae et al., 2004; Somia et al., 1999; Xu & Reed, 1998). Interestingly, BI-1 and LFG are also evolutionarily conserved, with orthologous genes present in the Arabidopsis genome (Chae et al., 2003; Reimers et al., 2006).

Gene duplication has been extensive in plant genomes. 65% of Arabidopsis genes are part of multigene families, compared to 38% for humans, and 30 % for yeast (Saccharomyces cerevisiae) (Zhang, 2003). Duplicated genes may have a number of fates. Paralogous genes are unlikely to maintain the same function unless it is advantageous to have excess amounts of the gene product (Nowak et al., 1997). One of the copies may become a pseudogene, which is either silenced, unexpressed or functionless (pseudogenisation). Functions of the parental gene may become divided among the daughter copies (subfunctionalisation). This can occur at a functional level, whereby one of each daughter gene specializes to carry out a particular function of the
parental gene. Subfunctionalisation can also be achieved by differential spatial or temporal expression of the genes. Division of gene expression is considered to be a general rule rather than exception and is thought to happen quickly after duplication events (Gu et al., 2002; Wagner, 2000). One member of a duplicate gene pair may also acquire a novel function through a process termed neofunctionalisation (Zhang, 2003).

Since the completion of the Arabidopsis genome sequencing (The Arabidopsis Genome Initiative, 2000), genome scale analysis of gene expression has become possible using microarrays. A wealth of gene expression data has become publicly available, and expression data analysis tools, such as Genevestigator, have become routine and powerful research tools in the Arabidopsis research community (Zimmermann et al., 2004). With regard to Arabidopsis gene families, expression data can be used to make predictions whether duplicated genes have gone through subfunctionalisation leading to divergent expression patterns. For example, Chong et al. (2010) used Genevestigator to carry out expression profiling of the Arabidopsis exocyst complex gene families. Despite the availability of microarray data, gene expression analysis using transgenic plants containing promoter-β-glucuronidase (GUS) gene fusions and RT-PCR analysis have remained widely used methods for analysis of gene expression patterns in Arabidopsis. Tissue-specific expression patterns of a number of multigene families have been established using these methods. This information can be used to make predictions of specific roles these genes may play in plant development (Foster et al., 2008; Takahashi et al., 2004).

Gubser et al. (2007) reported that a GAAP paralogue showing 38% sequence similarity to human GAAP exists in the Arabidopsis genome. In order to discover whether the GAAP gene family is conserved more widely in the plant kingdom, a phylogenetic analysis was carried out. A number of bioinformatics tools were used to make predictions of transmembrane topology, conserved domains and subcellular localisation of plant GAAPs. This information was used to make predictions about the possible conservation of function between plant GAAPs and their human and viral counterparts. Finally, a comprehensive gene expression analysis of Arabidopsis GAAP gene family was carried out using publicly available microarray data, RT-PCR and promoter-GUS fusions. Gene expression data was used to make predictions of the
roles these genes may play in plant development and to address the issue of redundancy.

3.2 Results

3.2.1 Phylogenetic analysis of GAAPs

Gubser et al. (2007) showed that GAAPs are highly conserved in evolution with closely related proteins in mammals, plants, insects, amphibia, and viruses. We carried out BLAST searches to identify GAAP orthologues within the plant kingdom. GAAP orthologues were identified from dicots, monocots and the lower plants, including Arabidopsis, rice (Oryza sativa), maize (Zea mays), soybean (Glycine max), rapeseed (Brassica napus), sorghum (Sorghum bicolor), yellow lantern chilli (Capsicum chinense), castor oil plant (Ricinus communis), grape vine (Vitis vinifera), poplar (Populus trichocarpa), Sitka spruce (Picea sitchensis) and moss (Physcomitrella patens and Selaginella moellendorffii). There is an apparent expansion of the GAAP gene family in several plant species; e.g. analysis of the fully sequenced and annotated genomes of Arabidopsis and rice reveal the presence of five and seven putative GAAPs, respectively (Table 3-1; Figure 3-1). Arabidopsis and rice GAAPs were designated as AtGAAP1-5 and OsGAAP1-7, respectively (Table 3-1).

<table>
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Table 3-1: Designation of Arabidopsis and rice GAAP homologues

Designation, locus, GenBank accessions references and size in amino acids as indicated for AtGAAPs and OsGAAPs (http://www.ncbi.nlm.nih.gov)
Figure 3-1: Phylogenetic analysis of GAAP and BI-1 proteins
Human GAAP (h-GAAP), viral GAAP (v-GAAP), Arabidopsis GAAPs (AtGAAP1-5), rice GAAPs (OsGAAP1-7), human Bax inhibitor-1 (hBI-1) and Arabidopsis Bax-inhibitor-1 amino acid sequences were aligned using ClustalW2 (Chenna et al., 2003).

Arabidopsis GAAPs are highly conserved in length and sequence identity (Table 3-1 and Figure 3-2). Arabidopsis GAAP paralogues show between 82 and 48% amino acid sequence identity with each other. Sequence alignment and phylogenetic analysis classify AtGAAP1, AtGAAP2 and AtGAAP3 into a distinct clade with 76-82% sequence identity within the group. AtGAAP4 and AtGAAP5 are distinct from this group, and from each other. AtGAAP4 shares the highest level of identity with h-GAAP (40%), followed by AtGAAP5 (37%), AtGAAP2 (36%), and AtGAAP1 and AtGAAP3 (34%) (Figure 3-2). The length of the protein is also evolutionarily well conserved: h-GAAP and v-GAAP consist of 238 and 237 aminoacids, respectively, and the length of AtGAAPs is between 235 and 256 amino acids (Table 3-1).
Figure 3-2: Comparison of deduced amino acid sequences of human GAAP, viral GAAP and Arabidopsis thaliana GAAP homologues

(A) ClustalW (Chenna et al., 2003) alignment of human GAAP (h-GAAP), viral GAAP (v-GAAP) and Arabidopsis (AtGAAP1-5). Identical residues are shown in black.

(B) Amino acid sequence identity and similarity between Arabidopsis thaliana (AtGAAP1-5), human (h-GAAP) and viral (v-GAAP) GAAPs. Percentage identity and similarity of sequences were calculated using Basic Local Alignment Search Tool (BLAST) (Altschul & Lipman, 1990)
Arabidopsis GAAPs also share high sequence identity with their rice counterparts. AtGAAP1-3 share between 64 and 73\% sequence identity with OsGAAP1-3 that form a distinct phylogenetic cluster. AtGAAP4 shares highest (61\%) sequence identity with OsGAAP4, whereas OsGAAP5-7 are more distantly related to AtGAAPs on the basis of sequence identity (Figure 3-1).

Human GAAP has been shown to share sequence similarity to Bax inhibitor-1 (Gubser et al., 2007). We found sequence conservation between AtGAAPs and Arabidopsis Bax inhibitor-1, with 25 \% sequence identity between AtGAAP4 and AtBI-1.

### 3.2.2 Predicted transmembrane topology, conserved domains and subcellular localisation of GAAP proteins

Pfam (Finn et al., 2008) and Interpro (Quevillon et al., 2005) databases were used to determine putative conserved domains of GAAP proteins. Both programs assigned all AtGAAPs and OsGAAPs to the protein family UPF0005, known to contain a subset of Bax-inhibitor-1 proteins. Characterised mammalian members of this protein family that have been shown to have anti-apoptotic function include human and viral GAAPs (Gubser et al., 2007), Bax inhibitor-1 (Chae et al., 2004; Xu & Reed, 1997) and Lifeguard (Somia et al., 1999). Three Arabidopsis genes have been assigned to this family in addition to AtGAAPs: At5g47120 (AtBI-1), At4g17580 and At5g47130. AtBI-1 has been characterized and shown to inhibit Bax-induced cell death in yeast (Sanchez et al., 2000). At4g17580 and At5g47130 genes code for proteins that share 59 and 56 \% sequence identity with AtBI-1. Altogether eight rice genes have been assigned to this family; OsBI-1 and OsGAAPs. OsBI-1 has been shown to function as an inhibitor of Bax-induced cell death in yeast (Kawai et al., 1999). Human, Arabidopsis and rice BI-1 proteins contain a Bax inhibitor-1 family signature motif (Prosite accession number PS01243). This signature motif was not found to be conserved in GAAP proteins. The InterProScan program additionally assigned all AtGAAPs and OsGAAPs to the PTHR23291:SF9 protein family. This family is assigned as Glutamate-/aspartate-binding peptide-related family. The only proteins assigned to this family are AtGAAPs and OsGAAPs.
Human and viral GAAPs have previously been predicted to have six or seven transmembrane domains (Gubser et al., 2007). The TMHMM Server (http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used in order to study the transmembrane topology of AtGAAPs and OsGAAPs. All Arabidopsis and rice GAAPs are predicted to have a seven transmembrane topology (Table 3-2).

Human and viral GAAPs have been shown to localise to the Golgi (Gubser et al., 2007). Three different prediction programs were used to study possible subcellular localisation of AtGAAPs and OsGAAPs (Table 3-2). Predictions of the localisation of GAAP proteins created by the different software were contradictory. Furthermore, the Subcellular Localisation using Local Alignments (SLP-L) (Matsuda et al., 2005) program generated very low reliability scores for the predicted subcellular localisation. Similarly, WoLF PSORT (Horton et al., 2007) reported low observed frequency values for all GAAPs. Also, SLP-L, WoLF PSORT, and ChloroP1.1 (Emanuelsson et al., 1999) predicted AtBI-1 to be localised to the PM, nucleus or cytosol, or chloroplast, respectively. It has been experimentally shown that AtBI-1 localises to the ER in plant cells. These results highlight the importance of confirming subcellular localisation of AtGAAPs experimentally.

Table 3-2: Subcellular localisation and transmembrane topology of AtGAAP, OsGAAP and AtBI-1 proteins as predicted by bioinformatics software

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<th>Protein</th>
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(A) Localisation predicted using WoLF PSORT (Horton et al., 2007)
(B) Localisation predicted using Subcellular Localisation using Local Alignments (SLP-L) (Matsuda et al., 2005) program. Reliability index (RI) is indicated in brackets. RI ranges from 1 to 10. As the RI increases, the prediction becomes more reliable.
(C) ChloroP1.1 program (Emanuelsson et al., 1999) predicts the presence or absence of chloroplast transit peptides (cTP)
(D) Number of transmembrane (TM) domains, as predicted by TMHMM Server (http://www.cbs.dtu.dk/services/TMHMM-2.0/)
3.2.3 *AtGAAP* gene expression analysis using Genevestigator

In order to gain insight into the function and possible redundancy in the *AtGAAP* gene family, gene expression analysis was carried out. *AtGAAPs* are included in the Arabidopsis whole genome Affymetrix probe array (Affymetrix ATH1 GeneChip® probe array) and hence a wealth of expression data exists for these genes (Redman et al., 2004). Publicly available microarray gene expression sets were analysed using Genevestigator, a microarray database coupled with expression-data analysis tools (Hruz et al., 2008; Zimmermann et al., 2004). *AtGAAP* gene expression in different organs and developmental stages was studied using the Genevestigator online meta-profile analysis tool.

The Genevestigator Anatomy search tool was used to look at the expression levels of *AtGAAPs* in different organs or tissues of the plant (Figure 3-3A). *AtGAAP4* showed strong and fairly uniform expression throughout the plant. *AtGAAP4* transcript abundance was similar in root, rosette and inflorescence tissue, however, *AtGAAP4* expression showed a marked increase in pollen. *AtGAAP2* was also expressed throughout all plant tissues (Figure 3-3A). *AtGAAP2* expression was highest in root tissue, followed by rosettes and with lower expression in inflorescence tissue. *AtGAAP1* shows a low level of expression in all tissues apart from pollen, where expression is comparatively high, and to a lesser extent in stamen and root endodermis (Figure 3-3A). *AtGAAP1* was very highly expressed in sperm cells in cell culture (Figure 3-3C). Microarray data indicated *AtGAAP3* expression to be almost exclusively seed specific, with signal intensity for other tissues very low or below background levels (Figure 3-3B). *AtGAAP5* expression was detected in the seed coat, siliqua and carpel. *AtGAAP5* was very weakly expressed in all tissues and in many experiments the signal was not above the background noise (Figure 3-3B).
Figure 3-3A: AtGAAP1, AtGAAP2 and AtGAAP4 gene expression in plant organs and tissues. AtGAAP expression pattern was obtained by searching results compiled from Arabidopsis microarray experiments using Genevestigator Anatomy tool. For full description of the figure, see page 60.
Figure 3-3B: AtGAAP3 and AtGAAP5 gene expression in plant organs and tissues

AtGAAP expression pattern was obtained by searching results compiled from Arabidopsis microarray experiments using Genevestigator Anatomy tool. For full description of the figure, see page 60.
Figure 3-3: AtGAAP gene expression in plant organs and tissues and cell culture

AtGAAP expression pattern was obtained by searching results compiled from Arabidopsis microarray experiments using Genevestigator Anatomy tool (Hruz et al., 2008; Zimmermann et al., 2004). Note that main categories contain all chips hybridized with RNA extracted from the entire organ and from individual or a combination of specific parts of the particular organ shown as indented subcategories. Numbers on the right of the image indicates the number of chips used for the particular category. Mean signal intensity is indicated by a dot and standard error of the mean by error bars.

(A) AtGAAP gene expression in plant organs and tissues. AtGAAP1, AtGAAP2 and AtGAAP4 expression patterns as indicated

(B) AtGAAP gene expression in plant organs and tissues. AtGAAP3 and AtGAAP5 expression patterns as indicated

(C) AtGAAP gene expression in plant cell culture. AtGAAP1, AtGAAP2, AtGAAP3, AtGAAP4 and AtGAAP5 expression patterns as indicated

Genevestigator Development tool was used to look at the expression of AtGAAPs at different growth stages from germination to plant senescence. AtGAAP2 and AtGAAP4 were expressed throughout all growth stages (Figure 3-4). AtGAAP4 showed a slightly enhanced expression during and after the emergence of flower buds. AtGAAP1 is expressed at low levels throughout the development of the plant with a significant increase in expression at late stages where flowering is complete (Figure 3-4). AtGAAP3 and AtGAAP5 expression levels are near or below the background levels for nearly all growth stage categories (Figure 3-4). AtGAAP3 shows a significant peak in expression at the very last stage of development before senescence of the plant and the very first stages of seed germination until the cotyledons are fully opened. This expression pattern clearly correlates with the results from Anatomy tool in that AtGAAP3 is almost exclusively expressed in the seed of the plants.
AtGAAP gene expression in different growth stages of the plant

AtGAAP expression pattern was obtained by searching results compiled from Arabidopsis microarray experiments using Genevestigator Development tool (Hruz et al., 2008; Zimmermann et al., 2004). The stages of plant development are based on age from seed germination to plant senescence according to the Boyes standard (Boyes et al., 2001). Each category contains all arrays from the corresponding developmental stages, including all organs available at that stage. Numbers at the bottom of the image indicates the number of chips used for the particular category. AtGAAP1, AtGAAP2, AtGAAP3, AtGAAP4 and AtGAAP5 expression patterns as indicated.

3.2.4 AtGAAP gene expression analysis using RT-PCR

Expression levels of AtGAAP genes in leaf and inflorescence tissue were analyzed using semi-quantitative RT-PCR in order to verify the results from Genevestigator analysis. Leaf and flower material was collected from 5-week old Arabidopsis plants of ecotypes Colombia-0 (Col-0) and Landsberg erecta (Ler). The level of transcript for ACT8 (At1g49240) was used as a control. AtGAAP2 and AtGAAP4 showed a high level of expression in both leaf and flower tissue (Figure 3-5, upper panel). No differences between the levels of AtGAAP2 and AtGAAP4 expression between the leaf
and flower tissue were seen using 35 PCR cycles so the experiment was repeated with 25 cycles (Figure 3-5, lower panel). Results indicated that AtGAAP4 is the most highly expressed of the AtGAAP genes and expression of AtGAAP4 is higher in inflorescence tissue than in leaf tissue. Approximately equal amounts of AtGAAP2 transcript were present in the leaf and inflorescence tissue. Transcript levels of AtGAAP1, AtGAAP3 and AtGAAP5 were significantly lower than those of AtGAAP2 and AtGAAP4 (Figure 3-5, upper panel). Also, the transcript levels of AtGAAP1, AtGAAP3, and AtGAAP5 were significantly higher in inflorescence tissue compared to leaf tissue. AtGAAP5 expression was higher than AtGAAP1 and AtGAAP3 in inflorescence tissue but undetectable in leaf tissue. However, the AtGAAP5 transcript could be detected in leaf tissue if a significantly higher amount of cDNA is used as template for PCR (data not shown). AtGAAP1 and AtGAAP3 were similarly expressed in flower tissue but in leaf material the abundance of AtGAAP1 transcript was slightly higher than AtGAAP3. The pattern of AtGAAP gene expression in ecotype Landsberg erecta was similar to that observed for Col-0, although the transcripts of AtGAAP1 and AtGAAP3 were undetectable in leaf tissue under the conditions used (data not shown).

![Figure 3-5: RT-PCR analysis of AtGAAP expression](image)

RT-PCR analysis of AtGAAP1, AtGAAP2, AtGAAP3, AtGAAP4 and AtGAAP5 expression in 5-week old wild type Col-0 inflorescence and leaf tissue. ACT8 expression provided a control for RT-PCR. PCR was carried out for 35 (upper panel) or 25 (lower panel) cycles.  

a Primer pairs used were specific for a particular AtGAAP paralogue and Actin 8 (ACT8).  
b Total RNA was extracted from either leaf or inflorescence tissue of wild type Colombia-0 plants. I, Inflorescence tissue; L, leaf tissue.
3.2.5 *AtGAAP* gene expression analysis using promoter-GUS fusions

In order to study tissue specificity of *AtGAAP* expression, promoter-GUS fusion constructs were generated. Approximately 1800bp genomic (promoter) region 5’ upstream and up to the start codon of *AtGAAPs* was cloned and fused upstream of the coding sequence of the GUS reporter gene in pGWB3 vector (Nakagawa *et al*., 2009; see section 2.6.1). Promoter region of *BFN1* (Bifunctional nuclease I) was also cloned into this vector for use as a positive control (Farage-Barhom *et al*., 2008). Six transgenic lines containing the promoter-GUS constructs each from two independent transformation experiments were selected for further analysis. Although some variation in the intensity and tissue specificity of GUS staining was observed between lines containing a particular promoter-GUS construct, we were able to establish a typical expression pattern for each *AtGAAP*.

Stable transgenic lines containing *BFN1* promoter fused to GUS and pGWB3 empty vector constructs were analysed in order to establish that the vector and the GUS histochemical staining method used provided reliable data. No GUS staining was observed in any of the transgenic lines containing the pGWB3 empty vector construct (Figure 3-6D-F). *BFN1* promoter expression was observed in senescing leaf tissue, floral organs including anthers, stigma, petals, sepals and stamen, and septum of mature siliques as reported previously (Figure3-6A-C) (Farage-Barhom *et al*., 2008).

Figure 3-6: Histochemical analysis of GUS expression in *BFN1* promoter-GUS and pGWB3 transgenic plants
Inflorescence tissue (A,D), rosette leaves (B,E) and siliques (C,F) of 6- week old Arabidopsis plants expressing the promoter-GUS fusions were subjected to histochemical staining for GUS activity.
(A-C) GUS reporter gene under the control of BFN1 promoter region.
(D-F) pGWB3 empty vector construct containing the gateway cassette and GUS protein.
3.2.5.1 Pattern of AtGAAP expression in inflorescence tissue

In order to observe organ specific expression patterns of AtGAAP genes in floral tissue, histochemical staining of inflorescence tissue of the transgenic lines containing AtGAAP promoter-GUS fusions was performed. A similar pattern of GUS staining was observed in AtGAAP1, AtGAAP2 and AtGAAP4 promoter-GUS transgenic lines. GUS staining was observed in sepals, petals, pistil, and stamen (Figure 3-7). Within the stamen, both anther and the filament showed GUS staining (Figure 3-7; K, L, N). Anthers were crushed in order to study GUS staining of individual pollen grains. Pollen grains of all transgenics showed clear GUS staining (Figure 3-7; P, Q, S). Within the pistil, strong GUS staining was observed in the stigma and the top (or neck) of the style (Figure 3-7; U, V, X, Z, AA, AC). Although weak GUS activity could also be observed in ovules within the ovary, this staining was not consistent throughout the analysed transgenic lines. The AtGAAP4 promoter also directed weak GUS activity in the petal and sepal abscission zones (AZ; Figure 3-7AH).

Although some overlap in the expression patterns was observed, AtGAAP5 expression was generally quite distinct from that observed for AtGAAP1, AtGAAP2 and AtGAAP4. AtGAAP5 showed the strongest expression in ovules and petal and sepal AZs (Figure 3-7; Y, AD, AI). Within the stamen, only the very tip of the filament showed GUS activity (Figure 3-7O). No GUS activity was observed in the anther or in the pollen grains within the anther (Figure 3-7T). In addition to the ovules, AtGAAP5 expression was detected in the tip of the style, but not stigma (Figure 3-7; Y, AD). AtGAAP5 GUS staining was also observed in the petals and sepals (Figure 3-7J).

No GUS staining was observed in floral organs of plants containing the AtGAAP3 promoter-GUS fusion construct (Figure 3-7C, H, M, R, W, AB, AG) even though a similar number of independent transgenics were analyzed.
Figure 3-7: Histochemical analysis of GUS expression in inflorescence tissue of AtGAAP promoter-GUS transgenic plants

Inflorescence tissue of 6-week old Arabidopsis plants expressing the promoter-GUS fusions was subjected to histochemical staining for GUS activity.

(A-E) Inflorescence tissue containing flower buds and young siliques
(F-J) Individual flower buds
(K-O) Stamen
(P-T) Stamen. Anther has been crushed to release pollen grain. Arrows indicate individual pollen grains.
(U-Y) Pistil
(Z-AD) Top of pistil, including stigma and top (neck) of style
(AE-AI) Base of pistil, including ovary. Arrows and arrowheads indicate sepal and petal abscission zones, respectively.

GUS gene expression is driven by AtGAAP promoters as indicated.
3.2.5.2 Pattern of AtGAAP expression in siliques

AtGAAP1, AtGAAP2, AtGAAP4 and AtGAAP5 showed similar expression patterns in siliques (Figure 3-8). AtGAAP4 and AtGAAP5 expression in siliques was generally stronger than AtGAAP1 and AtGAAP2 based on the intensity of GUS staining. GUS staining was observed in the valve, septum (Figure 3-8F-J, P-T) and silique abscission zone (Figure 3-8; K-O). The staining in the septum was strongest in the tip area (Figure 3-8; F-J). AtGAAP4 and AtGAAP5, and AtGAAP1 to a lesser extend, also drove GUS expression in other parts of the septum (Figure 3-8; P-T). A Similar trend was observed for GUS staining of the valves, the staining being most intense near the tip of the siliques (Figure 3-8; F-J). AtGAAP1, AtGAAP2 and AtGAAP5 GUS staining was very weak in the valves. AtGAAP GUS staining was also detected in the siliques pedicels (Figure3-8; A-E, Figure 3-8; K-O). Expression of AtGAAP1, AtGAAP2 and AtGAAP5 in the pedicels was generally weaker than that observed for AtGAAP4. AtGAAP genes are not expressed in mature seeds based on GUS staining (Figure 3-8; P-T). AtGAAP3 expression was not detected in the siliques by GUS staining (Figure 3-8; C, H, M, R).

3.2.5.3 Pattern of AtGAAP expression in rosette leaves

Mature rosette leaves of 6-week old plants were subjected to GUS staining in order to observe AtGAAP expression pattern in this tissue. Expression of AtGAAP2 and AtGAAP4 was detected throughout the rosette, and was particularly intense in the leaf vasculature and hydathodes (Figure 3-8; V, X, AA, AC). AtGAAP1 showed a similar expression pattern, but GUS staining was considerably weaker (Figure 3-8U, Z). AtGAAP5 expression was only detected in the hydathodes (Figure 3-8Y, AD). No AtGAAP3 expression was observed in the rosette leaves (Figure 3-8; W, AB).
Figure 3-8: Histochemical analysis of GUS expression in mature siliques and rosette leaves of *AtGAAP* promoter-GUS transgenic plants

Siliques and rosette leaves tissue of 6-week old Arabidopsis plants expressing the promoter-GUS fusions was subjected to histochemical staining for GUS activity.

(A-E) Silique

(F-J) Top of silique. Staining in the septum and valve can be observed.

(K-O) Base of silique, including silique abscission zone.

(P-T) Middle of silique containing seeds.

(U-Y) Rosette leaf

(Z-AD) Magnification of rosette leaf showing staining in veins and hydathodes. Hydathodes are indicated with arrows.

GUS gene expression is driven by *AtGAAP* promoters as indicated.
3.3 DISCUSSION

GAAPs form an evolutionarily conserved gene family with a single family member present in viruses, humans and many other eukaryotes (Gubser et al., 2007). Plants however show an apparent expansion of the gene family with five and seven homologous genes present in the Arabidopsis and rice genomes, respectively. Maintenance of GAAPs as a multigene family in plants suggests a positive selection for conservation of these genes. Arabidopsis GAAPs show between 34 and 40% sequence identity with human GAAP. AtGAAPs are also evolutionarily related to Arabidopsis Bax inhibitor-1. Sequence conservation may indicate evolutionarily conserved function for AtGAAPs as inhibitors of cell death.

AtGAAPs and OsGAAPs belong to uncharacterised protein family UPF0005. Although the functionality of the UPF0005 motif is uncharacterized so far, many proteins bearing this signature have been shown to have antiapoptotic functions (Reimers et al., 2006; Reimers et al., 2007). Human and viral GAAPs have been previously shown to contain the UPF0005 sequence motif (Gubser et al., 2007). However, the best characterised member of this family is Bax inhibitor-1, first characterized as an inhibitor of Bax-induced cell death in yeast (Xu & Reed, 1998). Several plant BI-1 orthologues, including Arabidopsis, rice and tomato BI-1, have been shown to function as inhibitors of cell death (Chae et al., 2003; Kawai et al., 1999; Sanchez et al., 2000). Another member of the UPF0005 family, Lifeguard (LFG), has been shown to protect cells against Fas-mediated cell death in mammalian cells (Somia et al., 1999). Although a LFG orthologue has been identified in the Arabidopsis genome, the function of this protein has not been characterised to our knowledge (Reimers et al., 2006). Phylogenetic analysis carried out by Hu et al. (2009) suggested that LFG and GAAP form a distinct protein family that does not include BI-1. The fact that Bax inhibitor-1 signature motif (PS01243) was not recognized on h-, At- and OsGAAP protein sequences by Pfam protein domain search supports this data.

Members of the UPF0005 protein family are predicted to contain 6 or 7 transmembrane domains (Gubser et al., 2007; Reimers et al., 2006; Walter et al.,
In the present study, analysis of AtGAAP and OsGAAP sequences predicted a protein topology consisting of seven transmembrane domains. Human and viral GAAPs have previously been shown to localize to the Golgi. The presence of transmembrane domains suggests that plant GAAPs may also be membrane localised. Bioinformatic analysis of predicted subcellular localization of AtGAAPs and OsGAAPs generated contradictory data for each AtGAAP highlighting the importance of experimental analysis of localisation of these proteins.

Extensive analysis of AtGAAP gene expression was carried out using the Arabidopsis microarray database Genevestigator, RT-PCR and analysis of transgenic plants expressing AtGAAP promoter-GUS fusions. Analysis provided clear evidence for expression of all five Arabidopsis GAAP paralogues. AtGAAP4 and AtGAAP2 showed the highest abundance of transcript throughout plant tissues according to all methods used. Microarray data indicated AtGAAP4 expression to be markedly higher than that of AtGAAP2 throughout plant tissues. RT-PCR results supported this data in the inflorescence tissue, however, level of AtGAAP4 and AtGAAP2 transcript in the rosette leaves was similar according to RT-PCR. Tissue specific expression patterns detected by promoter-GUS fusions were very similar for AtGAAP4 and AtGAAP2. Expression in the flowers was detected in the filament and anther, including individual pollen grains, and in the pistil, particularly in the stigma and neck of the style. Enrichment of expression of AtGAAP4 in the pollen and stamen was also evident from the microarray data, however, this trend was not apparent from the AtGAAP2 Genevestigator data. Petals and sepals also showed GUS staining for AtGAAP2 and AtGAAP4. GUS staining was detected throughout the rosette leaf, with particularly intense staining of the vasculature and hydathodes for AtGAAP2 and AtGAAP4. The level of AtGAAP4 expression was slightly higher than AtGAAP2, particularly in the siliques according to histochemical analysis.

According to microarray data, AtGAAP1 is expressed at low levels throughout plant tissues, apart from pollen, where AtGAAP1 expression is comparatively high, and in stamens to a lesser extent. This trend in expression was confirmed by RT-PCR, with higher AtGAAP1 transcript abundance in the inflorescence tissue than rosette leaves. Histochemical analysis confirmed increased expression levels of AtGAAP1 in the pollen and stamen, however, a notable increase in AtGAAP1 expression was also
detected in other floral organs. The tissue specific patterns were near identical to those
described for AtGAAP2 and AtGAAP4, although not as strong. In agreement with RT-
PCR and microarray data, AtGAAP1 expression in the rosette leaves was significantly
lower than that observed for AtGAAP4 and AtGAAP2 according to histochemical
staining, showing specificity in the veins and hydathodes.

According to Genevestigator, AtGAAP5 expression was much reduced compared to
AtGAAP1, AtGAAP2 and AtGAAP4; signal intensity values were near or below the
background levels for most tissues tested. AtGAAP5 expression was detected in the
testa (seed coat), and silique and carpel, particularly the ovary at lower levels.
However, RT-PCR analysis indicated that AtGAAP5 transcripts were easily detectable
in inflorescence tissue; in fact AtGAAP5 expression in the inflorescence tissue was
much higher than that of AtGAAP1. Also, relatively strong GUS staining was
observed in floral organs for AtGAAP5. In agreement with the tissue specificity (but
not intensity) of Genevestigator data, histochemical analysis revealed elevated
AtGAAP5 expression in the carpel, most prominently in the ovary, neck of the style,
and abscission zone of floral organs and also in the silique. In addition, AtGAAP5
GUS staining was observed in the petals and sepals and hydathodes of rosette leaves.
Both RT-PCR analysis and promoter-GUS fusions indicated that AtGAAP5 is
expressed at comparatively high levels in inflorescence tissue. Low signal intensity
values in microarray datasets for AtGAAP5 could be due to compromised probe
hybridisation efficiency.

Microarray data indicated AtGAAP3 expression to be almost exclusively seed
specific. However, AtGAAP3 expression could not be detected by GUS staining, not
even in the seed. Detection and localisation of GUS expression in the seed can be
difficult, especially in mature seeds (Stangeland & Salehian, 2002; Stangeland et al.,
2003). Longitudinal dissection of siliques prior to staining and use of improved
clearing methods as described by Stangeland and Sahelian (2002) could be used in
order to detect low levels and precise localisation of AtGAAP expression in the seed.
Results from RT-PCR were inconsistent with microarray and GUS data for AtGAAP3;
AtGAAP3 transcript was detectable in inflorescence tissue at levels similar to
AtGAAP1 using RT-PCR and also detectable weakly in the rosettes. It is possible that
certain regulatory promoter elements for AtGAAP3 expression are situated outside of
the promoter region used in the GUS construct used in this study. Low signal intensity values detected by microarray experiments could be due to a low probe hybridisation efficiency.

The expression patterns detected for a particular *AtGAAP* by RT-PCR, analysis of microarray data and promoter-GUS fusions provided informative data, however, the data obtained with different methods was sometimes inconsistent. This was particularly the case for *AtGAAP3*. RT-PCR analysis indicated high expression levels in the inflorescence tissue for *AtGAAP3*. In contrast, GAAP3 expression could not be detected by GUS staining and microarray analysis indicated seed-specific expression pattern. As mentioned previously, probe hybridization efficiencies can have an affect on the observed level of expression in microarray experiments (Zimmermann et al., 2004). Also, the fact that certain tissue specific trends observed with GUS staining were not reflected in the microarray data could be due to the developmental stage of the floral structures used for experiments. For example, GUS staining revealed strong expression for *AtGAAP1*, *AtGAAP2* and *AtGAAP4* in the stigma, a trend not observed in the microarray datasets. Microarray signal intensity values for expression of these genes in the stigma were compiled from only three arrays. It is possible that these arrays were done at a developmental stage when *AtGAAP* expression is low or absent, or has already ceased. This also raises the importance of analysis of expression patterns at different developmental stages of the floral structures. Gene expression during flower development and sexual reproduction occurs in a highly coordinated manner, both spatially and temporally. For example, expression of the *LHT* amino acid transporter family was found to be tissue and cell specific, and highly coordinated during floral development and the fertilisation process (Foster *et al.*, 2008).

Comparison of levels of expression between genes by RT-PCR can be misleading due to differences in primer annealing. Real-time quantitative RT-PCR would provide more comparative and reliable data. For instance, this method was used successfully to establish distinct expression patterns for five members of the *GA20ox* gene family involved in gibberellin biosynthesis (Rieu *et al.*, 2008).
Analysis of gene expression by promoter-GUS fusion relies on the assumption that cis-acting regulatory elements driving the expression of the gene are contained within the chosen promoter region. For our purpose, we chose to include approximately 1800bp of AtGAAP promoters based on the fact that this was the length of the genomic region between the AtGAAP start codon and the closest upstream gene for AtGAAP3 and AtGAAP4. Although proximal and distal cis-acting regulatory sequences are usually located within 1kb of the transcription initiation sites, some cis-acting sequences are further upstream (Taiz & Zeiger, 1998). Additionally, intragenic sequences and control elements that are situated thousands of basepairs away from the transcription initiation site are known to regulate eukaryotic genes (Taiz & Zeiger, 1998). For example, expression of the AtSUC gene that plays a role in pollen germination was initially thought to be pollen specific based on promoter-GUS fusions (Stadler et al., 1999). After microarray data revealed expression of AtSUC also in the roots, whole-gene GUS constructs were analysed. These constructs drove GUS expression in the roots, showing that expression in the roots was regulated by intragenic sequences (Schmid et al., 2005; Sivitz et al., 2007).

In summary, analysis of AtGAAP expression patterns has given us insight into the possible functions and redundancy of AtGAAP paralogues. Most importantly, gene expression data can be used to inform further experiments, for example, in which tissue to look for phenotypes and which atgaap mutant combinations would be of particular interest.
Chapter 4 : Isolation and characterisation of \textit{atgaap} mutant plants

4.1 Introduction

Programmed cell death is an essential part of vegetative and reproductive plant development and commonly occurs in response to abiotic and biotic stress triggers. Plant PCD shares several morphological and biochemical features with animal apoptosis. These include cell shrinkage and DNA fragmentation, involvement of Ca\textsuperscript{2+} fluxes and reactive oxygen species, and participation of proteases (Lam, 2008; Reape & McCabe, 2010; Williams & Dickman, 2008). Apoptotic pathways in animal cells are largely governed by the Bcl-2 family of proteins, members of which can either inhibit or promote apoptosis (Lawen, 2003). Plant genomes do not contain apparent homologues of Bcl-2 family members, although the function of Bcl-2 has been shown to be conserved in plants when expressed from a transgene (Dickman \textit{et al}., 2001; Kawai-Yamada \textit{et al}., 2001; Lacomme & Cruz, 1999; Mitsuhara \textit{et al}., 1999; Qiao \textit{et al}., 2002; Xu \textit{et al}., 2004). Interestingly, two families of cytoprotective proteins seem to be conserved in both animal and plant kingdoms: Bax inhibitor-1 and Golgi anti-apoptotic protein (Chae \textit{et al}., 2003; Gubser \textit{et al}., 2007).

GAAPs are evolutionarily conserved regulators of cell death. Overexpression of human GAAP inhibits apoptosis triggered by a variety of intrinsic and extrinsic apoptotic stimuli, whereas knock-down of h-GAAP induces cell death by apoptosis (Gubser \textit{et al}., 2007). Human GAAP modulates intracellular calcium fluxes. Overexpression of h-GAAP inhibits the influx of extracellular Ca\textsuperscript{2+} and decreases IP3-mediated release of calcium from intracellular Ca\textsuperscript{2+} stores, Golgi and ER. Knock-down of h-GAAP has the opposite effect on Ca\textsuperscript{2+} fluxes. Overexpression of h-GAAP also lowers the amount of free Ca\textsuperscript{2+} stored in the intracellular Ca\textsuperscript{2+} stores (de Mattia \textit{et al}., 2009). Ca\textsuperscript{2+} loading of the organelles can determine the sensitivity of cells to apoptotic stress; overloading sensitises cells to apoptosis whereas lowering the Ca\textsuperscript{2+} content of the stores protects cells from apoptosis. Increases in the amount of Ca\textsuperscript{2+} available for signalling may increase cytotoxic Ca\textsuperscript{2+} fluxes between the stores, cytosol
and mitochondria, whereas lowering the amount of Ca\textsuperscript{2+} in the stores may decrease these fluxes and thereby protect cells from apoptosis (Ma et al., 1999; Nakamura et al., 2000; Pinton et al., 2001; Pinton & Rizzuto, 2006; Rizzuto et al., 2009).

Human and plant BI-1 proteins are able to protect yeast cells against Bax-induced apoptosis, illustrating the conservation of BI-1 proteins throughout evolution (Kawai-Yamada et al., 2001; Kawai et al., 1999; Sanchez et al., 2000; Xu & Reed, 1998). Arabidopsis Bax-inhibitor-1 (AtBI-1) inhibits cell death triggered by both abiotic and biotic stress stimuli in plant cells (Kawai-Yamada et al., 2009; Watanabe & Lam, 2006). AtBI-1 binds calmodulin, and this interaction may be necessary for its cytoprotective function (Ihara-Ohori et al., 2007; Kawai-Yamada et al., 2009). AtBI-1 has also been shown to regulate intracellular Ca\textsuperscript{2+} fluxes and protect cells against ER stress, further demonstrating functional conservation with its animal counterpart (Watanabe & Lam, 2008b). Interestingly, GAAPs and BI-1 show significant sequence similarity with 28% identity between human BI-1 and GAAP, and 25% sequence identity between AtGAAP4 and AtBI-1 (Gubser et al., 2007; see chapter 3).

Reverse genetic approaches are powerful tools with which to analyse gene function in a number of organisms. However, redundancy is widely observed throughout genomes of higher organisms. Examples of apparently redundant genes come from a number of studies characterising gene families in plants and the genes involved may be governing any aspect of plant development and differentiation or responses to environment. Arabidopsis growth-regulating factor (AtGRF) gene family comprises of 9 members that code for putative transcription factors involved in plant growth (Kim et al., 2003). Knocking out AtGRF1-AtGRF3 individually had no effect on the phenotypes of plants compared to wild-type. Double mutants displayed minor, but significant, reduction in leaf growth, a phenotype that became much more pronounced in the triple mutant. Additionally, overexpression of AtGRF1 or AtGRF2 resulted in larger leaves (Kim et al., 2003). This highlights the fact that analysis of phenotypes caused by overexpression can provide an effective way of studying the function of members of multigene families in the absence of a KO phenotype. Pawlowski et al. (2006) studied the Arabidopsis late pollen actin (LPA) gene family, comprising of four genes. Knock out of individual genes did not result in obvious morphological phenotypes. However, knocking down expression of all four alleles with RNAi led to
defects in male and female fertility in a dosage dependent fashion with strongest epialleles being nearly sterile (Pawloski et al., 2006). Redundancy has also been reported for proteins involved with regulation of programmed cell death in plants (Kuroyanagi et al., 2005). Arabidopsis has 4 paralogues of vacuolar processing enzyme (AtVPE), αVPE, βVPE, γVPE and δVPE, which regulate PCD triggered by the fungal toxin fumoncin B1 (FB1). FB1 induces lesion formation on WT Arabidopsis leaves, and on αvpe, βvpe, and δvpe single KO mutants. No lesion formation, however, was observed on a VPE null mutant, which lacks all four VPE genes, whereas reduced lesion formation was observed on γvpe leaves. It therefore appears that γVPE is less dispensable for FB1-induced cell death, with the other three VPEs able to partially compensate for the loss of γVPE, thereby illustrating a degree of functional redundancy within this gene family (Kuroyanagi et al., 2005).

The AtGAAP gene family consists of five members. The aim of this chapter was to firstly identify single knock-out mutants for each AtGAAP in order to uncover a potential role in plant growth and development. To address the issue of possible redundancy within this gene family, we aimed to generate double mutants of AtGAAPs in all combinations and also plants that overexpress AtGAAPs. Phylogenetic and gene expression analysis presented in the previous chapter allowed us to make predictions about possible redundancy between specific members of this gene family. Based on this information, we also decided to generate certain triple mutant combinations. AtGAAPs show strong sequence similarity to proteins that function as inhibitors of cell death and are able to regulate intracellular Ca$^{2+}$ fluxes. In order to study whether this cytoprotective function is conserved in AtGAAPs, single KO mutants were exposed to a variety of stress treatments known to induce PCD, and phenotypes were analysed. In addition, intracellular Ca$^{2+}$ fluxes in WT and AtGAAP mutant plants were compared to study a potential role of AtGAAPs in Ca$^{2+}$ signalling.
4.2 Results

4.2.1 Isolation of atgaap mutant plants

To better understand the impact of the loss of AtGAAPs on the plant, we set out to isolate mutants that lacked a functional *AtGAAP* gene. Indexed insertion mutant database was searched and T-DNA insertion lines were identified for all AtGAAPs. A homozygous T-DNA insertion line was available for AtGAAP1 (SALK_066103), otherwise T3 segregating seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC). In addition, a homozygous gene trap transposon-tagged line for AtGAAP2 was obtained. Homozygous plants were identified using PCR to discriminate between the wild-type and mutant alleles. Up to 24 plants were genotyped for each insertion line and homozygous plants were identified for most insertion lines (Table 4-1). One homozygous plant from one insertion line was selected for further work for AtGAAP1, AtGAAP3, AtGAAP4 and AtGAAP5. These mutants were designated as *atgaap1*, *atgaap3*, *atgaap4* and *atgaap5* (Table 4-1). These insertion lines were in the Col-0 background. For AtGAAP2, two lines were selected for further analysis: T-DNA insertion line in the Col-0 background and a transposon-tagged line in Ler background, designated as *atgaap2-1* and *atgaap2-2*, respectively (Table 4-1).

**Table 4-1: Arabidopsis insertion lines used for the study**

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene</th>
<th>Insertion line</th>
<th>Insertion site</th>
<th>Name of mutant</th>
<th>Background</th>
<th>HM line</th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g03070</td>
<td>AtGAAP1</td>
<td>SALK_066103</td>
<td>exon4</td>
<td><em>atgaap1</em></td>
<td>Col-0</td>
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<tr>
<td></td>
<td></td>
<td>SALK_104306</td>
<td>3' UTR</td>
<td></td>
<td>Col-0</td>
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<tr>
<td>At3g63310</td>
<td>AtGAAP2</td>
<td>SALK_52507</td>
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<td><em>atgaap2-1</em></td>
<td>Col-0</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GT_93791</td>
<td>exon1</td>
<td><em>atgaap2-2</em></td>
<td>Col-0</td>
<td>✓</td>
</tr>
<tr>
<td>At4g02690</td>
<td>AtGAAP3</td>
<td>FLAG_513A10</td>
<td>5' UTR</td>
<td><em>atgaap3</em></td>
<td>Ws-0</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SAIL_567_D02</td>
<td>5'UTR</td>
<td></td>
<td>Col-0</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GABI_186E10</td>
<td>exon1</td>
<td></td>
<td>Col-0</td>
<td>✓</td>
</tr>
<tr>
<td>At4g15470</td>
<td>AtGAAP4</td>
<td>SALK_296B11</td>
<td>intron1</td>
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<td>Ws-0</td>
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</tr>
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<td>Col-0</td>
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</tr>
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<td>SALK_137440</td>
<td>intron1</td>
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<td></td>
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<td>exon1</td>
<td></td>
<td>Col-0</td>
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<tr>
<td></td>
<td></td>
<td>SALK_146311</td>
<td>5' UTR</td>
<td></td>
<td>Col-0</td>
<td>x</td>
</tr>
<tr>
<td>At4g14730</td>
<td>AtGAAP5</td>
<td>SALK_46652</td>
<td>5' UTR</td>
<td><em>atgaap5</em></td>
<td>Col-0</td>
<td>✓</td>
</tr>
</tbody>
</table>

Arabidopsis T-DNA or transposon-tagged lines were obtained for each AtGAAP. SALK, FLAG, SAIL and GABI lines are T-DNA insertion lines. GT refers to gene-trap transposon tagged line. Predicted insertion sites were located using T-DNA Express Arabidopsis Gene Mapping Tool (www.signal.salk.edu). Mutants that were selected for further work were designated as indicated, and insertion sites in these mutants were confirmed by sequencing. Lines homozygous for the insertions were identified using PCR to discriminate between the wild-type and mutant alleles. HM line, homozygous line; ✓, homozygous plant identified; x, no homozygous plants identified. Arabidopsis mutant backgrounds as indicated.
Figure 4-1 shows the genotyping results for these plants. Sequence analysis of the junction fragments obtained with T-DNA left border primer and gene-specific primers identified the exact position of the insertion in the selected lines. The insertion lies within the first exon of the gene in *atgaap2-2*, *atgaap3* and *atgaap4*, and within the last (fourth) exon in *atgaap1*. In *atgaap2-2* T-DNA insertion line the insertion is in the 3'UTR, 30 base pairs beyond the stop codon. T-DNA insertion site in *atgaap5* line is 26 base pairs upstream of the start codon in the 5'UTR (Table 4-1).

Figure 4-1: Genotyping T-DNA or transposon-tagged *atgaap1-5* mutant lines
Homozygous plants were selected using PCR-based markers that can discriminate between the wild-type and mutant alleles.

a Primers used were specific for a particular *AtGAAP* parologue
b Primers used were specific for the wild type or mutant *AtGAAP* allele
c Genomic DNA used as template was extracted from wild type and *atgaap* mutant backgrounds. Wild type: Col, Colombia-0; Ler, Landsberg erecta. Mutants: 1, *atgaap1*; 2-1, *atgaap2-1*; 2-2, *atgaap2-2*; 4, *atgaap4*, 5, *atgaap5*; 3, *atgaap3*. 
RT-PCR was carried out to see whether the insertions in the AtGAAP genes led to a lack of full-length transcript in the atgaap plants. Total RNA was extracted from inflorescence tissue as gene expression analysis had previously indicated higher AtGAAP transcript abundance in the inflorescence tissue compared to leaves (see Chapter 3). RT-PCR analysis indicated that AtGAAP1, AtGAAP3, AtGAAP4 and AtGAAP5 transcripts were not detectable in the respective homozygous mutant plants (Figure 4-2). The atgaap2-1 transposon-tagged line also showed a complete lack of transcript, but atgaap2-2 T-DNA insertion mutant showed AtGAAP2 expression levels similar to wild type plants (Figure 4-2). Therefore the atgaap2-1 line was selected for further study and was designated as atgaap2.

Figure 4-2: RT-PCR analysis of AtGAAP1-5 gene expression in the wild type and atgaap mutant plants
Total RNA was extracted from inflorescence tissue of wild-type and atgaap mutant plants. RT-PCR was carried out with gene specific primers to detect the presence or absence of AtGAAP transcript. Actin8 (ACT8) expression provided a loading control for RT-PCR. Lower bands in samples detecting AtGAAP1 and AtGAAP3 transcript from Col-0 tissue correspond to expected size for AtGAAP1/3 transcript. The size of the upper bands correspond to size expected from amplification of AtGAAP1/AtGAAP3 genomic DNA, and likely resulted from contamination of the RNA sample with genomic DNA.

a Primers used were specific for a particular AtGAAP paralogue or Actin8 as indicated.
b Total RNA was extracted from inflorescence tissue of wild type and atgaap mutant plants and cDNA synthesis was carried out using oligo-T primer. Wild type: Col, Colombia-0; Ler, Landsberg erecta. Mutants: 1, atgaap1-1; 2-1, atgaap2-1; 2-2, atgaap2-2; 4, atgaap4, 5, atgaap5; 3, atgaap3.
4.2.2 Phenotypic analysis of *atgaap* mutants

Homozygous *atgaap1, atgaap2, atgaap3, atgaap4* and *atgaap5* mutants were fertile and produced seeds that germinated and developed normally. Mutant plants were indistinguishable from wild-type plants throughout their life cycle when grown under either 10- or 16-hour day regime (Figure 4-3). Therefore, AtGAAP1-5 appear not to be essential for normal vegetative growth and development in *Arabidopsis*. It is possible that due to redundancy of function within the GAAP gene family there is no distinct phenotype associated with individual *atgaap* mutants.

![Figure 4-3: Phenotype of *atgaap* knock out mutants was indistinguishable from wild-type](image)

Rosettes of 4-week old plants grown under short-day conditions are shown. Flowering plants were germinated in the short-day for two weeks before moving them to long-day conditions for a further four weeks to induce flowering. Wild-type and mutant plants as indicated above the images. *atgaap1, atgaap3, atgaap4* and *atgaap5* were isolated from Col-0 background. *atgaap2* was isolated from Ler background. Wild type: Col-0, Colombia-0; Ler, Landsberg erecta. Ruler length: 20cm
4.2.3 Isolation of atgaap double mutants

We set out to generate double mutants for atgaaps in all combinations. Knockout lines for AtGAAP2, AtGAAP4 and AtGAAP5 were first isolated whereas atgaap1 and atgaap3 KO lines only became available from the stock centre in the autumn of 2008.

Crosses between atgaap mutant lines were performed in all combinations using each line as both a female or male donor (Table 4-2). F1 plants were tested for the presence of both mutant and wild-type allele. Screening of two to six individual plants from F1 generation by PCR revealed that crosses had been successful and all plants were confirmed F1 hybrids (Table 4-2).

Table 4-2. Isolation of plants that are heterozygous for two atgaap mutant alleles

<table>
<thead>
<tr>
<th>♂</th>
<th>♀</th>
<th>Desired genotype</th>
<th>Expected</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>atgaap1</td>
<td>atgaap2</td>
<td>atgaap1+/-atgaap2+/-</td>
<td>All</td>
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<td>atgaap1</td>
<td>atgaap1+/-atgaap2+/-</td>
<td>All</td>
<td>2/2</td>
</tr>
<tr>
<td>atgaap1</td>
<td>atgaap3</td>
<td>atgaap1+/-atgaap3+/-</td>
<td>All</td>
<td>2/2</td>
</tr>
<tr>
<td>atgaap3</td>
<td>atgaap1</td>
<td>atgaap1+/-atgaap3+/-</td>
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<td>atgaap2</td>
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<td>2/2</td>
</tr>
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<td>atgaap4</td>
<td>atgaap2+/-atgaap4+/-</td>
<td>All</td>
<td>4/4</td>
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<tr>
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<td>atgaap4</td>
<td>atgaap4+/-atgaap5+/-</td>
<td>All</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Crosses between atgaap KO mutants were performed as indicated in order to identify plants heterozygous for both mutations in the F1 generation. Plants were screened for the presence of wild-type and mutant alleles using PCR-based markers. Expected and observed frequency of plants heterozygous for two atgaap mutant alleles in F1 generation is indicated as shown.
4.2.3.1 atgaap2atgaap4 and atgaap2atgaap5

F2 seeds from selfed atgaap2+/-atgaap4+/- and atgaap2+/-atgaap5+/- F1 hybrids were screened by PCR in order to isolate a plant homozygous for both mutations. As the mutations are unlinked (on different chromosomes), 1 out of 16 plants in the F2 generation is expected to be homozygous for both mutations. One atgaap2atgaap4 and atgaap2atgaap5 double mutant plant were identified out of 36 and 28 F2 plants screened, respectively (Table 4-4).

4.2.3.2 Other double mutant atgaap combinations

As gaap1gaap2, gaap1gaap3, gaap1gaap4, gaap1gaap5, gaap2gaap3, gaap3gaap4 and gaap3gaap5 double mutants were being generated simultaneously, we decided to backcross F1 hybrid plants to the respective single KO mutant backgrounds in order to minimise the number of genotyping reactions. We expected one in four F1 plants from each backcross to be homozygous for one of the mutations (KO line used as a parent), and heterozygous for the other. These F1 plants were then allowed to self pollinate to generate the F2 generation in which 1:4 of the plants should be double mutants (Table 4-3).
Table 4-3: A genetic model outlining the strategy used for isolation of *atgaap* double mutants

**A**

♀AaBb x ♂aaBB

<table>
<thead>
<tr>
<th></th>
<th>AaBB</th>
<th>AaBB</th>
<th>aaBB</th>
<th>aaBb</th>
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<tbody>
<tr>
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<td>AaBB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>♂aaBb</td>
<td>AaBB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>♀aB</td>
<td>AaBB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>♂aaBB</td>
<td>AaBB</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

“A” and “a” represent the wild-type and mutant allele of one *AtGAAP* gene, and “B” and “b” represent the wild-type and mutant allele for another *AtGAAP* gene.

(A) 1:4 of plants in the F1 population from the indicated cross should be homozygous for “a” mutant allele and heterozygous for “b” mutant allele. In the first round of genotyping PCRs plants highlighted in yellow are identified using a primer set specific for “b”. These plants are then screened for the absence of “A” in order to identify the desired plants highlighted in dark yellow.

(B) 1:4 of plants in the F2 population derived from self-pollination of “aaBb” plant are expected to be homozygous for “a” and “b” mutant alleles. These plants can be identified by screening for the absence of “A” and “B” wild-type copies.

*AtGAAP1* and *AtGAAP2* are located on Arabidopsis chromosome one and three, respectively, whereas *AtGAAP3*, *AtGAAP4* and *AtGAAP5* are located on chromosome four and therefore genetically linked. Following the approach outlined above, a plant heterozygous for two *atgaap* mutant alleles was crossed with a plant that was homozygous for one of the mutations and WT for the other (Table 4-3). Between four and 12 F1 seedlings were screened in order to obtain the desired mutant combinations, namely *atgaap1+/+atgaap2/-, atgaap1+/+atgaap3/-, atgaap1+/+atgaap4/-, atgaap1+/+atgaap5/- and atgaap2+/+atgaap3/-. These plants were allowed to self-pollinate and F2 seedlings were screened to identify double mutants. Up to 12 F2 seedlings were screened in order to isolate *atgaap1/-atgaap2/-, atgaap1/-atgaap3/-, atgaap1/-atgaap4/-, atgaap1/-atgaap5/- and atgaap2/-atgaap3/- double mutants (Table 4-4).
Table 4-4: Isolation of atgaap double mutants

<table>
<thead>
<tr>
<th>Desired genotype</th>
<th>Expected</th>
<th>Observed</th>
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</thead>
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<tr>
<td>gaap2gaap4</td>
<td>F2 gaap2+/-gaap4+/- self-pollination</td>
<td>1:16</td>
</tr>
<tr>
<td>gaap2gaap5</td>
<td>F2 gaap2+/-gaap5+/- self-pollination</td>
<td>1:16</td>
</tr>
<tr>
<td>gaap1gaap2</td>
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<td>1:4</td>
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<td></td>
<td>F2 gaap1+/-gaap2/- self-pollination</td>
<td>1:4</td>
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<tr>
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<td>F1 gaap1+/-gaap3+/- gaap3/-</td>
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<td></td>
<td>F2 gaap1+/-gaap3/- self-pollination</td>
<td>1:4</td>
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<td>F1 gaap1+/-gaap4+/- gaap4/-</td>
<td>1:4</td>
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<tr>
<td></td>
<td>F2 gaap1+/-gaap4/- self-pollination</td>
<td>1:4</td>
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<td>gaap1gaap5</td>
<td>F1 gaap5+/-gaap1+/- gaap5/-</td>
<td>1:4</td>
</tr>
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<td></td>
<td>F2 gaap1+/-gaap5/- self-pollination</td>
<td>1:4</td>
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<td>F1 gaap3+/-gaap2+/- gaap3/-</td>
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</tr>
<tr>
<td></td>
<td>F2 gaap2+/-gaap3/- self-pollination</td>
<td>1:4</td>
</tr>
</tbody>
</table>

atgaap2atgaap4 and atgaap2atgaap5 double mutants were isolated from F2 population deriving from self-pollination of plants heterozygous for both mutant alleles as indicated. atgaap1atgaap2, atgaap1atgaap3, atgaap1atgaap4, atgaap1atgaap5 and atgaap2atgaap3 double mutants were isolated using the genetic strategy outlined in Table 3. Expected and observed frequency of the desired genotypes as indicated. Plants with genotypes highlighted in yellow were allowed to self pollinate in order to isolate double mutants in the following generation. Plants highlighted in green are double mutants for the indicated AtGAAPs.

4.2.3.3 atgaap3atgaap4 and atgaap3atgaap5

AtGAAP3, AtGAAP4 and AtGAAP5 are all located on Arabidopsis chromosome four. Physical distance between AtGAAP3 and AtGAAP5 is approximately 7300kb, and AtGAAP3 and AtGAAP5 are separated by approximately 7700kb. In order to estimate the genetic distance between these loci a genetic linkage map by Singer et al. (2006) was used. Three single feature polymorphism (SFP) markers that lie close to AtGAAP loci were selected. SFP marker C4_010 lies 1.2kb downstream of AtGAAP3, and CH_053 and CH_056 lie 129kb and 17 kb upstream of AtGAAP5 and AtGAAP4, respectively (Figure 4-4). Genetic distance between the markers close to AtGAAP3 and AtGAAP5 or AtGAAP4 is 28,3cM and 30,3cM, respectively. Hence we expect
approximately 30/100 chromosomes or 30/50 (1/1.7) plants to show a recombination event between AtGAAP3 and AtGAAP4/AtGAAP5 loci.

Figure 4-4: Schematic diagram of region of Arabidopsis chromosome four surrounding *AtGAAP3*, *AtGAAP4* and *AtGAAP5*

Physical distance between *AtGAAP3* and *AtGAAP4*/*AtGAAP5* as shown. Single feature polymorphism (SFP) markers (Singer et al., 2006) that lie downstream of *AtGAAP3* and upstream of *AtGAAP4* and *AtGAAP5* were used to estimate genetic distance between *AtGAAP* loci.

A cross between *atgaap4+/-atgaap3+/-* and *atgaap4-/-* was performed in order to isolate *atgaap4-/-atgaap3+/-* plant in the F1 population. Despite PCR screening 16 plants of the F1 population, such a plant was not identified. We then screened an F1 population from *atgaap3+/-atgaap4+/- X atgaap3-/-* cross in order to isolate *atgaap3-/-atgaap4+/-* plant. One plant out of 12 displayed the desired genotype. F2 population from self-pollination of *atgaap3-/-atgaap4+/-* was screened in order to isolate a double mutant. One out of eight screened plants was a *atgaap3-/-atgaap4-/-* double mutant (Table 4-5).

Single knockout plants for *atgaap3* and *atgaap5* were crossed to *atgaap5+/-atgaap3+/-* in order to isolate a plant that was homozygous for one of the mutations and heterozygous for the other. Despite screening 16 and 20 F1 plants, respectively, a plant with a crossover event was not identified (Table 4-5). One plant that was heterozygous for both transgenes was selected from both F1 populations in order to identify a crossover event in the F2 generation. We also proceeded to screen F1 populations from two more independent crosses of the abovementioned genotypes. One *atgaap3-/-atgaap5+/-* and one *atgaap3+/-atgaap5-/-* plant was identified. Self-
pollination of \textit{atgaap3+-atgaap5-} resulted in identification of \textit{atgaap3-/atgaap5-} double mutant in the F3 generation (Table 4-5).

Table 4-5: Isolation of \textit{atgaap3atgaap4} and \textit{atgaap3atgaap5} double mutants

<table>
<thead>
<tr>
<th>Generation</th>
<th>♀</th>
<th>♂</th>
<th>Desired genotype</th>
<th>Expected</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{atgaap3atgaap4}</td>
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<td></td>
</tr>
<tr>
<td>A F1</td>
<td>\textit{gaap4+/-atgaap3+/-}</td>
<td>\textit{gaap4-/-}</td>
<td>\textit{gaap3+/-atgaap4-/-}</td>
<td>1:4</td>
<td>0:16</td>
</tr>
<tr>
<td>B F1</td>
<td>\textit{gaap3+/-atgaap4+/-}</td>
<td>\textit{gaap3-/-}</td>
<td>\textit{gaap3+/-atgaap4+/-}</td>
<td>1:4</td>
<td>1:12</td>
</tr>
<tr>
<td>C F2</td>
<td>\textit{gaap3+/-atgaap4+/-}</td>
<td>\textit{self-pollination}</td>
<td>\textit{gaap3+/-atgaap4+/-}</td>
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<td>1:8</td>
</tr>
<tr>
<td>\textit{atgaap3atgaap5}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A F1</td>
<td>\textit{gaap5+/-atgaap3+/-}</td>
<td>\textit{gaap3-/-}</td>
<td>\textit{gaap3+/-atgaap5-/-}</td>
<td>1:4</td>
<td>0:16</td>
</tr>
<tr>
<td>B F1</td>
<td>\textit{gaap5-/-}</td>
<td>\textit{gaap5+/-atgaap3+/-}</td>
<td>\textit{gaap5-/-atgaap5+/+}</td>
<td>1:4</td>
<td>0:20</td>
</tr>
<tr>
<td>C F1</td>
<td>\textit{gaap5+/-atgaap3+/-}</td>
<td>\textit{gaap5-/-}</td>
<td>\textit{gaap3+/-atgaap5-/-}</td>
<td>1:4</td>
<td>0:8</td>
</tr>
<tr>
<td>D F1</td>
<td>\textit{gaap3+/-atgaap5+/-}</td>
<td>\textit{gaap3-/-}</td>
<td>\textit{gaap3+/-atgaap5-/-}</td>
<td>1:4</td>
<td>1:8</td>
</tr>
<tr>
<td>E F2</td>
<td>\textit{gaap5+/-atgaap3+/-}</td>
<td>\textit{self-pollination}</td>
<td>\textit{gaap5+/-atgaap3-/-}</td>
<td>1:16</td>
<td>0:8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>\textit{gaap3+/-atgaap5+/+}</td>
<td>1:8</td>
<td>1:8</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>\textit{gaap3+/-atgaap5-/-}</td>
<td>1:8</td>
<td>0:8</td>
</tr>
<tr>
<td>F F2</td>
<td>\textit{gaap5+/-atgaap3+/-}</td>
<td>\textit{self-pollination}</td>
<td>\textit{gaap5+/-atgaap3-/-}</td>
<td>1:16</td>
<td>0:8</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>\textit{gaap3+/-atgaap5-/-}</td>
<td>1:8</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>\textit{gaap3+/-atgaap5+/-}</td>
<td>1:8</td>
<td>0:8</td>
</tr>
<tr>
<td>G F3</td>
<td>\textit{gaap3+/-atgaap5-/-}</td>
<td>\textit{self-pollination}</td>
<td>\textit{gaap5+/-atgaap3-/-}</td>
<td>1:4</td>
<td>2:8</td>
</tr>
</tbody>
</table>

\textit{atgaap3atgaap4} and \textit{atgaap3atgaap5} double mutants were isolated using the genetic strategy outlined in Table 3. A plant that was heterozygous for both \textit{atgaaps} was crossed with a plant that was homozygous for one mutant allele and wild-type for the other in order to isolate \textit{atgaap+/atgaap-} plant in the F1 generation as indicated. Double mutant would be isolated in F2 population derived from self-pollination of this plant. Expected and observed frequency of the desired genotypes as indicated. Due to linkage of the loci in question, some reduction in the expected numbers was likely to occur. Plants with genotypes highlighted in yellow were allowed to self pollinate in order to isolate double mutants in the following generation. Plants highlighted in green are double mutants for the indicated \textit{AtGAAP}s.

\textit{atgaap3atgaap4} \textit{F1} populations from two independent crosses (A and B) had to be screened in order to isolate \textit{atgaap+/atgaap-} intermediate.

\textit{atgaap3atgaap5} \textit{F1} populations from four independent crosses (A, B, C, D) had to be screened in order to isolate \textit{atgaap+/atgaap-} intermediate. Also, one plant that was heterozygous for both transgenes (E and F) was selected from \textit{F1} populations (A and B) in order to isolate a plant with the desired genotype in the F2 population. \textit{atgaap3+/atgaap5-} was isolated, allowed to self pollinate, and \textit{atgaap3+/atgaap5-} double mutant was identified in the following generation.

4.2.3.4 \textit{atgaap4atgaap5}

\textit{AtGAAP4} and \textit{AtGAAP5} are situated on chromosome 4 within 400 kb from each other (Figure 4-5). In order to estimate the genetic distance between these loci, a genetic linkage map by Singer et al. (2006) was used. We identified five informative single feature polymorphism (SFP) markers that reside in the region surrounding \textit{AtGAAP4} and \textit{AtGAAP5}: C4_053, C4_054, C4_055, C4_056, C4_057. C4_053 lies 130kb upstream of \textit{AtGAAP5} on chromosome 4, C4_056 lies 11 kb downstream of
AtGAAP4, and the other 3 markers lie in between AtGAAP4 and AtGAAP5. The genetic distance between markers C4_053 (closest upstream marker to AtGAAP5) and C4_057 (closest downstream marker to AtGAAP4), was reported to be 2.5cM (Figure 4-5). Based on this, we expect 2.5/100 chromosomes, or 2.5/50 (1/20) plants to show a recombination event between genes close to these loci. The genetic distance between C4_054 (closest downstream marker to AtGAAP5) and C4_056 (closest upstream marker to AtGAAP4), was reported to be 1cM (Figure 4-5). Crossover event between these loci was likely to occur in 1/50 plants. Based on these data, we expected approximately 1/35 plants in the F2 population derived from self-pollination of atgaap4+/- atgaap5+/- F1 hybrids to be homozygous for one of the mutations and heterozygous for the other. Likewise, we expected to find a plant that was WT for one of the alleles and heterozygous for the other with the same frequency. F2 populations deriving from two independent crosses between atgaap4+/- and atgaap5+/- parents have been screened in order to identify a crossover event between AtGAAP4 and AtGAAP5 using PCR-based markers that can discriminate between the wild-type and mutant alleles. No recombination event has been identified despite screening 460 F2 plants.

![Figure 4-5: Schematic diagram of region of Arabidopsis chromosome four surrounding AtGAAP4 and AtGAAP5](image)

Physical distance between AtGAAP4 and AtGAAP5 as shown. Genetic distance between single feature polymorphism (SFP) markers (Singer et al., 2006) that reside in the region surrounding AtGAAPs as shown.
4.2.4 Phenotypic analysis of *atgaap* double mutants

All double mutant combinations for *AtGAAPs*, except for *atgaap4atgaap5*, were successfully isolated (Table 4-6).

<table>
<thead>
<tr>
<th></th>
<th>atgaap1</th>
<th>atgaap2</th>
<th>atgaap3</th>
<th>atgaap4</th>
</tr>
</thead>
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<td>✓</td>
<td>✓</td>
<td>X</td>
</tr>
<tr>
<td>atgaap4</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
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<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>atgaap2</td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Isolated *atgaap* double mutants as shown (✓). We were unable to isolate the *atgaap4atgaap5* double mutant (X).

*atgaap1*, *atgaap3*, *atgaap4* and *atgaap5* mutants are in the Col-0 background. Double mutants combining these mutant alleles were indistinguishable from wild-type Col-0 plants throughout their life cycle (Figure 4-6). *atgaap2* mutant is in the Ler background. Phenotypes of *atgaap2atgaap3* and *atgaap2atgaap4* double mutants were similar to Col-0, whereas *atgaap1atgaap2* and *atgaap2atgaap5* double mutants displayed a phenotype similar to Ler plants. Phenotypic differences were observed between individual plants, likely due to the combination of Col-0 and Ler background in these plants. Individuals from a cross between Ler and Col wild-type plants displayed segregation of similar characteristics to these double mutant plants (Figure 4-6). No obvious developmental defects were observed in any of the isolated double mutants.
Figure 4-6: Phenotype of atgaap double mutants was indistinguishable from wild-type
Rosettes of 4-week old plants grown under short-day conditions are shown. Flowering plants were germinated in the short-day for two weeks before moving them to long-day conditions for a further four weeks to induce flowering. Wild-type and mutant plants as indicated above the images. Mutants in the upper panel were isolated from Col-0 background. Mutants in the lower panel are of mixed Col-0/Ler background. atgaap2atgaap3 and atgaap2atgaap4 double mutants displayed Col-0-like phenotypes. atgaap1atgaap2 and atgaap2atgaap5 mutants displayed Ler-like phenotypes. Individuals from wild-type Col-0 x Ler cross are shown as controls for these plants.

4.2.5 Isolation of atgaap triple mutants

AtGAAP gene expression analysis using RT-PCR and publicly available microarray expression data indicated that generation of atgaap1/-/atgaap2/-/atgaap4/- and atgaap2/-/atgaap3/-/atgaap4/- triple mutants would be of particular interest for the following reasons: in these lines the expression of the most highly abundant AtGAAPs (AtGAAP2 and AtGAAP4) would be knocked-out in combination with AtGAAP1 or AtGAAP3 that show tissue specificity with elevated expression levels in pollen and stamen, or seeds, respectively. Experimental GUS-expression data was not available at the time this experiment was designed, and therefore decisions were based on publicly available microarray data.
Figure 4-7 details the approach used for isolation of \textit{atgaap1}-/-\textit{atgaap2}-/-\textit{atgaap4}-/- and \textit{atgaap2}-/-\textit{atgaap3}-/-\textit{atgaap4}-/- triple mutants. Table 4-7 lists the number of plants in each generation that had to be screened in order to identify a plant with the desired genotype. As discussed previously, \textit{AtGAAP3} and \textit{AtGAAP4} are both located on chromosome four, approximately 30cM apart, and we therefore prepared to screen almost twice the number of plants than expected based on Mendelian genetics to isolate a crossover event between \textit{AtGAAP3} and \textit{AtGAAP4}. \textit{atgaap1}-/-\textit{atgaap2}-/-\textit{atgaap4}-/- and \textit{atgaap2}-/-\textit{atgaap3}-/-\textit{atgaap4}-/- triple mutants were isolated in August 2010 and therefore only preliminary phenotypic observations have been made. The phenotype of the plants appeared wild-type and were indistinguishable from other plants in the segregating F3 generations (data not shown). However, these triple mutants will have to be grown side by side with wild-type plants in order to make more detailed and robust comparisons.

\textbf{Figure 4-7: Strategy used for isolation of \textit{atgaap} triple mutants}

Capital and lower case letters indicate wild-type and mutant alleles of particular \textit{AtGAAP} genes, respectively. Expected frequency of desired genotypes in each generation as indicated is based on Mendelian genetics. Some distortion in these ratios were expected to occur during isolation of \textit{atgaap2atgaap3atgaap4} triple mutant as \textit{atgaap3} and \textit{atgaap4} are genetically linked. \textit{atgaap1atgaap2atgaap4} triple mutant was isolated using the strategy outlined on the left. Plants with genotypes highlighted in red were allowed to self pollinate in order to look for the desired genotypes in the following generation. \textit{atgaap2atgaap3atgaap4} mutant was isolated using the strategy outlined on the right. This was due to the fact that we were unable to identify a plant with AabbCc genotype in the initial crosses screened. Plants with genotypes highlighted in blue were allowed to self pollinate in order to look for the desired genotypes in the following generation.
atgaap1atgaap2atgaap4 and atgaap2atgaap3atgaap4 triple mutants were isolated using the genetic strategy outlined in Figure 7. Expected and observed frequency of the desired genotypes in each generation as indicated. Due to linkage of AtGAAP3 and AtGAAP4 loci, some reduction in the expected numbers was likely to occur during isolation of atgaap2atgaap3atgaap4 triple mutant. Plants with genotypes highlighted in yellow were allowed to self pollinate in order to isolate plants with desired genotypes in the following generation as indicated. Plants highlighted in green are triple mutants for the indicated AtGAAPs.

(atgaap1atgaap2atgaap4) atgaap2atgaap4 double mutant was crossed to a plant heterozygous for atgaap4 and atgaap1. Two independent crosses (A and B) had to be screened in order to find an atgaap1+/atgaap2+/atgaap4-- intermediate. Screening of F2 population deriving from self-pollination of two atgaap1+/atgaap2+/atgaap4-- plants (C and D) resulted in discovery of two atgaap1+/atgaap2+/atgaap4-- plants. Triple mutants were isolated following self-pollination of these plants.

(atgaap2atgaap3atgaap4) atgaap2atgaap4 double mutant was crossed to a plant heterozygous for atgaap4 and atgaap3. We were unable to find atgaap2+/atgaap3+/atgaap4-- plants despite screening altogether 32 plants from two independent crosses (A and B). However, plants that were heterozygous for all three mutant alleles were isolated from these F1 populations and allowed to self-pollinate (E and F). At the same time two more independent F1 crosses were sown (C and D). Six plants with desired genotypes were identified as indicated. Self-pollination of atgaap2+/atgaap3+/atgaap4-- plant resulted in isolation of triple mutant in the following generation.

### Table 4-7: Isolation of atgaap1atgaap2atgaap4 and atgaap2atgaap3atgaap4 triple mutants

<table>
<thead>
<tr>
<th>Desired genotype</th>
<th>Expected</th>
<th>Observed</th>
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</thead>
<tbody>
<tr>
<td>A F1 gaap1+/-gaap4+/- gaap2-/-gaap4-/- 1:4 0:12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B F1 gaap2-/-gaap4-/- gaap1+/-gaap4-/- 1:4 4:12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C F2 gaap1+/gaap2+/gaap4-/- self-pollination gaap1+/gaap2+/gaap4-/- 1:8 2:8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>gaap1+/gaap2+/gaap4-/- 1:8 0:8</td>
</tr>
<tr>
<td>D F2 gaap1+/gaap2+/gaap4-/- self-pollination gaap1+/gaap2+/gaap4-/- 1:8 0:8</td>
<td></td>
<td></td>
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<tr>
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<td></td>
<td>gaap1+/gaap2+/gaap4-/- 1:8 0:8</td>
</tr>
<tr>
<td>E F3 gaap1+/gaap2+/gaap4-/- self pollination gaap1+/gaap2+/gaap4-/- 1:4 3:12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F F3 gaap1+/gaap2+/gaap4-/- self pollination gaap1+/gaap2+/gaap4-/- 1:4 2:12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Desired genotype</th>
<th>Expected</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>A F1 gaap2-/-gaap4-/- gaap4+//-gaap3+/- 1:4 0:12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B F1 gaap3+//-gaap4+/- gaap2-/-gaap4-/- 1:4 0:20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C F1 gaap4+//-gaap3+/- gaap2-/-gaap4-/- 1:4 0:8</td>
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<td></td>
</tr>
<tr>
<td>D F1 gaap2+//-gaap3+/- gaap2-/-gaap4-/- 1:4 2:8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E F2 gaap2+//-gaap3+/gaap4+/- self-pollination gaap2+//-gaap3+/gaap4+/- 1:64 1:32</td>
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<tr>
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<td>gaap2+//-gaap3+/gaap4+/- 1:16 1:16</td>
</tr>
<tr>
<td>F F2 gaap2+//-gaap3+/gaap4+/- self-pollination gaap2+//-gaap3+/gaap4+/- 1:64 1:32</td>
<td></td>
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<tr>
<td></td>
<td></td>
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<td>gaap2+//-gaap3+/gaap4+/- 1:16 1:16</td>
</tr>
<tr>
<td>G F3 gaap2-/-gaap3+/gaap4+/- self-pollination gaap2-/-gaap3+/gaap4+/- 1:4 2:8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
AtGAAP1, AtGAAP2 and AtGAAP3 form a distinct clade based on sequence alignment and phylogenetic analysis (see Chapter 3). Therefore generation of atgaap1-/atgaap2-/atgaap3-/- triple mutant would be of interest. atgaap1-/atgaap3-/- double mutant has been crossed to atgaap1-/atgaap2-/- and atgaap2-/atgaap3-/- double mutant plants in order to isolate a triple mutant. This research is ongoing.

4.2.6 Testing for PCD-related phenotypes in atgaap mutant plants

Human and viral GAAPs inhibit apoptosis triggered by a wide range of apoptotic stimuli and knock out of h-GAAP induces cell death in human cells (Gubser et al., 2007). We wanted to test whether the function of GAAP as an inhibitor of cell death is conserved in plants. Therefore we searched for a phenotype associated with AtGAAP knock-out mutation by exposing plants to various stress conditions that are known to trigger PCD and comparing the effect these stresses have on the wild-type and atgaap single knock-out mutant plants.

4.2.6.1 Responses of wild-type and atgaap mutants to Pseudomonas syringae pv tomato DC3000 (avrRpm1)

To examine the role of AtGAAPs on the regulation of PCD progression, the avirulent bacterial pathogen Pseudomonas syringae pv tomato DC3000 expressing avirulence gene avrRpm1 [Pst DC3000 (avrRpm1)] was selected as a biotic cell death inducer of Arabidopsis leaf cells. Inoculation with Pst DC3000 (avrRpm1) leads to the development of a form of programmed cell death known as the hypersensitive response (HR) around the infection site. Leaves were inoculated with $1 \times 10^7$ cfu and the progression of HR was monitored up to 48 hours by quantification of electrolyte leakage from the dying cells and by staining inoculated leaves with lactophenol trypan blue (visualisation of dead and dying cells). The extent of cell death as judged by trypan blue staining at 12 and 24hpi was indistinguishable in wild-type and atgaap mutant plants (Figure 4-8A). Ion leakage was measured at 12 hour intervals for 48 hours. Ion leakage from atgaap mutant leaves showed similar levels to that of wild-type leaves at all time points (Fig 4-8B).
Figure 4-8: Responses of wild-type and atgaap mutants to *Pseudomonas syringae* pv *tomato* DC3000 (avrRpm1)

Wild-type and mutants plants were inoculated with *Pseudomonas syringae* pv *tomato* DC3000 carrying *avrRpm1*.

(A) Extent of cell death was studied using trypan blue staining for cell death at 12 and 24 hours post inoculation. Leaves were inoculated with MgCl2 as negative control. Wild type and mutant plants displayed similar extend of cell death at both time points. MgCl2 did not induce cell death. Representative images are shown. Experiment was repeated three times with three replicates in each experiment.

(B) Electrolyte leakage induced by HR cell death. Electrical conductivity was measured in DC3000 (avrRpm1) and MgCl2 inoculated plants as described in Materials and Methods. Data are mean +/- S.E (error bars) of three plants each. Experiment was repeated twice with similar results.
4.2.6.2 Dark-induced senescence in wild-type and *atgaap* mutant plants

Senescence is a type of PCD that is regulated by both exogenous and endogenous factors. Light plays an important role in the regulation of senescence and leaf senescence can be induced by detaching leaves and placing them in the darkness (Guo & Crawford, 2005; Weaver & Amasino, 2001). Endogenous factors, such as the age of the leaves, is an important factor influencing the senescence response. Therefore comparison of leaves of identical age was used in the experiment. Rosette leaves 5-8 were detached from wild-type and *atgaap* mutant plants and kept in the dark for seven days. Development of senescence was analysed by observation of visible yellowing of the leaves. Wild-type and mutant leaves senesced at a similar rate and by day 7 most of the leaf area was chlorotic (Figure 4-9).

![Figure 4-9: Dark-induced leaf senescence in wild-type and *atgaap* mutant plants](image)

Rosette leaves 5, 6, 7 and 8 (in order from left to right) were detached from 4-week wild-type and mutant plants and placed in the dark. Images of leaves after 4 and 7 days of dark-treatment are shown. Images of detached leaves kept in the light for 7 days are shown as controls.
4.2.6.3 Responses of wild-type and *atgaap* mutants to heat stress

Exposing plant cells to elevated temperatures induces programmed cell death in a synchronous manner. Knock out of AtBI-1, which shares sequence conservation with AtGAAPs, results in increased sensitivity to heat stress (Watanabe and Lam, 2006). We therefore wanted to explore whether AtGAAPs play a role in the response to heat. Exposure of 5-week old plants to 55°C heat stress for 20 minutes has been reported to cause extensive cell death in Arabidopsis plants (Watanabe and Lam, 2006). These conditions did not result in extensive cell death under our experimental set up. However, increasing the temperature to 65°C resulted in extensive cell death in both wild-type and mutant plants. No consistent differences in the extent of tissue collapse between wild type and *atgaap* mutant plants could be observed (Figure 4-10).

![Figure 4-10: Heat-shock induced cell death in wild type and *atgaap* mutant plants](image)

Four-week old wild type and mutant plants were subjected to heat treatment at 65°C for 20 minutes. Plants were transferred back to the growth room under standard conditions after treatment. Photographs were taken before and 3 days after the treatment Wild-type and mutant plants as indicated.
4.2.7 Study of Ca$^{2+}$-related responses in atgaap mutant plants

Human GAAP has been shown to modulate intracellular calcium fluxes induced by both physiological and apoptotic stimuli (de Mattia et al., 2009). Human GAAP inhibits the influx of extracellular Ca$^{2+}$ and decreases IP3-mediated release of Ca$^{2+}$ from intracellular stores. We wanted to test whether the function of GAAP as modulator of calcium fluxes is conserved in plants. Stress treatments that are known to induce intracellular calcium fluxes were selected in order to monitor whether atgaap knock-out mutants show altered calcium signatures in response to stress treatments compared to wild-type plants.

4.2.7.1 Responses of wild-type and atgaap mutants to drought stress

Human GAAP has been shown to decrease IP3-mediated release of calcium from the intracellular stores. We were interested to find out whether this function is conserved in plants. Drought stress causes a rapid increase in cytosolic calcium concentrations in intact Arabidopsis seedlings. Furthermore, drought stress has been shown to cause release of calcium from the vacuole through IP3-dependent calcium channels (Knight et al., 1997). Therefore we decided to study whether the tolerance of atgaap mutant plants to drought stress is different from the wild-type. Wild type and atgaap mutant plants were grown on soil with sufficient water for seven weeks after which watering was stopped and plants were observed on a daily basis for 10 days. Wild-type and mutant plants showed similar levels of wilting throughout the experiment and after 10 days without water, the rosette leaves of both the wild type and mutant plants had wilted and the stems of all plants had lost their turgor (Figure 4-11). Control plants that were not subjected to drought treatment remained turgid and green (Figure 4-11).
Figure 4-11: Responses of wild-type and atgaap mutant plants to drought stress

Wild type and atgaap mutant plants were grown on soil with sufficient water for seven weeks and were then subjected to water withholding for 10 days. Representative images of wild-type and atgaap mutant plants after 10 days of drought treatment (drought) or without drought treatment (control). Each experiment consisted of 3 pots with 4 plants in each pot for wild-type and atgaap mutants. Experiment was repeated three times.
4.2.7.2 AtGAAPs as modulators of calcium fluxes

Plants respond to a variety of stimuli with increases in cytosolic free calcium. Changes in cytosolic calcium levels can be studied in plants expressing the apoaequorin gene. Reconstitution of functional aequorin occurs within the apoaequorin expressing plant cells upon application of its luminophore coelenterazine. Aequorin emits blue light upon binding of calcium ions. Emitted light can be measured using a luminometer, and related to cytoplasmic calcium levels (Knight et al., 1996).

We were interested to study whether atgaap mutants have an altered calcium signature in response to stress treatments by analysing \([\text{Ca}^{2+}]_{\text{cyt}}\) responses in wild-type and atgaap mutants transformed with the apoaequorin gene. We decided to study histamine-induced calcium fluxes as h-GAAP has been shown to reduce histamine-induced increases in cytosolic and mitochondrial Ca\(^{2+}\) concentrations. Overexpression of Arabidopsis Bax inhibitor-1 (AtBI-1) in aequorin expressing tobacco BY-2 cells has been shown to result in an attenuated rise in cytosolic calcium following treatment with cyclopiazonic acid (CPA) or H\(_2\)O\(_2\) (Ihara-Ohori et al., 2007). As AtGAAPs show sequence similarity to AtBI-1, we were interested to discover whether calcium fluxes induced by CPA and H\(_2\)O\(_2\) were altered in atgaap mutant plants compared to wild type. For reasons introduced in section 4.2.7.1, we were also interested to study mannitol (simulated drought stress) –induced calcium fluxes in wild-type and atgaap mutant seedlings.

4.2.7.2.1 Introduction of apoaequorin into wild-type and atgaap mutant plants

The pGIF2(HindIII)-35S-Aq plasmid carrying the apoaequorin gene driven by the strong constitutive 35S promoter was transformed into wild-type and atgaap single KO mutant plants via Agrobacterium-mediated transformation (see section 2.6.3). T1 transformants were selected using hygromycin and the presence of the transgene in these plants was confirmed by PCR. Eight lines per genotype were selected for for Col-0, atgaap1, atgaap3, atgaap4 and atgaap5 mutants. No T1 transformants were recovered for Ler and atgaap2 mutant.
4.2.7.2.2 In vitro reconstitution of Aequorin

In order to demonstrate reconstitution of aequorin in the transformed plants and to select wild-type and atgaap mutant lines that express apoaequorin protein at equivalent levels, the ability of these plants to produce blue light was quantified after addition of coelenterazine. Six T2 seedlings per line were used for in vitro reconstitution of aequorin. Using a luminometer, background fluorescence of each sample was measured prior to discharging all the reconstituted aequorin by addition of CaCl₂ in order to measure the total amount of reconstituted aequorin in these plants. Two independent transgenic lines were selected for wild-type and atgaap mutants based on their ability to produce light at comparable levels (Table 4-8). These two sets of independent transgenic lines will hereafter be referred to as belonging to Batch 1 or Batch 2 (Table 4-8).

Table 4-8: Total amount of reconstituted aequorin in WT and atgaap mutant plants expressing proaequorin

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<tr>
<th>Transgenic line</th>
<th>Aequorin luminescence</th>
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</thead>
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<tr>
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Six seedlings from eight T2 populations (A-H) transformed with proaequorin were used for wild type and atgaap mutant plants for in vitro reconstitution of aequorin as detailed in Materials and Methods. Sample background was measured before injecting CaCl₂ to discharge all the reconstituted aequorin. Background values were subtracted to give luminescence counts for each sample. Two transgenic lines were selected for further work for wild-type and atgaap mutants based on most similar luminescence counts. Two independent sets of transgenic lines highlighted in yellow or green will hereafter be referred to as Batch 1 and Batch 2.
4.2.7.2.3 Luminometric measurements of changes in $[\text{Ca}^{2+}]_{\text{cyt}}$

In vivo changes in cytosolic calcium concentrations are measured by placing a freshly reconstituted seedling into a luminometer cuvette containing water, injecting a solution of concentrated inducer into the cuvette, and then measuring luminescence counts with a luminometer. Arabidopsis plants expressing cytoplasmic aequorin (pMAC2) were used in order to study the calcium dynamics induced by the chosen treatments. Also, cytosolic calcium fluxes are known to be induced by a touch response caused by the injection of solution. We wanted to study whether calcium fluxes caused by the touch response overlap with the response evoked by the chosen inducers in order to establish a method for injection that would minimise interference from the touch response.

Touch-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ elevations were monitored by applying water (the amount to be used for the inducer) with a fast rate of injection (1 sec) and a slow rate of injection (over 4 seconds). Background luminescence counts were measured for ten seconds before injection at the chosen rate. High rate of injection caused a strong and rapid increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ starting immediately after application of water, followed by a rapid decrease to basal levels within 10 seconds (Figure 4-12). No rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ was observed after application of water at a slow rate (Figure 4-12).

Next, mannitol was applied to the seedlings to give a final concentration of 0.444M. Rapid transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ was observed immediately after application of mannitol, followed by a rapid decrease over approximately 20 seconds. A plateau phase of slightly elevated $[\text{Ca}^{2+}]_{\text{cyt}}$ followed for approximately one minute (Figure 4-12). Both touch and mannitol treatment caused a rapid rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ immediately after application of inducer. A slow rate of injection was used for mannitol treatment in following experiments to give a negligible touch-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation in the seedlings.

$\text{H}_2\text{O}_2$ was applied to the seedlings to give a final concentration of 3mM using both high and slow rate of injection. An immediate rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ near identical to the previously observed touch response was observed upon fast injection (Figure 4-12). This transient peak was not evoked upon slow rate of injection (data not shown). Strong and rapid increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ starting after approximately 20 seconds and
peaking after 30 seconds followed by a gradual decline over the next three minutes was observed for all H₂O₂ treated samples. As H₂O₂ induced calcium fluxes were clearly distinguishable from the touch response caused by fast rate of injection, H₂O₂ was applied using fast rate of injection in all following experiments.

Luminometry of Arabidopsis seedlings treated with histamine and cyclopiazonic acid revealed no rises in [Ca²⁺]ₙ (Figure 4-12). As optimisation of the treatment was not possible due to time constraints, these inducers were not used for further studies.

Figure 4-12: Touch, mannitol, H₂O₂, histamine, and cyclopiazonic acid – induced [Ca²⁺]ₙ responses in Arabidopsis seedlings

All inducers were added 10 sec after the beginning of the trace. “fast” and “slow” refer to the rate of injection of the inducer, which was applied over 1 or 4 seconds, respectively. Representative traces are shown from six individual seedlings tested per treatment.
In order to study the effect of AtGAAPs on calcium response induced by oxidative stress, changes in H$_2$O$_2$-induced calcium fluxes were measured in apoaequorin expressing *atgaap* and wild-type plants. In these and subsequent experiments, measurements were performed on six seedlings from two independent apoaequorin-expressing lines, referred to as batch 1 and batch 2. Results for the two batches are shown separately with traces showing means of six individual seedling responses. Reconstituted seedlings were treated with 3 mM H$_2$O$_2$ and changes in cytoplasmic Ca$^{2+}$ levels were monitored using a luminometer. A clear Ca$^{2+}$ response was detected in Col-0 plants, with a rise in [Ca$^{2+}$]$_{cyt}$ starting 10 seconds after H$_2$O$_2$ application and reaching a maximum after 50 seconds, resulting in [Ca$^{2+}$]$_{cyt}$ peak values of 0.31 μM in batch 1 and 0.24 μM in batch 2 (Figure 4-13). The Ca$^{2+}$ response of *atgaap* mutants was very similar to that of wild-type plants in batch 1, although *atgaap3*, *atgaap4* and *atgaap5* showed slightly reduced [Ca$^{2+}$]$_{cyt}$ at the peak point (Figure 4-13:C, E, G). In batch 2, both the timing and magnitude of the [Ca$^{2+}$]$_{cyt}$ peak was enhanced in *atgaap1*, *atgaap3* and *atgaap5* mutants compared to wild-type (Figure 4-13:B, D, H). [Ca$^{2+}$]$_{cyt}$ peak was reached 20 seconds earlier in these mutant plants compared to wild type and peak values of 0.33 μM, 0.36 μM and 0.34 μM were reached in *atgaap1*, *atgaap3* and *atgaap5* mutants, respectively, compared to a peak value of 0.24 μM in the wild-type plants. The timing of [Ca$^{2+}$]$_{cyt}$ rise in *atgaap4* mutant plant was similar to that observed for wild-type, but the magnitude of the response was increased compared to wild-type for the entire duration of the of the experiment (Figure 4-13F). [Ca$^{2+}$]$_{cyt}$ values measured for *atgaap5* also remained above those of the wild-type plant for the duration of the experiment (Figure 4-13H). [Ca$^{2+}$]$_{cyt}$ of *atgaap1* and *atgaap3* mutants declined to wild-type levels 50 and 90 seconds after stress treatment, respectively (Figure 4-13B, D).
Figure 4-13: H₂O₂-induced [Ca²⁺]_{cyt} responses in Col-0 and atgaap mutant seedlings. Graphs show [Ca²⁺]_{cyt} response to 3 mM H₂O₂. H₂O₂ was added 10 seconds after the beginning of the trace. Traces shown are averages of measurements of six seedlings and error bars indicate standard error of the mean. Seedlings from two T2 transgenic lines expressing apoaequorin were used for wild type and atgaap mutants. A, C, E, G: Batch 1; B, D, F, H: Batch 2.
4.2.7.2.5 Mannitol-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ responses in Col-0 and atgaap mutant seedlings

The effect of AtGAAPs on calcium response induced by mannitol treatment (simulated drought stress) was investigated. Measurements were performed as described above. As shown in Figure 4-14, rapid elevation in $[\text{Ca}^{2+}]_{\text{cyt}}$ appeared immediately after mannitol application, peaking at 4 seconds in wild-type and atgaap mutant seedlings. A slight increase of $[\text{Ca}^{2+}]_{\text{cyt}}$ at peak point was observed in the atgaap4 mutant compared to the wild-type in batch 1 (Figure 4-14 E). atgaap5 mutant showed a decrease of $[\text{Ca}^{2+}]_{\text{cyt}}$ at peak point in batch1 compared to wild-type plants (Figure 4-14 G). However, $[\text{Ca}^{2+}]_{\text{cyt}}$ rose to similar levels at peak point in wild type, atgaap4 and atgaap5 mutants in batch 2 (Figure 4-14;F, H). No significant differences of $[\text{Ca}^{2+}]_{\text{cyt}}$ at peak point was observed for atgaap1 and atgaap3 mutant plants compared to the wild-type (Figure 4-14;A, B, C, D).

After reaching the peak point $[\text{Ca}^{2+}]_{\text{cyt}}$ showed rapid decline to approximately 0.5μM over a period of 5 seconds in wild-type and atgaap mutant seedlings. Some fluctuations in $[\text{Ca}^{2+}]_{\text{cyt}}$ followed before $[\text{Ca}^{2+}]_{\text{cyt}}$ reached a plateau phase of slightly elevated $[\text{Ca}^{2+}]_{\text{cyt}}$. $[\text{Ca}^{2+}]_{\text{cyt}}$ was reduced in atgaap1 mutant compared to wild-type in both batches around the 20 second timepoint after the application of mannitol (Figure 4-14A, B). A secondary peak with $[\text{Ca}^{2+}]_{\text{cyt}}$ rising to 0.53 μM at 35 seconds after mannitol application was observed for atgaap3 in batch1, however, secondary peak was absent in atgaap3 in batch 2 (Figure 4-14C, D). $[\text{Ca}^{2+}]_{\text{cyt}}$ remained significantly lower in atgaap5 mutant compared to wild type for 25 seconds after mannitol application in batch 1 (Figure 4-14G). Also, a delayed and reduced secondary peak in $[\text{Ca}^{2+}]_{\text{cyt}}$ was observed for atgaap5 mutant with significantly higher calcium concentration than that of wild-type around 50 to 60 seconds after mannitol application in batch 1 (Figure 4-14G). Wild-type plants and atgaap5 mutants showed indistinguishable $[\text{Ca}^{2+}]_{\text{cyt}}$ traces in batch 2 (Figure 4-14H).
Figure 4-14: Mannitol-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} responses in Col-0 and atgaap mutant seedlings. Graphs show [Ca\textsuperscript{2+}]\textsubscript{cyt} response to 0.444 M mannitol. Mannitol was added 10 seconds after the beginning of the trace. Traces shown are averages of measurements of six seedlings and error bars indicate standard error of the mean. Seedlings from two T2 transgenic lines expressing apoaequorin were used for wild type and atgaap mutants. A, C, E, G: Batch 1; B, D, F, H: Batch 2.
4.2.8 Overexpression of AtGAAP-G/YFP fusion protein leads to developmental phenotypes

If there is redundancy of function within the *AtGAAP* gene family, overexpression may be able to generate a phenotype rather than knocking out individual or combinations of genes. Genomic *AtGAAP* sequences without the stop codon were cloned into pENTR™/D-TOPO® entry vector in the Invitrogen GATEWAY system to allow for rapid and efficient transfer of the sequences into multiple vector systems. *AtGAAP* sequences were recombined into binary destination vector (pEG101 or pGWB5) for strong, constitutive expression under the cauliflower mosaic virus 35S promoter. These vectors also introduce a green (pGWB5) or yellow (pEG101) fluorescent protein tag to the C-terminus of AtGAAPs. 35S-*AtGAAP-G/YFP* constructs were transformed into Arabidopsis plants by floral dip (Clough & Bent, 1998). Respective knockout mutants were transformed where available at the time, namely *atgaap2, atgaap4* and *atgaap5* in order to test for functionality of the proteins by rescuing possible phenotypes associated with knocking out these genes, in addition to studying possible phenotypes caused by overexpression. 35S-*AtGAAP1-YFP* and empty vector constructs were transformed into wild type Col-0 plants. No data is presented for *AtGAAP3*, as cloning of this construct failed despite several attempts with distinct point mutations leading to amino acid change arising during the cloning procedure. Initial attempts to select *AtGAAP4-GFP* T1 transformants failed. Although these transgenic lines have since been isolated, we were unable to thoroughly analyse these lines due to time constraints. Therefore, data is presented for transgenic lines expressing *AtGAAP1-YFP*, *AtGAAP2-YFP* and *AtGAAP5-YFP* fusion constructs.

One to five T1 plants from three independent transformations were selected for each *AtGAAP* and up to 16 plants from segregating T2 populations were analysed for expression level of the fusion proteins and associated phenotypes. Expression level of the fusion protein was analysed by imaging YFP fluorescence of rosette leaves under a stereofluorescence microscope. This method relies on the assumption that the level of YFP fluorescence correlates with the expression level of the *AtGAAP-YFP* fusion protein. Images were captured using identical settings and fluorescence levels were
quantified by measuring pixel intensity of these images using ImageJ software. These arbitrary fluorescence units will hereafter be referred to as fluorescence units (fu). Fluorescence units presented throughout this thesis are directly comparable.

Twelve T2 plants from three independent T1 transformants carrying the empty vector constructs were screened for background YFP fluorescence and any potential developmental phenotypes. Level of background YFP fluorescence ranged between 1600 and 3000 fluorescence units (fu). Values within this range were also recorded for wild-type Col-0 plants. No developmental defects were observed in plants carrying empty vector constructs.

For the purpose of the following experiments T2 plants that showed fluorescence values within the range experimentally observed for empty vector controls were considered not to contain the transgene, although the absence of the transgene was not experimentally confirmed in these plants. In the following paragraphs, T2 plants with background fluorescence values will be referred to as WT, atgaap2, or atgaap5, depending on the background that the construct was transformed into.

4.2.8.1 Overexpression of AtGAAP1-YFP fusion protein leads to dwarfism

Arabidopsis Col-0 ecotype was transformed with Agrobacterium carrying 35S-AtGAAP1-YFP construct. Two T1 plants each from three independent batches of transformations were selected and 12-16 T2 plants were analysed for the level of AtGAAP1-YFP expression as judged by YFP fluorescence of rosette leaves. Plants were analysed for any obvious visible phenotype that potentially correlated with the level of YFP fluorescence. Figure 4-15A shows a representative panel of 6-week old short-day grown plants with varying levels of YFP fluorescence. Plants are numbered from one to fourteen based on decreasing amount of YFP fluorescence. Values in brackets represent YFP fluorescence units (fu). Plant number 14 is a wild-type Col-0 plant. All plants that had fluorescence values above 8000fus displayed a distinct phenotype in all six batches. The rosette was smaller and more compact than wild-type. Also, the leaves of these plants were visually thicker and wider than those of the wild type, as shown in Figure 4-15B. The size of the rosette did not seem to be
directly proportional to the amount of YFP fluorescence. The small and compact appearance of the rosette became even clearer with age. Figure 4-16 shows three 10-week old plants with varying YFP fluorescence levels next to a wild-type plant. Altogether 80 plants were analysed, 27 of which showed fluorescence values of above 8000 fus. 31 plants showed fluorescence values between 4500 and 8000. Some of these plants exhibited the above described phenotype to a lesser extent, others appeared wild-type in appearance. More detailed analysis should be carried out to confirm this visual observation. Twenty-two plants showed fluorescence values below 2100 fus and were considered to be wild-type. These plants were wild-type in appearance.
Figure 4-15: Overexpression of AtGAAP1-YFP fusion protein leads to dwarfism
(A) A representative panel of 6-week old T2 plants expressing varying levels of AtGAAP1-YFP fusion protein, as judged by the amount of YFP fluorescence of rosette leaves. Fluorescence levels were quantified by ImageJ software. Values in brackets represent arbitrary fluorescence units (fu). Plant number 14 is a wild-type. (B) Plants expressing AtGAAP1-YFP have small and compact rosette and wide and thick leaves compared to wild-type.
Figure 4-16: Overexpression of AtGAAP1-YFP fusion protein leads to dwarfism
Rosette phenotype of 10-week old plants expressing varying amounts of AtGAAP1-YFP fusion protein. Plant numbers correspond to those in Figure 4-15. Fluorescence levels were quantified by ImageJ software. Values in brackets represent arbitrary fluorescence units (fu). Plant number 14 is wild-type.

11 week-old short-day grown plants that showed YFP values of above 8000fus and had started to flower were analysed for differences in flower morphology and flowering stem architecture. Although some variation in the developmental stages of inflorescence stems of individual plants were observed, all 21 wild-type plants had produced healthy flowering stems at this stage. Out of 27 plants with YFP fluorescence values above 8000fus, only four plants had a healthy flowering stem, and this was always less developed than in the wild-type (4-17A). Six plants had a stem with an aborted terminal bud (Figure 4-17B) and 17 plants did not produce a flowering stem at all.
4.2.8.2 AtGAAP2-YFP overexpression affects cauline branch architecture

Knock out mutants of atgaap2 (*Landsberg erecta*) were transformed with 35S-AtGAAP2-YFP fusion construct. Two or three T1 plants each from three independent transformations were selected and up to 16 T2 plants, altogether 110 plants, were analysed for level of YFP fluorescence and associated mutant phenotype.

Morphology of cauline branches was altered in plants overexpressing AtGAAP2-YFP fusion protein when compared to the atgaap2 mutant background. Plants were divided into four categories based on the level of YFP fluorescence and the severity of the phenotype observed, as summarised in Table 4-9. YFP fluorescence values above 16200, between 10700 and 14400, between 7000 and 9800 and between 1800 and 3000, are hereafter referred to as to be high, medium, low and background, respectively. atgaap2 plants that emitted background fluorescence displayed a relatively straight stature as illustrated in Figure 4-18A. Cauline branches of plants that emitted medium and high fluorescence levels were crooked and the angles of cauline branches relative to the stem axis were widened. The angles of cauline branches of plants with medium fluorescence ranged from slightly widened angles to
near horizontal (Figure 4-18B). Cauline branches of plants emitted high fluorescence levels were pointing downwards (Figure 4-18C). The angles of siliques relative to the stem axis were also widened in plants emitting medium and high fluorescence. Siliques of the control atgaap2 plants were pointing upwards, whereas siliques of plants with medium and high expression levels were pointing near horizontally, or downwards, respectively (Figure 5-18; D-F). Plants that emitted low fluorescence values were generally atgaap2-like in appearance, although some seemed to have slightly widened angles of cauline branches.

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<td>Medium (10700-14400)</td>
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<td>Medium</td>
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<tr>
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<td>No (1800-3000)</td>
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Fluorescence levels were quantified by ImageJ software. Values in brackets represent arbitrary fluorescence units (fu).
Figure 4-18: Alteration of cauline branch architecture by overexpression of AtGAAP2-YFP fusion protein. Plants overexpressing AtGAAP2-YFP fusion protein have wider angles between the main stem and cauline branches and wavy cauline branches (B and C) compared with atgaap2 plants (A). The angles of siliques relative to the stem axis were widened upon AtGAAP2-YFP expression (E and F) compared to atgaap2 mutant (D). (A) and (D) atgaap2 plant; (B) and (E) Transgenic plant expressing AtGAAP2-YFP fusion protein to medium level; (C) and (F) Transgenic plant expressing AtGAAP-YFP fusion protein to high level. Amount of YFP fluorescence (quantified by ImageJ software) displayed by the plants was used as a measure for the expression level of the fusion protein.
4.2.8.3 Overexpression of AtGAAP5-YFP leads to severe growth defects

AtGAAP5-YFP construct was transformed into atgaap5 (Col-0 background). Two to five T1 plants from 3 independent transformations were selected and five to 31 T2 plants per individual T1 transformant, altogether 181 plants, were analysed for YFP fluorescence and associated phenotypes. Level of YFP fluorescence of all plants was significantly lower than that observed for individuals with lowest recovered fluorescence levels of AtGAAP1-YFP and AtGAAP2-YFP fusion proteins. It was not possible to quantify the level of YFP fluorescence of AtGAAP5-YFP transformants reliably due to the generally low levels of YFP fluorescence and dramatic differences in the size of the rosette leaves used for detection of fluorescence. Therefore plants were simply classified as atgaap5, where no YFP fluorescence was observed, and low expressors, where YFP signal was detected. 52 plants were classified as wild-type, and 129 plants as low expressors.

Plant morphology was dramatically altered in all plants expressing AtGAAP5-YFP fusion protein. Rosettes were small in size compared to atgaap5 and plants displaying a range of sizes could be identified from all three independent batches. A representative panel of 5-week old plants displaying varying degrees of the developmental phenotype is shown in Figure 4-19A. Senescence of lower rosette leaves developed earlier in plants expressing AtGAAP5-YFP compared to atgaap5 plants. As an illustration, Figure 4-19B and Figure 4-19C display the entire leaf set of a representative example of the atgaap5 mutant and an AtGAAP5-YFP overexpressor, respectively. The oldest rosette leaves of AtGAAP5 overexpressors without visible signs of yellowing, as indicated by arrows in Figure 4-19C, usually had brown lesions (Figure 4-19D). These lesions were clearly visualised as regions of dead cells with trypan blue staining (Figure 4-19E). Trypan blue staining for dead cells was most pronounced around the site of the lesions, however, staining was also observed throughout these leaves. No cell death was observed in atgaap5 leaves of similar age (Figure 4-19F).
Figure 4.19 Overexpression of AtGAAP5-YFP leads to dwarfism, enhances senescence and development of spontaneous lesions on the rosette leaves

(A) A Panel of 5-week old plants expressing AtGAAP5-YFP fusion protein (2-7) next to atgaap5 plant (1). Expression of AtGAAP5-YFP lead to a dwarf phenotype and induced senescence of lower rosette leaves.

(B) Rosette leaves of 5-week old atgaap5 plant

(C) Rosette leaves of a 5-week old plant expressing AtGAAP5-YFP

(D) Some rosette leaves of plants expressing AtGAAP5-YFP fusion protein had brown lesions (arrows). These leaves are indicated by arrowheads in Figure 6C.

(E) Leaf in Figure 6D after trypan blue staining for cell death. Brown lesions are clearly visible as regions of dead cells.

(F) atgaap5 leaves did not have dead cells based on trypan blue staining. Stained leaves were of similar age to the ones used for Figure 6D and 6E.
The plants shown in Figure 4-19A were again analysed when 11-weeks old. Plants were classified into three categories; atgaap5 mutants that did not show YFP fluorescence (plant no1 in Figure 4-19A), AtGAAP5-YFP expressors that showed some growth defects (plant no 2-4 in figure 4-19A), and AtGAAP5 expressors with severe growth defects (Plant no 5-7 in Figure 4-19A). These categories contained 52, 48, and 81 plants, respectively. Flowering phenotype of 11-week old plants grown under short days was analysed. All 52 plants that showed no YFP fluorescence had produced flowering stems at this age. 30 out of 48 plants with some growth defects had flowering stems at this stage, although they were shorter than that observed for plants with no YFP fluorescence (Figure 4-20A). Only 4 out of 81 plants with severe growth defects were flowering, with most plants having died at this stage. The stems of these plants were very short as seen in Figure 4-20A. AtGAAP5-YFP expression also affected the phenotype of the cauline leaves. Cauline leaves of all plants expressing AtGAAP5-YFP showed signs of senescence including yellowing and browning of the cauline leaves (Figure 4-20B). In addition, brown lesions could be detected in cauline leaves of plants expressing AtGAAP5 fusion protein (Figure 4-20C). Cauline leaves of atgaap5 plants showed no signs of senescence (Figure 4-20B). The appearance of rosettes of 11-week old plants that had previously been classified as belonging to category 2, 3, 4 or 5 in severity (Figure 4-19) of the phenotype was compared to that of atgaap5. Extensive senescence of the lower rosette leaves was evident compared to atgaap5. Also, the leaves had a curled lamina and the appearance of the rosette was bushier compared to atgaap5 (Figure 4-20D).
Figure 4.20: Growth defects of 11-week old plants overexpressing AtGAAP5-YFP

(A) Phenotype of the inflorescence stem was affected by overexpression of AtGAAP-YFP fusion protein. *atgaap5* plant (left) next to plants overexpressing AtGAAP5-YFP fusion protein.

(B) Cauline leaves of plants expressing AtGAAP5-YFP fusion protein showed induced senescence compared to *atgaap5* plants. Cauline leaves at the base of the lowest cauline branch are shown for *atgaap5* (left) and AtGAAP5-YFP overexpressor (right).

(C) Close up image of a cauline leaf of AtGAAP5-YFP overexpressor. Brown lesions are visible (arrows).

(D) Rosette of 11-week old *atgaap5* plant (left) and a plant overexpressing AtGAAP5-YFP (right). Rosette leaves of the overexpressor had a curled lamina and the appearance of the rosette was bushier compared to *atgaap5*. 
4.3 Discussion

To investigate the role of the five AtGAAPs in Arabidopsis growth and development, we identified T-DNA or transposon insertion mutants for each gene. No AtGAAP transcripts were detected in these mutant lines, indicating the identified mutants are knockout (null) alleles. Inactivating individual AtGAAP genes did not reveal any obvious morphological phenotypes.

To study potential redundancy within the AtGAAP gene family, double mutant plants were generated. We successfully isolated all double mutant combinations apart from atgaap4atgaap5. AtGAAP3, AtGAAP4 and AtGAAP5 are all located on chromosome 4, with a genetic distance of approximately 30cM separating AtGAAP3 and the closely linked AtGAAP4/5 (Singer et al., 2006). We had to screen a larger number of plants than predicted based on the genetic distance between these loci in order to identify a plant with a recombination event between AtGAAP3 and AtGAAP4/5. Self pollination of this plant resulted in recovery of double mutants with the predicted frequency in the progeny. Therefore the initially observed lack of recombination was likely not due to the effect of combining atgaap mutant alleles. Growth and development of all double mutants appeared normal.

We set out to generate three triple mutant combinations to address the possible issue of redundancy further. We wanted to knock-out the expression of AtGAAP2, and AtGAAP4, the most highly expressed AtGAAPs in combination with either AtGAAP1 or AtGAAP3, which show flower, or seed specific expression, respectively. atgaap1atgaap2atgaap4 and atgaap2atgaap3atgaap4 triple mutants did not display any obvious morphological phenotypes. As observed previously for atgaap3atgaap4 double mutant, we had to screen a larger number of plants than expected in order to identify a recombination event between AtGAAP3 and AtGAAP4, which are genetically linked. However, a triple mutant was identified with the expected frequency in the following generation, suggesting that this observation was not due to combining atgaap mutant alleles. Generation of atgaap1atgaap2atgaap3 triple mutant is in under way. This triple mutant is of particular interest as AtGAAP1, AtGAAP2 and AtGAAP3 protein sequences are highly
conserved, showing 88-91% sequence similarity suggesting conservation of function within this clade. AtGAAP4 and AtGAAP5 are distinct from this clade, and from each other based on amino acid sequence similarity.

Generation of double mutants of \textit{AtGAAP4} and \textit{AtGAAP5} that are genetically linked has proved problematic. Based on the genetic distance between these loci we expected approximately 1/35 plants in the F2 population derived from self-pollination of \textit{atgaap4-1 \times atgaap5-1} hybrids to contain a crossover event. Despite genotyping over 460 F2 plants, we were unable to identify a plant with a recombination event in this interval. The fact that a crossover event leading to the combination of wild-type alleles of AtGAAP4 and AtGAAP5 on the same chromosome was not observed indicates that the result is not due to any putative deleterious effect of combining these \textit{atgaap} mutant alleles, but could be due to a strong suppression of recombination in this interval. It is possible that a background mutation in either \textit{atgaap4} or \textit{atgaap5} line is causing the observed suppression of recombination at this interval. An independent null line for \textit{atgaap4} has been identified in the Ws-0 background that could be used to test this, however additional \textit{atgaap5} KO lines are not available. An alternative method for studying the effect of KO of \textit{AtGAAP4} and \textit{AtGAAP5} would be to silence one of these genes in the null background of the other.

Human and viral GAAPs function as inhibitors of apoptosis (Gubser et al., 2007). In order to study whether this function is conserved in the Arabidopsis \textit{GAAP} genes, we exposed \textit{atgaap} null mutants to various stress conditions that are known to trigger PCD in plants. Analysis of single \textit{atgaap} null mutants did not uncover a PCD-related phenotype when challenged with an incompatible pathogen or exposed to heat stress. Also, response of \textit{atgaap} and wild-type leaves to dark-induced senescence was indistinguishable. These data indicate that either these genes have no role in abiotic and biotic stress adaptation to the stimuli tested or the function of one or more \textit{AtGAAP} genes is redundant. It is possible that more sensitive and detailed analysis need to be carried out in order to identify potential subtle differences. In addition to observing visible yellowing of leaves, total chlorophyll content could be measured in order to quantify the level of dark-induced senescence in wild-type and mutant leaves (Guo & Crawford, 2005; Weaver & Amasino, 2001). To quantify the effects of heat shock, mutant seedlings could be assessed for seed germination, hypocotyl
elongation, root growth and seedling survival, methods commonly used to assess thermotolerance defects (Hong & Vierling, 2000; Hong & Vierling, 2001).

Alterations in cytosolic calcium concentration play a role in apoptosis control mechanisms (Ihara-Ohori et al., 2007). Human GAAP has recently been shown to function as a regulator of calcium fluxes (de Mattia et al., 2009). In order to explore potential effects of AtGAAPs on calcium fluxes induced by cell death stimuli, we measured changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) in response to \(\text{H}_2\text{O}_2\) using apoaequorin expressing wild-type and \textit{atgaap1, atgaap3, atgaap4} and \textit{atgaap5} mutant seedlings. Two panels of independent transformants with comparable total aequorin luminescence were selected for analysis. \textit{atgaap} mutants showed a much larger \(\text{H}_2\text{O}_2\)-induced increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) than wild-type plants in one of the panels. However, this trend was not seen for the other independent panel of transformants. Although inconclusive, this preliminary result may suggest a potential role of AtGAAPs as regulators of calcium fluxes within the plant cell although further experiments are needed to test the robustness and reproducibility of this phenotype across independent transformants. \(\text{H}_2\text{O}_2\)-induced calcium fluxes are influenced by the stress levels of the plants. If plants used for calcium measurements have been previously stressed, possible differences in calcium signatures are less likely to be picked up (Prof M. R. Knight, Durham University, personal communications). Seedlings used for these experiments had been under severe mechanical stress as plates with 7-day old seedlings were transported from London to Durham by train. The panel of plants that showed clear differences in \(\text{H}_2\text{O}_2\)-induced calcium response between wild-type and \textit{atgaap} mutant plants had undergone a longer recovery period than the panel that showed no differences.

Human GAAP has been shown to decrease IP3-mediated calcium release from intracellular stores (de Mattia et al., 2009). We used drought stress stimuli in order to determine whether this function is conserved in AtGAAPs as drought –induced cytosolic calcium rises have been linked to release of calcium from the vacuole through IP3-dependent calcium channels in plants (Knight et al., 1996, 1997). No obvious phenotypic differences were detected between the drought response of wild-type and \textit{atgaap} mutant plants. To detect more subtle differences, mannitol (drought) –induced \([\text{Ca}^{2+}]_{\text{cyt}}\) fluxes in wild-type and \textit{atgaap} mutant plants were studied. Significantly increased \([\text{Ca}^{2+}]_{\text{cyt}}\) at peak point was observed for \textit{atgaap4} mutant
compared to wild type. Conversely, attenuated $[\text{Ca}^{2+}]_{\text{cyt}}$ at the peak point was observed for \textit{atgaap5} compared to wild-type. These differences were only observed for one of the two independent transgenic lines tested. Although these results are potentially interesting, no conclusions can be drawn without repeating this experiment. It is also puzzling that the closely related \textit{AtGAAP4} and \textit{AtGAAP5} would have opposite effects. A more sensitive analysis of the role of \textit{AtGAAPs} during mannitol-induced calcium release from the vacuole could be carried out using plants expressing aequorin in the cytosolic microdomain adjacent to the vacuolar membrane, instead of the cytosolic aequorin reporter used for this study (Knight et al., 1996; Knight et al., 1997).

In the absence of a phenotype associated with knocking out \textit{AtGAAP} expression, we were interested to study whether overexpression of \textit{AtGAAP} instead produces a phenotype. Stable transgenic lines expressing YFP –tagged \textit{AtGAAP}s under the strong constitutive 35S promoter were used for this study as they were concomitantly used for subcellular localisation studies. In this study, the level of YFP fluorescence was used as an indicator of the level of overexpression of these proteins in the transgenic lines. However, we must confirm whether the level of YFP fluorescence is correlated with \textit{AtGAAP}-G/YFP protein expression levels. Furthermore, as fluorescent tags may interfere with the function of GAAP proteins, functionality of these tagged proteins must be confirmed. In the absence of a phenotype associated with \textit{atgaap} null alleles, this has thus far not been possible.

Overexpression of \textit{AtGAAP1}, \textit{AtGAAP2} and \textit{AtGAAP5} -YFP fusion proteins caused morphological defects in \textit{Arabidopsis}. Unexpectedly, overexpression of individual GAAP members led to distinct developmental phenotypes rather than a range of overlapping phenotypes, as might have been expected from this highly conserved family. Overexpression of \textit{AtGAAP1}-YFP fusion protein was associated with dwarfism, and development of leaves that were wider than those of the wild-type. Also, defects in flowering were observed under short day conditions; most overexpressors did not produce flowering stems and where stems were produced they displayed aborted terminal buds or showed delayed flowering. The observed phenotype was reminiscent of that of brassinosteroid signalling mutants, which exhibit dwarfed stature and wide leaves (Li & Nam, 2002; Nam & Li, 2002; Noguchi
et al., 1999). Analysis of the levels of this hormone in the AtGAAP1-YFP overexpressors or exogenous application of brassinolides to rescue the phenotype could be carried out in order to find out whether this phenotype is associated with brassinosteroid. More detailed analysis of the phenotype should be carried out, including measuring the length of the petioles and measuring the width/length ratio of the leaves. Parameters used for studying vegetative phase change and floral transition, such as counting the days to floral transition and counting the leaf number at bolting under long day conditions should also be carried out to further characterise this mutant.

Overexpression of AtGAAP2-YFP fusion protein led to widened angles of cauline branches and siliques relative to the stem axis. Whether this effect is due to general developmental defects or gravitropic effects is yet to be confirmed. Auxin and cytokinin, amongst other hormones are known to play a role in the control of branching (Turnbull, 2005). However, the phenotype observed was not reminiscent of the typical hormone-related branching mutants (Turnbull, 2005). A phenotype very similar to that observed in this study was reported for mutation of the AtSAC1 gene in the fragile fiber 7 (fra7) mutant that causes alterations in cell morphogenesis, cell wall synthesis and actin organization (Zhong et al., 2005). SAC proteins modulate levels of phosphoinositides and AtSAC1 has also been shown to localise to the Golgi, indicating possible links with the GAAPs.

Overexpression of AtGAAP5-YFP fusion protein led to a severe dwarf phenotype. Also, enhanced leaf senescence and development of spontaneous lesions was observed in both rosette and cauline leaves of these plants. This phenotype resembles that of lesion mimic mutants (LMM) of Arabidopsis. LMMs form spontaneous lesions in the absence of pathogen challenge and mutated genes are thus thought to correspond to repressors of PCD (Lorrain et al., 2003). It is interesting that overexpression of AtGAAP5, homologous to a known inhibitor of apoptosis in mammalian cells, would lead to a phenotype typically associated with KO of a repressor of cell death. Observed phenotype could be due to a possible dominant-negative effect of AtGAAP5-YFP.
Chapter 5: Subcellular localisation of AtGAAP

5.1 Introduction

The secretory pathway, also called the endomembrane system, of eukaryotic cells is a highly dynamic network of membrane-bound compartments. It consists of the nuclear envelope (NE), the endoplamic reticulum (ER), the Golgi apparatus and the vacuoles/lysosomes. It also includes various post-Golgi intermediate organelles, referred to as endosomes in animal cells. In plant cells, post-Golgi compartments include the prevacuolar compartment/multivesicular body (PVC/MVB) (Foresti & Denecke, 2008). Small membrane-bound secretory vesicles shuttle between the compartments, budding from one organelle and fusing with another, thereby delivering their cargo and membrane material (Bonifacino & Glick, 2004). Biosynthetic products are delivered from the ER to the Golgi, where they are sorted for transport to the plasma membrane or the lysosome/vacuole. This forward traffic is referred to as anterograde transport. The same pathway functions also in the opposite direction, whereby plasma membrane proteins and extracellular material can be internalised through endocytosis followed by transport to the lysosome/vacuole or to the Golgi for sorting and recycling. Membrane lipids and trafficking machinery are continuously being recycled between the organelles (Zouhar et al., 2010).

The ER is responsible for many specialised functions including protein synthesis, folding and modification and lipid and polysaccharide synthesis. Proteins, lipids and other molecules are further modified in the Golgi. The Golgi plays a major role in sorting these molecules for transport to other organelles, mainly the lysosome/vacuole or the plasma membrane (Alberts et al., 1994). Golgi is also a major site for biosynthesis of cell wall polysaccharides in the plant cell (Perrin et al., 2001). The Golgi consists of multiple stacked cisternae, flattened, membrane-enclosed compartments. In animal cells multiple Golgi stacks are linked, forming a single complex (Alberts et al., 1994). In plant cells individual Golgi stacks are distributed throughout the cytoplasm, migrating over the ER network (Boevink et al., 1998). Lysosomes are organelles that contain hydrolytic enzymes and are responsible for intracellular digestion of macromolecules. Plant vacuoles are related to animal cell
lysosomes but are extremely versatile in function. Like their animal counterpart, plant vacuoles contain hydrolytic enzymes and can degrade cellular substrates. They can store defence compounds and other secondary metabolites, and may specialise in accumulating storage proteins. The central vacuole can occupy most of the cell volume and is essential for turgor maintenance and can drive cell expansion. Distinct vacuole types may function in the same cell, for example, one specialised in digestion and one for storage (Marty, 1999).

Evidence is accumulating that indicates the involvement of the endomembrane system in regulation of apoptosis in animal cells and programmed cell death in plant cells. In animal cells, the ER is known to regulate apoptotic responses. Certain stress conditions lead to accumulation of unfolded and misfolded proteins in the ER lumen due to reduced folding capacity of the ER, a phenomenon known as ER stress. If adaptive responses fail, the cell launches an apoptotic response (Boyce & Yuan, 2006). ER stress is also able to induce PCD in plants (Watanabe & Lam, 2008b; Zuppini et al., 2004). Studies from both animal and plant systems indicate a role for the evolutionarily conserved cytoprotective protein Bax-inhibitor-1 in protecting cells against ER stress-induced cell death (Watanabe & Lam, 2009).

Vacuole-mediated cell death has been proposed as a novel mechanism utilised by plant cells and absent in animal cells (Hatsugai et al., 2006). Vacuolar processing enzyme (VPE), a vacuole-localised protease which exhibits caspase-like activity, has been shown to regulate pathogen-induced PCD in plant cells (Hatsugai et al., 2004; Kuroyanagi et al., 2005). VPE mediates the development of PCD in both tobacco and Arabidopsis plants. VPE deficient tobacco plants are unable to undergo cell death in response to tobacco mosaic virus infection (Kuroyanagi et al., 2005). Similarly, fungal toxin induced PCD was completely abolished in VPE deficient Arabidopsis plants (Hatsugai et al., 2004). Tonoplast rupture preceded the appearance of cell death symptoms and VPEs were required for the vacuolar collapse. It has been proposed that VPE activates target proteins, which provoke tonoplast rupture thus leading to release of hydrolytic enzymes from the vacuole to the cytosol ultimately leading to cleavage of cellular targets and PCD (Hatsugai et al., 2006).
It has been proposed that the Golgi, like the ER, may be able to sense stress signals and activate signalling cascades to adapt, or if the stress is too severe, activate apoptosis (Hicks & Machamer, 2005; Maag et al., 2003; Machamer, 2003). Evidence from research on mammalian cells support this idea. The Golgi fragments during apoptotic event (Mukherjee et al., 2007). Fragmentation is largely due to proteolytic cleavage of Golgi structural proteins, such as golgins, by caspases (Short et al., 2005). Initiator caspase-2 has been shown to localise to the Golgi and nucleus (Mancini et al., 2000). Caspase-2 is able to cleave Golgi structural protein Golgin-160. Expression of a cleavage resistant form of golgin-160 delayed the apoptotic response triggered by certain stress stimuli, for example the ER stress inducer thapsigargin, and death receptor Fas. However, an unaltered apoptotic response was observed in response to other apoptotic stimuli, suggesting that cleavage of Golgin-160 is only required for the progression of apoptosis induced by a subset of proapoptotic stimuli (Maag et al., 2005). Furthermore, the Caspase-2 cleavage fragment of Golgin-160 is targeted to the nucleus when ectopically expressed (Hicks & Machamer, 2002). Although evidence is lacking, it has been postulated that this fragment could influence apoptotic response through regulation of gene expression (Hicks & Machamer, 2002; Machamer, 2003). Another golgin, vesicle transport protein p115 is cleaved by caspases-3 and -8 during apoptosis (Chiu et al., 2002). Expression of a noncleavable mutant p115 delayed Golgi disassembly during apoptosis. Furthermore, C-terminal caspase fragment of p115 (CTF) translocates to the nucleus upon over-expression and its expression was sufficient to induce apoptosis independently of Golgi fragmentation (Chiu et al., 2002). Mukherjee and Shields (2009) further demonstrated that nuclear import of CTF was an early apoptotic event and that nuclear import of CTF was required for its pro-apoptotic function. The role of Golgi in apoptotic mechanisms is further supported by the recent identification of Golgi-localised anti-apoptotic proteins by Gubser et al. (2007). GAAPs protect cells against a variety of proapoptotic stimuli and it may mediate this through regulation of intracellular Ca\(^{2+}\) fluxes (de Mattia et al., 2009). Involvement of the Golgi in regulation of programmed cell death in plants has not been demonstrated to our knowledge. Characterisation of plant GAAP orthologues may shed light on the involvement of Golgi in PCD in plants.
Human and viral GAAPs have been shown to localise to the Golgi, but also to the ER when expressed at higher levels (Gubser et al., 2007). The aim of this chapter was to study intracellular localisation of AtGAAPs. Colocalisation of fluorescent protein-tagged AtGAAPs with organelle marker proteins was studied in Nicotiana benthamiana. Additionally, subcellular localisation of AtGAAPs was studied in stable transgenic Arabidopsis plants expressing fluorescent protein–tagged AtGAAPs.

5.2 Results

5.2.1 Analysis of plant organelle morphology using fluorescently labelled marker proteins

As a prelude to characterizing the subcellular localization of GAAP fluorescent protein fusions, we wanted to familiarize ourselves with the characteristic morphologies of plant organelles. Therefore, a set of in vivo organelle markers generated by Nelson et al. (2007) were obtained (see section 2.10.1). Markers for the Golgi apparatus, mitochondria, endoplasmic reticulum (ER), peroxisomes, plastids, the tonoplast and plasma membrane (PM) were obtained. Markers had been constructed by either fusing well-established short targeting signals to the fluorescent protein coding region, or, in the case of the tonoplast and PM markers, by fusion of fluorescent proteins to full length proteins (Nelson et al., 2007).

Marker proteins were transiently expressed in Nicotiana benthamiana leaf tissue using Agrobacterium-mediated transformation. The inoculated area was analyzed using confocal laser scanning microscope over a four day period post inoculation. Characteristic morphology of the Golgi and the tonoplast were additionally studied in transiently transformed Arabidopsis leaf epidermal cells. Tonoplast localisation was also studied in a stable Arabidopsis line expressing CFP-labelled tonoplast marker.

Figure 5-1(A-F) shows representative images of organelle markers in N. benthamiana leaf epidermal cells three days post inoculation. Punctate organelles, namely the Golgi, mitochondria, peroxisomes and plastids, were visualised in the plane of the cortical cytoplasm underlying the PM. Fluorescently labelled Golgi is observed as a
series of discrete mobile punctate structures within the cell (Figure 5-1A). Individual Golgi stacks mostly appeared as small (<1 μm) perfectly round discs. Appearance of Golgi stacks inside Arabidopsis leaf epidermal cells was similar to that observed for N. benthamiana (Figure 5-1I). Mitochondria displayed a similar appearance to the Golgi, with high copy numbers inside each cell (Figure 5-1B). However, instead of perfectly round shape typical of the Golgi, mitochondria were often elongated in appearance and hence easily distinguishable from the Golgi stacks. Golgi and mitochondria could also be distinguished from each other by their actin-myosin-dependent movement patterns in the cytoplasm (data not shown). Peroxisomes were imaged as small, round structures inside the cells (Figure 5-1C). They were approximately five times larger than the Golgi and mitochondria and present in lower copy numbers inside the cell. Plastids were the largest of the small and round organelles, typically approximately 10 μm in diameter and present in low copy numbers in leaf epidermal cells (Figure 5-1D).

In contrast to the small, punctate organelles described above, ER, tonoplast and PM displayed a long and extended morphology within the epidermal cells. The appearance of the ER depended on the position of the focal plane within the cell. A reticular network that stretched throughout the cytoplasm was seen when ER was viewed in the plane of the cortical cytoplasm underlying the PM (Figure 5-1E). When viewed in a medial plane, the ER appeared as a stippled line in the thin layer of cytoplasm situated in between the PM and the tonoplast (Figure 5-1F).

Marker proteins for the tonoplast and the PM appeared to localise to the ER upon transient expression in Nicotiana benthamiana. For this reason morphology of the tonoplast was viewed in a stable Arabidopsis line expressing a CFP-tagged tonoplast marker protein. A GFP-tagged receptor-like kinase was used as a marker for the plasma membrane in Arabidopsis (Natalia Dinischiotu and Bart Feys, personal communications). The tonoplast, the delimiting vacuolar membrane, could be seen as a continuous line along the outline of the epidermal cells when focusing near the centre of the cell (Figure 5-1H). A clear gap between the tonoplasts of neighbouring cells could be seen (Figure 5-1H, white arrow); this gap contains the cell wall, plasma membrane, and the cytoplasm that is pushed against the plasma membrane by the central vacuole. Transvacuolar strands, tonoplast-delimited cytoplasmic channels that
traverse the lumen of the central vacuole, were clearly visible in some cells (Figure 5-1H, red arrow). These strands were constantly being remodelled. Spherical structures of varying sizes were observed inside the vacuolar lumen of some epidermal cells (Figure 5-1H, arrowhead). These structures, referred to as ‘bulbs’ in the literature, are invaginations of the tonoplast (Saito et al., 2002). The bulbs could be seen moving inside the lumen of the vacuole. The plasma membrane could be seen as a continuous layer along the outline of the cell (Figure 5-1G). A very narrow, non-fluorescent cell wall space could be seen between the plasma membranes of some neighbouring cells. However, this gap was very narrow and often indistinguishable. The PM marker could be distinguished from the tonoplast marker by the narrower gap between neighbouring cells, and the absence of bulbs and transvacuolar strands in the case of the PM marker.
Figure 5-1: Characteristic morphologies of plant organelles

Fluorescent markers specific for individual organelles were transiently expressed in *Nicotiana benthamiana* (A-F) or Arabidopsis (I) leaf tissue or viewed in stable transgenic Arabidopsis lines (G-H). (A) and (I) Golgi appeared as small (<1μm) round spots throughout the cells. (B) Mitochondria were found throughout the cells as small (<1μm), elongated spots. (C) Peroxisomes appeared as round (approximately 3μm) spots and were present in lower copy numbers than Golgi and mitochondria. (D) Plastids appeared as large (approximately 10μm) spherical structures and were present in low copy numbers inside the cells. (E) Endoplasmic reticulum (ER) viewed in the plane of the cortical cytoplasm appeared as a reticular network. (F) ER appeared as a stippled line when viewed in a medial plane. (G) Plasma membrane appeared as a uniform line along the cell surface. (H) Tonoplast marker appeared as a continuous line along the outline of the cells. A clear gap (containing the cell wall and cytoplasm) between tonoplast membranes of neighbouring cells was visible (white arrow). Spherical structures, ‘bulbs’, which are invaginations of the tonoplast could be seen moving inside the lumen of the vacuole (arrow head). Tonoplast marker also labelled transvacuolar strands (red arrow). Images were captured with confocal laser scanning microscope. Scale bar = 15 μM.
5.2.2 Generation of Arabidopsis transgenic lines expressing AtGAAP-YFP/GFP fusion proteins

Human and viral GAAPs are resident in the Golgi (Gubser et al., 2007). In order to study localization of AtGAAPs within the plant cell, transgenic plants expressing fluorescent protein-tagged versions of AtGAAPs were generated. Constructs were generated that carried a green (AtGAAP4) or yellow (AtGAAP1, AtGAAP2 and AtGAAP5) fluorescent protein (GFP/YFP) reporter fused in frame to the C-terminus of AtGAAP. Expression of the fusion construct was driven by the strong constitutive 35S promoter. Five separate attempts to clone AtGAAP3 construct failed, with a different mutation leading to an amino acid change occurring with each attempt. AtGAAP-G/YFP fusion constructs were transformed into Agrobacterium for transient expression in Nicotiana benthamiana and stable transformation of Arabidopsis plants. Arabidopsis gaap knockout plants, where available at the time (atgaap2, atgaap4 and atgaap5), were transformed with the G/YFP fusion constructs, and wild type Col-0 plants were transformed with AtGAAP1-YFP and pGWB5 (35S-GFP) and pEG101 (35S-YFP) control GFP/YFP constructs. The control vector constructs (pGWB5 and pEG101) used in this study contain unfused fluorescent tags as Gateway cassette spans the region between the promoter and the fluorescent tag. Initial attempts to recover AtGAAP4-GFP T1 transformants failed. Although such plants have now been recovered, transgenic lines have not been thoroughly analysed due to time constraints. Therefore data is only presented for AtGAAP1-YFP, AtGAAP2-YFP and AtGAAP5-YFP stable transgenic lines.

5.2.3 Subcellular localization of AtGAAPs in Nicotiana benthamiana

Human and viral GAAPs have been shown to localize to the Golgi (Gubser et al., 2007). Based on the high sequence conservation within the GAAP gene family, we predicted AtGAAPs also to be resident in the Golgi. To test this prediction, fluorescent protein-tagged AtGAAPs were co-expressed with a Golgi-CFP marker protein in Nicotiana benthamiana leaf tissue. Nicotiana benthamiana leaf tissue was co-infiltrated with equal amounts of Agrobacterium cells containing AtGAAP-G/YFP fusion constructs and Agrobacterium cells harbouring the Golgi-CFP marker plasmid.
Inoculated area was analysed for G/YFP and CFP fluorescence using confocal laser scanning microscope one to four days post-inoculation.

5.2.3.1 Identifying cross-talk between fluorochromes

Spectral properties of some fluorochromes overlap causing cross talk when doing simultaneous scanning. In order to identify possible cross talk between YFP and CFP, leaf discs that had been co-inoculated with AtGAAP5-YFP and Golgi-CFP were analysed using simultaneous scanning for both fluorochromes. The 514nm and 458nm laser lines, close to excitation maximum of YFP and CFP, respectively, were switched on and scan parameters were adjusted to simultaneously record emission from YFP and CFP. Golgi was clearly visible in images from both YFP and CFP channels. 458nm laser line was reduced to zero in order to detect possible bleed through to CFP channel. Under these settings, Golgi was clearly visible in YFP channel, and no signal was detected in the CFP channel, indicating no bleed through from the YFP channel. Next, the 458nm laser line was reset to optimal settings, and the 514nm laser line was reduced to zero. Under these settings, Golgi was clearly visible in CFP channel. Additionally, fluorescence could be detected in YFP channel, although it was considerable weaker than that seen under optimal settings. Observed cross-talk indicated either that the 458nm laser line was also exciting YFP, or that the emission slot for YFP was also detecting CFP.

To overcome the problem of cross talk, all co-localisation images shown in this thesis were taken in sequential scanning mode, rather than simultaneously, eliminating the problem of cross-talk.
5.2.3.2 AtGAAPs localise to the Golgi one and two days post inoculation

Sequential scans were performed on leaf epidermal cells of plants co-infiltrated with *Agrobacterium* carrying AtGAAP-G/YFP and Golgi-CFP constructs one and two days post-inoculation. Small, punctate structures distributed throughout the epidermal cells could be observed with both G/YFP and CFP channel when focusing on the cortical cytoplasm that lies immediately adjacent to the outer periclinal cell walls. When images from G/YFP and CFP channels were merged, it became evident that the fluorescent areas corresponded to the same structures. Figure 5-2 shows co-localisation of AtGAAP1, AtGAAP2, AtGAAP4 and AtGAAP5 with the Golgi marker protein in an area of approximately three epidermal cells. Magnification of selected Golgi stacks for all samples are shown in Figure 5-3. No fluorescence could be observed in plants inoculated with control vector constructs (Figure 5-2M, 5-2P, 5-3M, 5-3P). Hence it can be concluded that AtGAAP1-YFP, AtGAAP2-YFP, AtGAAP4-GFP and AtGAAP5-YFP fusion proteins localised to the Golgi at one and two days post-inoculation.
Figure 5-2: AtGAAP-G/YFP fusion proteins co-localise with Golgi-CFP marker in *Nicotiana benthamiana* leaf epidermal cells two days post-inoculation

(A-L) Low-magnification confocal images of G/YFP and CFP fluorescence in leaf epidermal cells of plants co-infiltrated with *Agrobacterium* carrying AtGAAP-G/YFP and Golgi-CFP constructs two days post-inoculation. AtGAAP-G/YFP fluorescence, Golgi-CFP fluorescence and merged images are shown.

(M-R) Low-magnification confocal images of (M) YFP and (P) GFP fluorescence in epidermal cells of leafs infiltrated with pEG101 and pGWB5 control vector constructs. (N,Q) Corresponding bright field images and (O,R) merged images are also shown. Scale bar = 15 μM.
Figure 5-3: AtGAAP-G/YFP fusion proteins co-localise with Golgi-CFP marker in *Nicotiana benthamiana* leaf epidermal cells two days post-inoculation
(A-L) High-magnification confocal images of G/YFP and CFP fluorescence in leaf epidermal cells of plants co-infiltrated with *Agrobacterium* carrying AtGAAP-G/YFP and Golgi-CFP constructs two days post-inoculation. AtGAAPG/YFP fluorescence, Golgi-CFP fluorescence and merged images are shown as indicated.
(M-R) High-magnification confocal images of (M) YFP and (P) GFP fluorescence in epidermal cells of leafs infiltrated with pEG101 and pGWB5 empty vector constructs. (N,Q) Corresponding bright field images and (O,R) merged images are also shown. Scale bar = 15 μM.
5.2.3.3 AtGAAP4 and AtGAAP5 localise to the Golgi three and four days post-inoculation

Subcellular localisation of AtGAAP proteins in relation to the Golgi marker was analysed three and four days post-inoculation. AtGAAP4-GFP and AtGAAP5-YFP fusion proteins co-localised exclusively with the Golgi marker protein, as shown in Figure 5-4. Localisation observed was identical to the pattern observed one and two days post-inoculation. No GFP or YFP signal was detected from plants inoculated with the corresponding empty vector constructs (Figure 5-4M, 5-4P).
Figure 5-4: AtGAAP4-GFP and AtGAAP5-YFP fusion proteins co-localise with Golgi-CFP marker in *Nicotiana benthamiana* leaf epidermal cells three days post-inoculation.

Plants were co-infiltrated with *Agrobacterium* carrying AtGAAP-G/YFP and Golgi-CFP constructs and viewed under confocal microscope three days post-inoculation. 
(A-C) Low- and (D-F) high-magnification confocal images of AtGAAP4-GFP and Golgi-CFP fluorescence and merged images as indicated. 
(G-I) Low- and (J-L) high-magnification confocal images of AtGAAP5-GFP and Golgi-CFP fluorescence and merged images as indicated. 
(M-R) High-magnification confocal images of (M) GFP and (P)YFP fluorescence in epidermal cells of leafs infiltrated with pGWB5 and pEG101 control vector constructs. 
(N, Q) Corresponding bright field images and (O, R) merged images are also shown. Scale bar = 15 μM.
5.2.3.4 AtGAAP1 and AtGAAP2 localise to the Golgi and the tonoplast three and four days post-inoculation

When AtGAAP1-YFP and AtGAAP2-YFP fluorescence was viewed under photomultiplier gain and offset settings used for viewing AtGAAP5-YFP fluorescence, distinct spots co-localising with the Golgi-marker could be observed when focusing on an optical section passing through the cortical cytoplasm (Figure 5-5). However, majority of YFP fluorescence in these cells came from overexposed structures distinct from the Golgi, especially when viewing the section passing through the centre of the cell. Photomultiplier gain for the YFP channel was reduced to identify these structures and to capture images where these structures were not overexposed (Figure 5-6).
### Figure 5-5: AtGAAP1-GFP and AtGAAP2-YFP fusion proteins co-localise with Golgi-CFP marker in *Nicotiana benthamiana* leaf epidermal cells three days post-inoculation

Plants were co-infiltrated with *Agrobacterium* carrying AtGAAP-G/YFP and Golgi-CFP constructs and viewed under confocal microscope three days post-inoculation.

(A-C) Low- and (D-F) high-magnification confocal images of AtGAAP1-YFP and Golgi-CFP fluorescence and merged images as indicated.

(G-I) Low- and (J-L) high-magnification confocal images of AtGAAP2-YFP and Golgi-CFP fluorescence and merged images as indicated. Scale bar = 15 μM.
The majority of YFP fluorescence of AtGAAP1 and AtGAAP2-tagged constructs did not co-localise with fluorescence of the Golgi marker protein as shown in Figure 5-6A-C and 5-6G-I, respectively. Instead, outline of the cells was clearly visible and ring structures were observed inside the epidermal cells. When cells were viewed under higher magnification (Figure 5-6D-F, 5-6J-L), it became evident that the YFP fluorescence pattern was similar to that observed for the tonoplast marker (Figure 5-1H). Cell wall space between two neighbouring cells was clearly visible (Figure 5-6D, 5-6J, arrows) and ring-like structures resembling the bulbs described by Saito et al. (2002) were present in most epidermal cells. Images shown in Figure 5-6 were taken from an optical section passing through the centre of the cells. CFP signal originating from the Golgi bodies in the thin layer of cortical cytoplasm situated in between the large central vacuole and the plasma membrane can be seen in Figure 5-6B, E, H and K. Although YFP fluorescence signal from the tonoplast mostly dominated the images taken for AtGAAP1-YFP and AtGAAP2-YFP, some YFP fluorescence also co-localised with CFP signal from the Golgi stacks as visualised in the merged images taken at high magnification (Figure 5-6F, 5-6L, arrows). A similar pattern was observed four days post-inoculation. However, only a very weak signal colocalising with the Golgi marker could be detected, especially for AtGAAP2-YFP.

In order to confirm the tonoplast localisation of AtGAAP1-YFP and AtGAAP2-YFP, these constructs were co-infiltrated into N. benthamiana leaf tissue with the tonoplast-CFP marker protein on three separate occasions. This experiment failed as each time the tonoplast marker protein mislocalised to the ER.

Characteristic morphology of the tonoplast has been well documented in the literature (Nelson et al., 2007; Saito et al., 2002). We have also previously observed this characteristic morphology in epidermal cells of stable Arabidopsis plants expressing the tonoplast-CFP marker protein (Figure 5-1H). Based on this data, we conclude that the fluorescence signal emitted by AtGAAP1-YFP and AtGAAP2-YFP three and four days post Agrobacterium-mediated expression derived mainly from the tonoplast, with some signal emerging from the Golgi.
Figure 5-6: AtGAAP1-YFP and AtGAAP2-YFP fusion proteins localise to the tonoplast and the Golgi three days post inoculation

Plants were co-infiltrated with Agrobacterium carrying AtGAAP-G/YFP and Golgi-CFP constructs and viewed under confocal microscope three days post-inoculation.

(A-C) and (G-I) Low-magnification confocal images of AtGAAP1-YFP, AtGAAP2-YFP and Golgi-CFP fluorescence and merged images as indicated. Majority of AtGAAP1-YFP fluorescence was detected in structures distinct from the Golgi.

(D-F) and (J-L) High-magnification confocal images of AtGAAP1-YFP, AtGAAP2-YFP and Golgi-CFP fluorescence and merged images as indicated. AtGAAP1-YFP (D) and AtGAAP2-YFP (J) fluorescence pattern was characteristic of the tonoplast. Arrows denote the cell-wall space between two neighbouring cells. Bulbs, circular extensions of the tonoplast are visible. (F) and (L) Some YFP fluorescence also co-localised with CFP signal from the Golgi stacks, as indicated by arrows. Scale bar = 15 μM.
5.2.4 Subcellular localization of AtGAAP-G/YFP fusion proteins in transgenic *Arabidopsis* lines

In order to study subcellular localisation of AtGAAPs in *Arabidopsis* cells, we generated stable transgenic Arabidopsis plants expressing yellow fluorescent protein–tagged AtGAAPs under the constitutive cauliflower mosaic virus (CaMV) 35S promoter. AtGAAP-G/YFP fusion constructs were transformed into *gaap* mutant backgrounds, where available at the time, including *atgaap2* and *atgaap5*, in order to also test for functionality of the fusion proteins. Wild type Col-0 plants were transformed with AtGAAP1-YFP and pEG101 and pGWB5 control vector constructs. Three independent T1 primary transformants harbouring the fusion constructs were selected for each AtGAAP. At least 12 T2 plants from each line were analysed for expression level and subcellular localisation of the fusion proteins.

5.2.4.1 Selection of transgenic plants with varying expression levels of AtGAAP-G/YFP fusion proteins

Expression levels of the AtGAAP-G/YFP fusion proteins were analysed by viewing detached rosette leaves under UV light using a stereofluorescence microscope. In order to quantify the expression levels of the fusion proteins, microscope settings were adjusted so that fluorescence from the plant with the highest fluorescence/expression level was not overexposed. Digital images were acquired from all plants using these settings, and fluorescence was quantified using ImageJ software.

The T2 population of AtGAAP1-YFP and AtGAAP2-YFP transgenic lines showed a broad range of fluorescence levels (Figure 5-7). Images for these plants were acquired using identical settings on the stereofluorescence microscope. For AtGAAP5-YFP, only plants with very low expression levels were recovered. For AtGAAP5, images of the leaves shown in Figure 5-7 were acquired with higher magnification and longer exposure time compared to AtGAAP1 and AtGAAP2. The fluorescence units shown in Figure 5-7 are directly comparable for all transgenic lines shown as these were calculated from images taken with identical settings. Digital images of YFP
fluorescence of rosette leaves and fluorescence units of representative plants with the highest, lowest and a medium level of fluorescence are shown in Figure 5-7. Plants exhibiting such levels of fluorescence are hereafter referred to as low, medium and high expresser.

![Fluorescence Images](image)

Figure 5-7: Selection of stable transgenic Arabidopsis lines with varying expression levels of AtGAAP-YFP fusion proteins

Stereofluorescence microscope images of plants expressing (A-D) AtGAAP1-YFP, (E-H) AtGAAP2-YFP, and (I-L) AtGAAP5-YFP to high, medium and low levels as indicated. Images (A-H) were taken with identical settings. Images (I-L) were taken with higher magnification and longer exposure time. The values represent arbitrary fluorescence units (fu) as quantified by ImageJ software. Fluorescence values are directly comparable between all samples.

5.2.4.2 AtGAAP1 localises to the Golgi at low expression levels, and the tonoplast at medium and high expression levels

Subcellular localisation of AtGAAP1 was studied in plants with varying expression levels of the AtGAAP1-YFP fusion protein. Figure 5-8 summarizes the pattern of AtGAAP1-YFP fluorescence in leaf epidermal cells of plants that expressed the transgene at low levels. Fluorescence was observed in a series of discrete mobile punctate structures typical of the plant Golgi apparatus. Outline of the cells was also visible as a haze of YFP fluorescence. No fluorescence was detected in plants expressing the corresponding empty vector construct (Figure 5-8D).
Figure 5-8: AtGAAP1-YFP localizes mainly to the Golgi in stable Arabidopsis lines at low expression levels

Low- (A-B), medium- (E-F) and high- (G-H) magnification images of YFP fluorescence in leaf epidermal cells of stable transgenic Arabidopsis lines that express AtGAAP1-YFP transgene at low levels. (A, E, G) Images were taken from a section passing through the cortical cytoplasm. (B, C, F, H) Images were taken from a section passing through the centre of the cells. (A-C), (E-F) and (G-H) correspond to different optical sections of the same cells. (B and C) YFP fluorescence image and bright field image of the same position. (D) No YFP-fluorescence was detected in cells expressing pEG101 empty vector construct. Scale bar = 15 μM.

Figure 5-9 shows representative images of YFP fluorescence within epidermal cells of plants that express the transgene to medium levels. The pattern of YFP fluorescence in plants that express the AtGAAP1-YFP fusion protein at medium levels was very distinct from that observed for the low expressers. The fluorescence observed mostly showed typical tonoplast patterns; fluorescence signal outlining the epidermal cells was uniform in both intensity and thickness (Figure 5-9B, E, F, G). Cell wall space between two neighbouring cells was clearly visible (Figure 5-9E, F, G, arrows). Fusion proteins also localized in vacuolar structures that resemble the bulbs described by Saito et al. (2002) (Figure 5-9B, F). These bright and spherical structures with varying diameter were observed in all epidermal cells studied. A highly fluorescent single membrane seemingly emerging from the tonoplast and appearing to divide the cell into sections could be observed in some cells (Figure 5-9B, E arrowheads). This structure was not apparent in the majority of cells and usually only one copy was observed per cell. When cells were viewed in a medial plane, punctate structures resembling the Golgi could be observed (Figure 5-9A, D). However, vacuolar bulbs
were also visible at this plane of view, and it is possible that the small structures are small vacuolar bulbs instead of the Golgi.

Figure 5.9: AtGAAP1-YFP localises mainly to the tonoplast at medium expression levels in stable Arabidopsis lines
YFP fluorescence images of leaf epidermal cells of stable transgenic Arabidopsis lines that express AtGAAP1-YFP transgene at medium levels.
(A-C) Low-magnification YFP fluorescence or brightfield images taken at the same position of the sample. (A) Section passing through the cortical cytoplasm, (B) Section passing through the centre of the cells. (C) Brightfield image.
(D-G) High-magnification confocal images of AtGAAP1-YFP fluorescence. (A) Section passing through the cortical cytoplasm. Golgi and small vacuolar bulbs visible. (E-F) Section passing through the middle of the cell. Typical tonoplast morphology is observed. Arrows denote cell wall space between neighbouring cells. Arrowheads denote a single membrane that is possibly an extension of the tonoplast. Scale bar = 15 μM.

Figure 5-10 shows representative images of the pattern of YFP fluorescence observed in cells that express AtGAAP1-YFP fusion protein to high levels. As for the medium expressor, a typical tonoplast pattern was observed in optical sections passing through the middle of the cell (Figure 5-10B, E, F). A highly fluorescent single membrane, previously described for the medium expresser, could be observed in almost all cells (Figure 5-10B, E, F). Furthermore, most cells were divided into several, typically four to six, sections by this single membrane. Arrows in Figure 5-10B point to three such membranes within a single epidermal cell. In contrast to that observed for the medium expresser, bulbs were not visible in sections passing through the centre of the cell (Figure 5-10B, E, F). However, small bulbs were visible when viewing the cell in the
medial plane (Figure 5-10A, D). Also, larger bulbs could be observed in the lumen of some large epidermal cells (Figure 5-10G). As observed for the medium expressor, Golgi-like structures could be observed in sections passing through the cortical cytoplasm (Figure 5-10A, D). It is, however, difficult to distinguish whether these correspond to the Golgi or small vacuolar bulbs.

Figure 5-10: AtGAAP1-YFP localises mainly to the tonoplast at high expression levels in stable Arabidopsis transgenic lines

YFP fluorescence images of leaf epidermal cells of stable transgenic Arabidopsis lines that express AtGAAP1-YFP transgene at high levels.
(A-C) Low magnification YFP fluorescence or brightfield images taken at the same position of the sample. (A) Section passing through the cortical cytoplasm, (B) Section passing through the center of the cells. Arrows denote single membranous structures that divides this particular epidermal cell to four sections (C) Brightfield image.
(D-G) High-magnification confocal images of AtGAAP1-YFP fluorescence. (A) Section passing through the cortical cytoplasm. Golgi and small vacuolar bulbs visible. (E-F) Section passing through the middle of the cell. Typical tonoplast morphology is observed. Highly fluorescent single membranes apparently emerging from the tonoplast are also present. (G) Tonoplast bulbs were present in large epidermal cells. Scale bar = 15 μM.

In addition to the structures described above, transvacuolar strands could be observed in large epidermal cells of all AtGAAP1-YFP expressing plants, regardless of expression level. In plants expressing the transgene at low levels, Golgi-like structures could be observed moving along these strands (Figure 5-11A, B). In high expressers,
cytoplasmic strands seemed to contain vacuolar bulbs (Figure 5-11C). In medium expressors, both Golgi, and bulbs could be observed moving along transvacuolar strands (Figure 5-11D, E, F).

Figure 5-11: Transvacuolar strands in large epidermal cells of AtGAAP1-YFP expressing plants
YFP fluorescence was detected in transvacuolar strands of plants expressing AtGAAP1-YFP transgene to (A,B) low, (C) high, and (D-F) medium levels. (A,B,D,E) Golgi and (C,F) vacuolar bulbs move along these tonoplast-delimited cytoplasmic tunnels. Scale bar = 15 μM.

5.2.4.3 AtGAAP2-YFP fusion protein localises mainly to the tonoplast

Subcellular localisation of AtGAAP2-YFP fusion protein was studied in transgenic Arabidopsis plants expressing the fusion protein at low, medium and high levels. The membrane of the central vacuole was clearly visible in all transgenic lines (5-12 A,B,C). Additionally, highly fluorescent bulbs of varying sizes were present in the cells of the medium expressor (5-12B). Highly fluorescent single membranes dividing the cells into a number of sections predominates the image of the high expressor (5-12C).
Figure 5-12: AtGAAP2-YFP fusion protein localises mainly to the tonoplast in stable Arabidopsis lines

(A-C) Low magnification images of YFP fluorescence in Arabidopsis leaf epidermal cells expressing AtGAAP2-YFP transgene to low (A), medium (B), and high (C) levels. Fluorescent tonoplast is visible in all samples. (B) Vacuolar bulbs are present in medium expressor. (C) Highly fluorescent single membrane, apparently an extension of the tonoplast is visible in high expressor.

(D-F) Corresponding bright field images of leaf epidermal cells. Scale bar = 15 μM.

Figure 5-13 shows more detailed images of the pattern of YFP fluorescence in these plants. YFP fluorescence highlighted the tonoplast of the central vacuole in all transgenic lines expressing the AtGAAP2-YFP transgene. Non-fluorescent cell wall space between neighbouring cells was clearly detected (Figure 5-13A, B, C, arrows). A number of vacuolar bulbs of varying sizes were present in the cells of the medium expresser (Figure 5-12B and 5-13E). Small to medium sized bulbs, typically one to three, could also be detected in a number of cells of the low expresser (Figure 5-12A and 5-13D). No large bulbs characteristic of the medium expressor were present in the low expressor. Only the largest epidermal cells of the high expressor contained bulbs (Figure 5-13F, 5-13I). Transvacuolar strands, along which bulbs appeared to be moving, could be observed in large epidermal cells of all AtGAAP2-YFP transgenics.
(Figure 5-13G, H, I, arrowheads). All epidermal cells of the high expressor were divided into several sections by highly fluorescent single membranes (Figure 5-12C, 5-13C, L). This membrane appears to be an extension of the tonoplast and was previously described for AtGAAP1-YFP. This structure was also present in a small number of cells of the medium expressor, with one cell containing typically only a single membrane (Figure 5-13K). Very few (less than 1%) of the cells of the low expressor contained this single membrane. However, some cells of the low expressor contained short, highly fluorescent strands that appeared to be connected to the tonoplast at one end (Figure 5-13J, arrowhead). These could be partially formed versions of the single membranous structure. Golgi-like structures could be observed in images taken from an optical section passing through the cortical cytoplasm of medium and high expressor (Figure 5-13N and 5-13O). No Golgi-like structures could be detected in cells of the low expressor. No YFP signal could be detected in plants transformed with the empty vector construct (Figure 5-13M).
Figure 5-13: AtGAAP2-YFP fusion protein localises mainly to the tonoplast in stable Arabidopsis lines

Confocal microscopy of YFP fluorescence in stable Arabidopsis lines expressing AtGAAP2-YFP transgene to (left panel) low, (centre panel) medium, and (right panel) high levels.

(A-C) Fluorescent tonoplast is visible. Arrows denote cell wall space between neighbouring cells.

(D-F) Vacuolar bulbs were present in all samples. Only a few cells of the low expressor contained bulbs. Every cell of medium expressor had several bulbs. Only large epidermal cells of the high expressor contained bulbs.

(G-I) Transvacuolar strands were present in large cells of all plants.

(J-L) Highly fluorescent single membranes divided cells of the high expressor into several sections. Some cells of the medium expressor contained this single membrane. Some cells of the low expressor contained a fluorescent strand that appeared to be connected to the tonoplast membrane at one end (arrow).

(N,O) Golgi-like structures could be seen in cells of the medium and high expressors in a section passing through the cortical cytoplasm.

(M) No YFP signal was detected in plants expressing pEG101 empty vector construct. Scale bar = 15 μM.

As described previously, the most highly fluorescent cellular structure of lines that expressed AtGAAP2-YFP to high levels was a single membrane appearing to emerge from the tonoplast (Figure 5-12C, 5-13C, 5-13L), whereas for medium expressors, vacuolar bulbs exhibited the highest YFP signal (Figure 5-12B, 5-13E). In epidermal cells of plants that expressed AtGAAP2-YFP at medium levels, structures were observed that appeared to be an intermediate between the single membrane and vacuolar bulb features. The images in Figure 5-14 suggest that the single membranous structure is transforming into a vacuolar bulb. We attempted to observe the disengagement of these structures from the tonoplast membrane, but due to bleaching of the YFP signal, it was not possible to follow this proposed process from start to the end.
5.2.4.4 AtGAAP5-YFP localises to the Golgi

Subcellular localisation of AtGAAP5 was studied in transgenic Arabidopsis plants expressing AtGAAP5-YFP fusion protein. In contrast to transgenic lines expressing AtGAAP1-YFP and AtGAAP2-YFP fusion proteins, we were unable to recover plants that expressed AtGAAP5-YFP fusion protein to medium or high levels. YFP fluorescence of all AtGAAP5-YFP lines was weaker than that of the lowest AtGAAP1-YFP and AtGAAP2-YFP transgenic lines recovered. Some variation in the level of AtGAAP5-YFP expression was observed between independent transgenic lines, but the subcellular pattern of fluorescence observed was indistinguishable between all the lines.

AtGAAP5-YFP expression showed a distinct punctate pattern of YFP-fluorescence typical of the Golgi (Figure 5-15). Feint YFP fluorescence could also be detected around the outline of the cells. This feint fluorescence did not appear to correspond to the pattern previously observed for the tonoplast. Transvacuolar strands could be observed in large epidermal cells, with distinct clusters of Golgi bodies travelling along these strands as shown by arrowheads in Figure 5-15J and 5-15K. No fluorescence was observed for cells expressing the control vector construct (Figure 5-15L).
Figure 5-15: AtGAAP5-YFP fusion protein localises to the Golgi in transgenic Arabidopsis lines
Confocal images of Arabidopsis plants expressing AtGAAP5-YFP fusion protein.
(A-C) Low-, (D-F) Medium-, and (G-I) High-magnification images of leaf epidermal cells expressing AtGAAP5-YFP transgene. Left and centre panel show images taken in an optical section passing through the cortical cytoplasm or middle of the cell, respectively. Right panel shows bright field images of the corresponding cells. (J, K) Arrows denote transvacuolar strands with Golgi bodies. (L) Plants transformed with pEG101 empty vector construct showed no YFP fluorescence. Scale bar = 15 μM.
5.3 DISCUSSION

Human and viral GAAP proteins have been shown to localise to the Golgi in stable human cell lines and transient transfection assays. Upon higher expression levels, the protein can also be detected in the endoplasmic reticulum (ER) (Gubser et al., 2007). In order to study subcellular localisation of AtGAAP proteins, fluorescent-protein labelled AtGAAPs were generated. Subcellular localisation of these proteins was analysed in planta upon transient expression in Nicotiana benthamiana leaf tissue and in stably transformed Arabidopsis plants.

Before submerging ourselves into analysis of subcellular localisation of AtGAAPs, we wanted to familiarise ourselves with the characteristic morphologies of plant organelles. Using a set of in vivo organelle markers, distinct fluorescence patterns were observed for the Golgi, mitochondria, peroxisomes, plastids, ER, plasma membrane (PM) and the tonoplast.

Based on sequence conservation within the GAAP gene family, we predicted AtGAAP localisation to mirror that of their human and viral counterparts. To test this prediction, co-localisation studies of AtGAAPs were performed with a Golgi marker protein in Nicotiana benthamiana leaf tissue. This analysis was carried out for AtGAAP1, AtGAAP2, AtGAAP4 and AtGAAP5, but not AtGAAP3. AtGAAPs colocalised exclusively with the Golgi marker 1 and 2 days post-inoculation. The same pattern was observed for AtGAAP4 and AtGAAP5 3 and 4 days post-inoculation. Interestingly, localisation of AtGAAP1 and AtGAAP2 was strikingly different 3 and 4 days post-inoculation; fluorescence emerged predominantly from structures resembling the tonoplast, although weak signal from the Golgi could also be detected.

Distinguishing whether a fluorescence signal emerges from the membrane of the central vacuole or the PM can be difficult (Held et al., 2008; Nelson et al., 2007). This is due to the fact that these membranes are juxtaposed and fluorescence signal from both membranes highlight the outline of the cells. Confirmation that AtGAAP1 and AtGAAP2 indeed localised to the tonoplast could be confirmed by colocalisation
studies with either a tonoplast or a PM marker protein. This was not possible with the marker protein set used in this study as both PM and tonoplast markers were found to mislocalize to the ER upon transient expression in *Nicotiana benthamiana* leaf tissue under our conditions. However, we are confident that the fluorescence signal observed for AtGAAP1/2-YFP three and four days post-inoculation emerged from the tonoplast. Firstly, bright and spherical structures could be detected in the vacuolar lumen. These structures were identical to structures described as ‘bulbs’ in the literature which represent extensions of the tonoplast (Hunter et al., 2007; Nelson et al., 2007; Saito et al., 2002). Also, transvacuolar strands could be observed in cells expressing AtGAAP1 and AtGAAP2. Unlike the tonoplast, the plasma membrane does not enter the interior of the cell, and is not found in transvacuolar strands (Nelson et al., 2007). We also observed this experimentally in Arabidopsis plants expressing the marker proteins. Secondly, we could observe a clear non-fluorescent space between two neighbouring cells upon AtGAAP1/2-YFP expression. The space between the tonoplasts of two neighbouring cells is relatively prominent as this space contains the cell wall and cortical cytoplasm of the neighbouring cells. Unlike the tonoplast, the space between the plasma membranes of two neighbouring cells contain only the cell wall, and hence this non-fluorescent space is less prominent and often indistinguishable (Nelson et al., 2007). Therefore we conclude that the fluorescence signal emitted by AtGAAP1-YFP and AtGAAP2-YFP three and four days post *Agrobacterium*-mediated expression derived mainly from the tonoplast, with some signal emerging from the Golgi.

Therefore we conclude that AtGAAP1, AtGAAP2, AtGAAP4 and AtGAAP5 localise mainly to the Golgi when expressed transiently in *Nicotiana benthamiana* leaf tissue. However AtGAAP1 and AtGAAP2, but not AtGAAP4 and AtGAAP5, also accumulate in the tonoplast, including membrane of the central vacuole, transvacuolar strands, and bulbs at later timepoints following transformation.

In order to study subcellular localisation of AtGAAPs in Arabidopsis cells, we generated stable transgenic Arabidopsis plants expressing AtGAAP1-, AtGAAP2- and AtGAAP5-YFP fusion proteins. We also wanted to determine whether the expression level of the fusion protein had an effect on subcellular localisation. For this purpose, a panel of plants with a range of YFP fluorescence levels were selected for
analysis. This analysis relied on the assumption that the level of YFP fluorescence reflects the amount of AtGAAP-YFP fusion protein within the cell. However, whether this is the case would have to be tested experimentally by Western blot. Dixit et al. (2006) primarily recommend the analysis of dimly expressing lines instead of brightly expressing lines for subcellular localisation studies as the likelihood of wild-type behaviour of the tagged proteins is higher in the former.

AtGAAP1 and AtGAAP2-YFP transgenic lines showed a broad range of fluorescence levels. Interestingly, all transgenic lines recovered for AtGAAP5-YFP showed weak fluorescence. This could indicate that expression of AtGAAP5-YFP above a certain threshold is lethal to the plant. AtGAAP5-YFP expression showed a distinct punctate pattern of YFP-fluorescence typical of the Golgi. Feint YFP fluorescence could also be detected around the outline of the cells. This could correspond to cytosolic localisation of a fraction of the fusion protein. A near identical pattern was observed for cells that expressed AtGAAP1-YFP at low levels. In contrast, cells that expressed AtGAAP2-YFP at low levels showed typical tonoplast patterns; vacuolar limiting membranes, transvacuolar strands and some bulbs were visible. We were unable to recover AtGAAP2-YFP transgenics with fluorescence levels as low as that observed for AtGAAP5-YFP and AtGAAP1-YFP. Based on the indistinguishable expression pattern of AtGAAP1-YFP and AtGAAP2-YFP constructs in Nicotiana benthamiana, we predict that AtGAAP2-YFP will also localize to the Golgi upon low expression levels in Arabidopsis plants. Screening of more primary transformants may confirm this.

Expression of AtGAAP1- and AtGAAP2-YFP at medium expression levels resulted in near-identical subcellular localisation consisting of a typical tonoplast pattern including transvacuolar strands and bulbs. Typically, both the number and size of bulbs was higher in AtGAAP2-YFP lines compared to AtGAAP1-YFP. This could be due to the fact that the genetic background of the plant was different, Col-0 and Ler, for AtGAAP1 and AtGAAP2, respectively. A highly fluorescent single membrane seemingly emerging from the tonoplast could also be observed in some cells of both AtGAAP1-YFP and AtGAAP2-YFP expressing lines. This membrane divided the cells into sections. No obvious identification for this structure was found in the literature. However, a close examination of images presented by Saito et al (2002) in
the paper originally describing bulbs reveals that this structure was also visible in cells expressing γ-TIP-GFP. We also observed structures that appeared to be intermediate between the single membrane and vacuolar bulb features. This suggests that the bulbs emerge from the tonoplast membrane through this intermediary structure. This theory is further supported by ultrastructural analysis performed by Saito et al. (2002) who reported that the membrane of the bulbs was in fact connected to the limiting vacuolar membrane.

Expression of AtGAAP1 and AtGAAP2 at high levels also resulted in near-identical subcellular localisation. Along with the limiting vacuolar membrane, the highly fluorescent single membranes were the most prominent structures observed. Several of these membranes were typically present in each cell, dividing the cell into a number of sections. The number of bulbs was generally reduced compared to a medium-level expresser, and were typically only present in large epidermal cells.

As described previously, AtGAAP1 and AtGAAP2 localised to the tonoplast upon higher expression levels. A striking feature of these cells was the presence of highly fluorescent ‘bulbs’. Bulbs were first described by Saito et al. (2002) and have since been observed by various groups (Hunter et al., 2007; Nelson et al., 2007). The fluorescence intensity of the bulb membrane has been reported to be higher than that of the vacuolar limiting membrane (Hunter et al., 2007; Saito et al., 2002). This feature was also apparent in AtGAAP1/2-YFP expressing cells. Saito et al. (2002) studied membrane dynamics of plant vacuoles in stably transformed Arabidopsis plants using a GFP-tagged γ-TIP (tonoplast intrinsic protein) driven by the strong 35S promoter. γ-TIP is commonly used as a marker for the lytic vacuolar membrane (Frigerio et al., 2008; Hunter et al., 2007; Saito et al., 2002). Saito et al.(2002) observed the bulbs mainly in enlarging cells of cotyledons and hypocotyls of young seedlings whereas these structures were absent in fully expanded cells. Hunter et al. (2007) confirmed this observation while studying localisation of YFP-tagged α-, γ-, and δ-TIP in Arabidopsis plants; bulbs were observed in cotyledons and young rosette leaves, but not in mature leaves. Although the function of bulbs is unclear, their presence in enlarging cells led the authors to speculate that bulbs could serve as a reservoir of vacuolar membrane in expanding cells. For our study, we used young rosette leaves of 4-5 week old plants. Our study therefore illustrates that these
structures may also be present in leaves of mature plants. However, for our analysis we used young rosette leaves of 4-5 week old plants in which cells were presumably still enlarging.

In order to confirm that the bulbs were authentic structures instead of artificial products caused by overexpression of γ-TIP-GFP, Saito et al. (2002) carried out ultrastructural analysis. Bulbs were present in both wild type and γ-TIP-GFP expressing cells, confirming that these structures were authentic. Examination by electron microscopy revealed that bulbs consisted of intricately folded double membrane that was continuous with the limiting vacuolar membrane. It is not clear how these structures are formed, but the single membraneous structures observed during our analysis that we propose to be an early form of these structures could shed light on this process. Cells that expressed GAAP1 and GAAP2 at medium levels contained a number of bulbs and only a few single membraneous structures whereas higher expression levels lead to a decrease in the number of bulbs and an increase in the number of single membranes. This could indicate that overaccumulation of AtGAAPs in the vacuole prevents the formation of the bulbs. This theory would have to be confirmed by ultrastuctural analysis. It is also possible that both the single membranes and bulbs are present within these cells at all times and the level of AtGAAP expression determines whether these proteins accumulate in bulbs or the single membranous structures. Ultrastructural analysis performed by Saito et al. (2002) revealed that larger structures were observed more frequently in γ-TIP-GFP expressing cells compared to wild-type. This could indicate that overaccumulation of fluorescent fusion proteins alters the native structure of the bulbs.

The function of bulbs is still unclear, but Saito et al. (2002) postulate that bulbs could serve as a reservoir of vacuolar membrane in expanding cells or alternatively as a store for hydrolytic enzymes. Interestingly, another vacuolar protein analysed by Saito et al. (2002), AtRab75c, did not localise to the bulbs, it was only observed in the membrane of the central vacuole. The presence of bulbs in these lines was confirmed by ultrastuctural analysis. This may indicate sorting mechanisms whereby some vacuolar proteins are excluded from the bulb structures whereas others proteins are concentrated in them.
In summary, AtGAAP1 and AtGAAP5, and possibly AtGAAP2, localise to the Golgi at low expression levels. AtGAAP1 and AtGAAP2 localise to the tonoplast upon medium and high expression levels. AtGAAP5 expression at medium and high levels might be lethal for the plants. We relied on the characteristic morphologies of the organelles for this analysis. In order to confirm that the observed structures indeed were the Golgi and the tonoplast, transient co-expression of marker proteins in the AtGAAP-YFP transformants could be carried out.

Although the subcellular analysis of AtGAAPs performed during this work was very informative, and is thought to reflect the localisation of native AtGAAPs, we are aware of the possible pitfalls of the approach used. Immunohistochemistry with antibodies specific for the protein of interest is considered to be the most reliable method for analysis of subcellular localisation of proteins as native protein under normal conditions can be detected using this method (Nelson et al., 2007). However, raising antibodies is time consuming and expensive and unlikely to yield AtGAAP -specific antibodies due to the high sequence conservation within this family. We therefore chose to study localisation using fluorescent protein fusions, a method commonly used in plant research. The presence of the fluorescent tag might affect the localisation or function of the protein. Therefore, functionality of the fusion protein should ideally be confirmed via complementation analysis (Dixit et al., 2006). In the absence of a phenotype for single atgaap mutants, this was not possible in our case. Strong constitutive promoters, such as the 35S promoter used in this study, are commonly used in subcellular localisation studies (Dixit et al., 2006; Hunter et al., 2007). However, overaccumulation of the protein may lead to detrimental phenotypes. This was observed upon overexpression of AtGAAP1-YFP, AtGAAP2-YFP and AtGAAP5-YFP fusion proteins in stable Arabidopsis transformants, as discussed in Chapter 4. High expression levels may still allow the protein to localize to its native location, but after saturation may lead to ectopic accumulation (Dixit et al., 2006; Sparkes et al., 2006). This was reported for ST, a Golgi marker that is synthesised at the ER and then moves to it’s final destination, the Golgi (Sparkes et al., 2006). Overaccumulation of ST-GFP leads to saturation of the trafficking pathway resulting in mislocalisation of the marker protein to the ER. This could be observed in neighbouring cells upon transient expression in tobacco leaf tissue; cells exhibiting weak fluorescence showed Golgi-localisation of ST-GFP whereas cells with higher
fluorescence level accumulated ST-GFP in the ER (Sparkes et al., 2006). This was also observed for AtGAAP1 and AtGAAP2 three days post-inoculation in N. benthamiana; most cells exhibited high fluorescence levels and localisation of AtGAAP in the tonoplast. The occasional cell with weak fluorescence levels was observed and the fluorescence in these cells emerged from the Golgi. It is therefore vital to follow the localisation of the protein at frequent intervals after transient expression (Sparkes et al., 2006). For stable lines, selecting the lines with lowest level of fluorescence is most informative, as these lines report wild-type behaviour with higher fidelity compared to highly fluorescent lines (Dixit et al., 2006). Using a native promoter of the protein of interest can reduce overexpression-related mislocalisation (Dixit et al., 2006). Use of native promoter can additionally provide valuable data of temporal and spatial expression pattern of the proteins of interest (Dixit et al., 2006; Hunter et al., 2007). Hunter et al. (2007) studied subcellular localisation of α-, γ-, and δ-TIP in stably transformed Arabidopsis plants using 35S and endogenous promoters. TIP fusions localised to the tonoplast with both promoters. However, using the endogenous promoters, the authors showed that α-, γ-, and δ-TIP expression is tissue and development specific.

Bearing all of the above in mind, we consider the true localisation of AtGAAPs to be the Golgi as this was the localisation of all AtGAAPs under low expression levels. Localisation of AtGAAP1 and AtGAAP2 to the tonoplast may be an artefact of overexpression. This would have to be confirmed in stable transgenic Arabidopsis plants by testing expression levels with western blot, or studying the subcellular localisation using the endogenous promoters. It is, however, intriguing that AtGAAP1 and AtGAAP2, but not AtGAAP4 and AtGAAP5 localised to the tonoplast upon higher expression levels. Overexpression of AtGAAP4 and AtGAAP5 above a certain threshold may be lethal. It would also be interesting to study localisation of AtGAAP3. AtGAAP1, AtGAAP2 and AtGAAP3 form a distinct clade based on sequence identity. It may be possible that these sequences contain a sorting signal necessary for transport to the tonoplast. Further work is necessary to analyse this.
Chapter 6: General discussion

Programmed cell death (PCD) plays an essential role in eukaryotes during development and in response to pathogens and abiotic stress signals. Although precise mechanisms governing PCD in plant cells remain largely obscure, some players of the molecular machinery are conserved between animals and plants. Golgi anti-apoptotic protein is proposed to be an inhibitor of cell death that is conserved throughout evolution. Human and viral GAAPs have been shown to inhibit cell death induced by a variety of pro-apoptotic stimuli and human GAAP is essential for cell survival (Gubser et al., 2007). Physiological importance of plant GAAPs is still unclear. In this study, we aim to characterise Arabidopsis thaliana GAAP orthologues.

Phylogenetic analysis revealed that GAAPs represent a multigene family in plants, with five and seven members present in the sequenced Arabidopsis and rice genomes, respectively. AtGAAP1-AtGAAP3 represent a distinct clade based on sequence conservation, which could imply conservation of function within members of this clade. AtGAAP4 and AtGAAP5 are distinct from this group and from each other, which may indicate diversified functions for these proteins. It has been proposed that sequences of genes governing plant development are less likely to diversify throughout evolution due to evolutionary pressure for functional conservation, compared to genes involved in more specialised processes, such as disease resistance genes, which are under evolutionary pressure to evolve distinct functions and recognition specificities (Fritz-Laylin et al., 2005). AtGAAPs are evolutionarily related to GAAP proteins from a number of species and also to Arabidopsis Bax inhibitor-1. AtGAAPs share a signature motif UPF0005 with proteins that have been shown to function as inhibitors of cell death, including mammalian and viral GAAP, Bax inhibitor-1, and Lifeguard (Chae et al., 2003; Gubser et al., 2007; Reimers et al., 2006; Reimers et al., 2007; Somia et al., 1999). Members of the UPF0005 gene family are predicted to contain six or seven transmembrane domains which is also the case for AtGAAPs (Gubser et al., 2007; Reimers et al., 2006; Walter et al., 1995). In summary, bioinformatic analysis has revealed a number of similarities that AtGAAPs share with proteins that have been shown to function as inhibitors of cell death. This
may indicate an evolutionarily conserved function for AtGAAPs as inhibitors of cell death.

Interestingly, GAAP, BI-1 and LFG orthologues have been identified in various plant genomes. BI-1 shows functional conservation throughout evolution; human BI-1 and its plant counterparts have been shown to function as inhibitors of cell death (Chae et al., 2003; Kawai et al., 1999; Sanchez et al., 2000). High sequence conservation across plant and animal kingdoms seems to be the exception rather than the rule for characterised cell death regulators, further highlighting the probability of functional conservation between GAAP orthologues. For example, orthologues of Bcl-2 family of proteins, members of which either inhibit or promote apoptosis in mammalian cells, have not been identified in plants. However, the function of these proteins is conserved in plants when expressed from a transgene (Kawai-Yamada et al., 2001; Lacomme & Cruz, 1999). Also, homologues of key executioners of mammalian cell death, caspases, have not been identified from plant genomes. However, caspase-like proteases do play a role in regulation of PCD in plants (Woltering, 2010).

The endomembrane system is involved in the regulation of apoptosis in animals and PCD in plants. It has been suggested that the Golgi may sense stress signals and either initiate recovery mechanisms, or initiate apoptosis if the stress is too severe (Hicks & Machamer, 2005; Machamer, 2003). Characterisation of human and viral GAAPs further demonstrates that the Golgi has an important role in the regulation of apoptosis in animal cells (Gubser et al., 2007). Human and viral GAAPs localise to the Golgi, but also to the ER when expressed at higher levels (Gubser et al., 2007). In order to find further evidence to support a similar conserved function for AtGAAPs, we studied subcellular localisation of AtGAAPs. AtGAAP-YFP fusion proteins co-localised with a Golgi marker upon transient expression in Nicotiana benthamiana leaf tissue. AtGAAP1- and AtGAAP2-YFP, but not AtGAAP4- and AtGAAP5-YFP, localised to the tonoplast, including the membrane of the central vacuole, transvacuolar strands, and bulbs under higher expression levels. Subcellular localisation studies in transgenic Arabidopsis lines further supported these observations. Further studies are necessary to confirm whether localisation to the tonoplast reflects the true localisation of AtGAAP1 and AtGAAP2 proteins or is an
artefact caused by overexpression. Thus, AtGAAPs localise to the Golgi under low expression levels, like their human counterpart.

The retention of GAAPs as a multigene family in plants suggests a positive selection for conservation of these genes. Gene expression analysis provided clear evidence for expression of all five AtGAAP paralogues, indicating that this gene family has not undergone pseudogenisation. Subfunctionalisation through differential spatial or temporal expression of genes is a common phenomenon (Gu et al., 2002; Wagner, 2000). In order to study whether this was the case for AtGAAPs, we carried out extensive gene expression analysis using Genevestigator, RT-PCR, and promoter-GUS fusions. These data would also facilitate predictions on possible redundancy between individual members of this gene family. A summary of the observed general trends and specific expression patterns is presented in the following paragraphs.

AtGAAP4 showed clearly the highest and most uniform abundance of transcript throughout plant tissues, followed by AtGAAP2. Expression of AtGAAP1 and AtGAAP5 was much lower, with significantly more expression in the inflorescence tissue than rosettes. AtGAAP2 and AtGAAP4 were expressed throughout the rosette leaves, whereas AtGAAP1 and AtGAAP5 expression was specific in the vasculature and hydathodes, or only hydathodes, respectively. AtGAAP1, AtGAAP2 and AtGAAP4 showed near identical expression pattern in the inflorescence tissue, although the expression of AtGAAP1 was not as strong as the latter two. Expression in the flowers was detected in the filament and anther, including individual pollen grains, and in the pistil, particularly in the stigma and neck of the style for AtGAAP1, AtGAAP2, and AtGAAP4. Expression of all AtGAAPs apart from AtGAAP3 could be detected in the petals and sepals. AtGAAP5 expression was elevated in the carpel, most prominently in the ovary, neck of the style, and abscission zone of floral organs. AtGAAP1, AtGAAP2 and AtGAAP4 and AtGAAP5 showed similar expression patterns in siliques, with the latter two showing highest expression levels. Most prominent expression was detected in the silique abscission zone, but particularly AtGAAP4 and AtGAAP5 were also expressed in the septum and valves. Gene expression analysis was very informative, however, the data obtained with different methods for a particular AtGAAP was sometimes contradictory. This was particularly the case for AtGAAP3; RT-PCR analysis indicated that AtGAAP3 is expressed in the
flower at relatively high levels whereas a low level of transcript is detected in the leaves. In contrast, microarray data indicated AtGAAP3 expression to be seed specific and AtGAAP3 expression could not be detected at all using histochemical staining of plants carrying AtGAAP3 promoter-GUS fusion construct.

A relatively uniform and high level of expression of AtGAAP2 and AtGAAP4 throughout different tissues could indicate a more general role during cell homeostasis. A near identical pattern of expression in floral tissues observed for AtGAAP1, AtGAAP2 and AtGAAP4 may indicate a redundant role during floral specific processes. For example, high expression levels of these genes in the anther and pollen could indicate the involvement of these genes in programmed cell death (PCD) known to occur during the development of the anther and during pollen maturation. For example, the tapetum, a layer of cells surrounding the microspores within the anther undergoes PCD during pollen development (Goldberg et al., 1993; Parish & Li, 2010). It would be interesting to carry out a more detailed analysis of the GUS stained pollen grains to see whether AtGAAP1, AtGAAP2 and AtGAAP4 were expressed specifically in the tapetal cells. Interestingly, expression of AtBI-1 under a tapetum-specific promoter delays PCD in tapetal cells (Kawanabe et al., 2006). The abscission process is responsible for controlled separation of plant organs from the main plant body, a process thought to involve PCD (Farage-Barhom et al., 2008; Lers et al., 2006). Expression of all AtGAAPs except AtGAAP3 in the abscission zone of the silique, and expression of AtGAAP4 and AtGAAP5 in the abscission zone of floral organs could indicate a role for these proteins in dehiscence-related PCD processes. AtGAAP5 expression in the ovary may indicate a specialised role for AtGAAP5 during embryogenesis. Elevated expression of AtGAAP3 in the seed, particularly the seed coat, could indicate a role in seed development. Programmed cell death is known to play a role in seed development. The seed coat, consisting of outer and inner integuments, surrounding the embryo and endosperm undergoes PCD during seed development (Nakaune et al., 2005). A member of the VPE family, δVPE has been shown to be specifically and transiently expressed in the inner integument. This has been shown by immunofluorescence analysis with anti-δVPE antibody, but also histochemical analysis of δVPE-promoter-driven GUS constructs. δVPE-deficiency delays the PCD of cell layers of the inner integument (Nakaune et al., 2005). It would be interesting to carry out a more detailed analysis of developing seeds of the
proAtGAAP3-GUS line to see whether AtGAAP3 expression can be detected in the seed coat as predicted based on Microarray data. If so, detailed analysis of the integuments could give us insight into the possible function of AtGAAP3 in this process. AtGAAP promoter-GUS transgenic lines provide a valuable resource for studying whether AtGAAP gene expression is stress responsive. Farage-Barhom et al. showed that expression of a putative senescence associated nuclease (BNF1) is upregulated in senescing leaves through analysis of promoter-GUS fusions. Kawai-Yamada et al. (2009) showed that AtBI-1 expression was elevated surrounding HR lesions triggered by Pst (avrRpt2) inoculation and went on to show that atbi-1 KO leads to increased sensitivity to this avirulent pathogen. Most importantly, gene expression data can be used to inform further experiments, for example, in which tissue to look for phenotypes and which atgaap mutant combinations would be of particular interest.

Reverse genetic approaches are powerful tools with which to analyse gene function. However, redundancy is widely observed throughout genomes of higher organisms and can complicate studies employing reverse genetics strategies. This is especially true for members of large gene families, such as MADS-box transcription factors and receptor-like proteins, which have 107 and 57 members in Arabidopsis, respectively (Parenicova et al., 2003; Wang et al., 2008). Reverse genetics approaches commonly utilise either the isolation of insertion mutant alleles in the gene of interest, or downregulating the expression of the gene of interest through RNA interference (RNAi). Multiple members of gene families can be knocked out by crossing individual T-DNA insertion lines. RNAi can be used to target expression of multiple genes simultaneously. Both strategies have been successfully employed in the characterisation of gene families with apparently redundant functions (e.g. VPE and actin)(Kuroyanagi et al., 2005; Pawloski et al., 2006). On the other hand, both methods have been relatively unsuccessful in identifying phenotypes related to members of large gene families, such as receptor-like proteins in Arabidopsis(Ellendorff et al., 2008; Wang et al., 2008). We decided to use the former approach to study the effect of loss-of-function of AtGAAPs for several reasons. Firstly, T-DNA or transposon tagged lines were readily available for each AtGAAP gene. We confirmed that no AtGAAP transcript could be detected in these plants indicating that they were null alleles. Secondly, AtGAAPs are part of a relatively
small gene family and AtGAAP loci are sufficiently dispersed throughout the genome to allow crossing of individual insertion lines to obtain knock-out lines for multiple AtGAAPs. Thirdly, gene expression analysis and sequence conservation within this gene family allowed us to make powerful predictions about possible redundancy within members of this gene family. Finally, RNAi typically leads to knock-down instead of knock-out of gene expression. We were cautious that knock-down by RNAi may not be sufficiently strong to compromise AtGAAP function in certain tissues.

During this work, we isolated KO alleles for each AtGAAP. We also set out to generate double mutant lines in all combinations. We predicted the atgaap2atgaap4 double mutant to be of particular interest as AtGAAP2 and AtGAAP4 are highly expressed in nearly all tissues and growth stages based on available microarray data and RT-PCR analysis. Also, as expression of AtGAAP1, AtGAAP3 and AtGAAP5 seemed tissue specific, we hypothesized that they were unlikely to complement for the loss of AtGAAP2 and AtGAAP4 in most tissues. We successfully generated all double mutant combinations apart from atgaap4atgaap5. AtGAAP4 and AtGAAP5 are genetically linked and according to our results there seems to be a strong suppression of recombination in the chromosomal region between the two genes. AtGAAP4, followed by AtGAAP5, share the highest level of identity with h-GAAP. Therefore generation of this double mutant would have been of particular interest. On the other hand, within the AtGAAP gene family, AtGAAP4 and AtGAAP5 are the most diversified based on amino acid sequence identity. It may imply that they also display diversified functions that other AtGAAPs could not complement. We also set out to generate three triple mutant combinations to address the possible issue of redundancy further. We wanted to knock-out the expression of AtGAAP2 and AtGAAP4, the most highly expressed AtGAAPs in combination with either AtGAAP1 or AtGAAP3, which showed tissue specificity in the flower, or seed, respectively. We reasoned that AtGAAP2 or AtGAAP4 might be able to complement for the loss of AtGAAP1 or AtGAAP3 in the specific tissues, and knocking out all three might lead to developmental defects during flower development or seed development or germination. Results from promoter-GUS analysis further strengthens the interest in the atgaap1atgaap2atgaap4 mutant combination, as the expression of these genes is near identical in the inflorescence tissue whereas no obvious AtGAAP3 or AtGAAP5 expression was observed in some of these organs, for example pollen
and stigma. The \textit{atgaap1atgaap2atgaap3} triple mutant is also of particular interest as these genes form a distinct clade implying conservation of function within this clade. We successfully generated all abovementioned mutants apart from \textit{atgaap1atgaap2atgaap3}, which is under way. No obvious developmental phenotypes could be observed in any of the mutant combinations, suggesting that functional redundancy plays a major role in the \textit{AtGAAP} gene family. More detailed analysis of the developmental phenotypes should be carried out concentrating on the tissues where \textit{AtGAAPs} are highly expressed, for example pollen and the inflorescence tissue where GAAPs show relatively high expression levels.

Human and viral GAAPs function as inhibitors of cell death (Gubser et al., 2007). Also, AtGAAPs show sequence conservation with \textit{AtBI-1}, which has been shown to protect cells against a number of stress stimuli that induce PCD in plants. \textit{atbi1 KO} mutants show increased sensitivity to cell death induced by heat stress, fungal toxin fumoninc B1, and avirulent bacterial pathogens (Watanabe & Lam, 2006). In order to study whether a similar cytoprotective function is conserved in AtGAAPs, \textit{atgaap} null mutants were exposed to various stress conditions that trigger PCD in plants. We did not uncover a PCD-related phenotype upon exposure of single \textit{atgaap} null mutants to heat stress and incompatible bacterial pathogens. The response of \textit{atgaap} mutants to dark-induced senescence was also indistinguishable from wild-type plants. It is possible that \textit{AtGAAPs} do not function as inhibitors of the PCD pathways tested during this analysis or that AtGAAPs play redundant roles in stress responses. Analysis of the available double and triple mutants may clarify the latter. Detailed analysis using more sensitive methods may reveal more subtle differences between wild-type and KO mutant plants. Analysis of possible upregulation of \textit{AtGAAP} gene expression in response to stress stimuli could be tested using the promoter-GUS transgenic lines generated during this work. This analysis would give us insight into what stress signalling pathways \textit{AtGAAPs} may be involved in and aid us in choosing relevant stress treatments to test on mutant combinations.
A rapid rise in cytosolic calcium concentration is a common response of organisms to numerous stress stimuli. The role of calcium in the regulation of apoptosis in animals has been well documented and its role in PCD of plant cells is becoming increasingly evident. Human GAAP and BI-1 are able to regulate cytosolic Ca\(^{2+}\) fluxes (Chae et al., 2004; de Mattia et al., 2009; Kim et al., 2008; Westphalen et al., 2005; Xu et al., 2008). Overexpression of these proteins results in reduced levels of releasable luminal Ca\(^{2+}\) in the Golgi and ER and their cytoprotective activity has been proposed to be correlated with free Ca\(^{2+}\) concentrations in the internal stores. Ihara-Ohori et al. (2007) demonstrated that AtBI-1 may also be involved in controlling Ca\(^{2+}\) homeostasis at the ER. In order to explore potential effects of AtGAAPs on Ca\(^{2+}\) fluxes induced by cell death stimuli, we measured changes in \([Ca^{2+}]_{\text{cyt}}\) in response to H\(_2\)O\(_2\) using apoaequorin expressing wild type and atgaap1, atgaap3, atgaap4, atgaap5 mutant seedlings. A KO of AtGAAP resulted in elevated \([Ca^{2+}]_{\text{cyt}}\) compared to wild-type plants upon H\(_2\)O\(_2\) treatment. However, this trend was not detected for another independent panel of apoaequorin transformants. These preliminary data are promising and may indicate a role for AtGAAPs as regulators of intracellular Ca\(^{2+}\) fluxes. Further experiments are needed to test the reproducibility of the phenotype across independent transformants.

Human GAAP has been shown to decrease IP3-mediated calcium release from intracellular stores through IP3 receptors (de Mattia et al., 2009). Although plant genomes do not contain sequences similar to animal IP3 receptors the role of exogenous IP3 in releasing Ca\(^{2+}\) from intracellular stores has been reported (DeWald et al., 2001; Schroeder et al., 2001). Drought–induced cytosolic calcium rises have been linked to release of calcium from the vacuole through IP3-dependent calcium channels in plants (Knight et al., 1996; Knight et al., 1997). In order to study whether AtGAAPs may be involved in IP3- and Ca\(^{2+}\) -related stress responses, we exposed atgaap and wild-type plants to drought stress. The Response of atgaap mutants to drought stress was indistinguishable from WT plants. Mannitol (simulated drought stress)-induced \([Ca^{2+}]_{\text{cyt}}\) rises were measured in atgaap KO and WT plants. atgaap4 and atgaap5 mutants showed an increased or reduced \([Ca^{2+}]_{\text{cyt}}\) at peak point, respectively, compare to wt plants. These differences were only observed in one of the two independent transgenic lines tested highlighting the necessity for repetition.
Also, it is puzzling that these closely related homologues would have an opposite effect.

We plan to explore the possible role of AtGAAPs in Ca\(^{2+}\) signalling further. Repeating the abovementioned experiments and testing a number of other treatments known to induce \([Ca^{2+}]_{cyt}\) may unravel a role for AtGAAPs as regulators of Ca\(^{2+}\) signalling. The Proaequorin gene has now also been transformed into atgaap double KO mutants. It may also be interesting to study developmental processes that involve Ca\(^{2+}\) signalling in atgaap and wild-type plants at the phenotypical level. For example, Ca\(^{2+}\) and IP3 signalling play a role in pollen development, including pollen viability, germination and pollen tube elongation (Kudla et al., 2010). As AtGAAP1, AtGAAP2 and AtGAAP4 are highly expressed in pollen, analysis of the triple mutant for defects in these processes would be of great interest. A further putative function of AtGAAPs as Ca\(^{2+}\)-channels could be tested through complementation of Ca\(^{2+}\)-channel defective yeast mutants (Urquhart et al., 2007).

An alternative to using loss-of-function mutants in the analysis of multigene families exhibiting functional redundancy, lies in the use of gain-of-function plants. This method has been successfully used to identify genes involved in both developmental and stress-induced responses. The role of CRK13, a member of the receptor like kinase (RLK) family of Arabidopsis in defence-related responses has been shown through overexpression analysis. KO of CRK13 showed no obvious developmental phenotype and did not alter the plant’s response to bacterial pathogens. However, overexpression of CRK13 led to HR-like cell death, activation of defence responses, and enhanced resistance to bacterial pathogens (Acharya et al., 2007). Role of AtGRF family in leaf growth has been shown through analysis of double and triple mutants and overexpressors (Kim et al., 2003). In the absence of a phenotype associated with knocking out AtGAAP expression, we tested the effect of overexpressing AtGAAP1, AtGAAP2 and AtGAAP5 in transgenic Arabidopsis plants. We carried out this analysis using stable Arabidopsis lines expressing AtGAAP-YFP fusions as these lines were used concomitantly for subcellular localisation studies. The data presented must be viewed with caution for several reasons. Firstly, level of YFP fluorescence was used as a measure for overexpression levels. Whether this corresponded to protein levels as assumed should be tested experimentally. Also, functionality of the
fusion proteins needs to be tested via complementation analysis, an approach not possible due to absence of a phenotype associated with loss-of-function alleles. Promisingly, it is known that the C-terminal YFP-tag does not interfere with AtBI-1 function, and since GAAPs belong to the same UPF005 protein family, it is conceivable that the GAAP-YFP fusions are functional. (Watatanabe and Lam, 2006).

Strikingly, overexpression of individual GAAP members led to distinct developmental phenotypes, rather than a range of overlapping phenotypes, as might have been expected from this highly conserved family. Dwarf plants with a compact rosette and round leaves were obtained upon AtGAAP1-YFP overexpression. The observed phenotype was reminiscent of that of brassinosteroid signalling mutants bri1-301, bri1-5 (weak bri1 alleles) and bak1 (Li & Nam, 2002; Nam & Li, 2002; Noguchi et al., 1999). This observation is of particular interest and relevance in the light of a recent report by Yamagami et al. (2009). Yamagami et al. (2009) identified AtGAAP2 (denoted as Brz-insensitive-long hypocotyls 4 (BIL4)) as a mediator of cell elongation in brassinosteroid (BR) signalling. BIL4 was identified from a screen of activation-tagged lines as a line that showed insensitivity to Brz, a BR biosynthesis inhibitor. BIL4 transcript was shown to be upregulated in the bil4-1D mutant. Wild-type plants show short hypocotyls and open cotyledons when grown in the dark in the presence of Brz, whereas bil4-1D showed longer hypocotyls and closed cotyledons. Light grown bil4-1D plants showed narrow, outward curving leaves, a feature the authors suggest resembles that of BRI1 and BSU1 overexpressing plants, which display upregulation of BR-signalling. This seems an odd comparison. Although leaves of BRI1 and BSU1 overexpressers display epinasty, the size of the rosette is similar to wild-type (Mora-Garcia et al., 2004; Wang et al., 2001), unlike bil4-1D, which displays a severely dwarfed rosette according to the figures presented (Yamagami et al., 2009). Unfortunately, no quantitative data on the leaf size is presented by the authors. Yamagami et al., (2009) also compare the leaf shape to that of WT plant exposed to excessive amounts of BR. The number of inflorescences and branches also increased in bil4-1D compared to WT. The authors remarked that this phenotype is similar to what happens when overexpressing the BR biosynthesis gene, DWF4. DWF4 overexpressing lines display an increased number of branches, but also significantly increased plant height compared to WT (Choe et al., 2001), in contrast to bil4-1D, which displays a significantly decreased height, compared to WT.
Yamagami et al. (2009) state that the height of \textit{bil4-1D} plant was reduced by 54\% compared to WT, a phenotype the authors also observed in plants treated with very high levels of BR. BIL4 overexpressing lines were generated by introducing untagged BIL4 cDNA under the control of the constitutive 35S promoter. The \textit{bil4-1D} phenotype was largely recapitulated in plants overexpressing BIL4. It is not clear whether the observed phenotype is reproducible across a number of independent transformants. The authors propose BIL4 to mediate cell elongation in BR signalling although no data for cell elongation at single cell level are presented. In stark contrast, the phenotype of 35S-AtGAAP2-YFP plants generated during this work did not resemble that of BIL4 overexpressors. Instead, overexpression of AtGAAP2-YFP fusion protein led to widened angles of cauline branches and siliques relative to the stem axis. There could be a number of reasons for the observed difference in phenotypes. Firstly, Yamagami et al., (2009) used an untagged AtGAAP2 construct, whereas our analysis was carried out with YFP-tagged AtGAAP2. The YFP-tag might interfere with the function of AtGAAP2. Secondly, analysis of BIL4 overexpression was carried out in Col-0 background as opposed to Ler used for our analysis. Thirdly, Yamagami et al., (2009) used the BIL4/AtGAAP2 cDNA, whereas our construct contained AtGAAP2 genomic sequence which may contain additional control elements. In the light of the results presented by Yamagami et al. (2009) and the possible BR-related phenotype we observed upon AtGAAP1-YFP overexpression, the link between BR signalling and AtGAAPs should certainly be pursued further. Untagged AtGAAPs under the 35S promoter have been transformed into WT Col-0 plants in order to rule out interference of AtGAAP function caused by the YFP tag.

Overexpression of AtGAAP5-YFP fusion protein led to a severe dwarf phenotype, enhanced leaf senescence and development of spontaneous lesions in both rosette and cauline leaves. This phenotype resembles that of lesion mimic mutants (LMM), which display spontaneous PCD and have been widely used as models for unravelling cell death signalling pathways (Lorrain et al., 2003). LMMs typically display constitutively active defence responses and enhanced resistance to pathogens. It would be interesting to study whether AtGAAP5-YFP overexpressors also display these features. LMMs are thought to be affected in genes controlling suppression of PCD as KO of these genes leads to induced PCD. It is, however, puzzling that overexpression of AtGAAP5, a putative repressor of cell death, would lead to a
phenotype typically associated with KO of a suppressor of cell death. Interestingly, characterisation of certain LMMs has shown a potential link between calcium related processes and cell death control. *copine1/bonzai1 (cpn1/bon1)* was identified as a null mutant exhibiting a humidity- and temperature-sensitive LMM phenotype with spontaneous HR cell death, defence gene activation and enhanced resistance to pathogens (Jambunathan et al., 2001). Copines represent a highly conserved protein family with C2 domains which exhibit Ca\(^{2+}\)-dependent phospholipid binding activity. Overexpression of BON1 with its functional partner, BAP1, another C2-domain containing protein, in *Nicotiana benthamiana* or Arabidopsis leaf tissue leads to inhibition of PCD induced by Bax, paraquat and incompatible pathogens (Yang et al., 2007). The phenotype of another LMM, *cpr22*, results from a fusion between two cyclic nucleotide gated channels, whose function as a calcium channel has been confirmed (Yoshioka et al., 2001; Urquhart et al., 2007). h-GAAP has been postulated to exert it’s antiapoptotic function through the regulation of intracellular Ca\(^{2+}\) fluxes. Further characterization of AtGAAP5 overexpressors could provide evidence for a function of AtGAAPs as regulators of cell death possibly through effects on Ca\(^{2+}\) signalling.

Endomembrane trafficking is an intricately controlled process within the plant cells and its disturbance can lead to detrimental defects (Sallese et al., 2009). During this work, we were able to correlate the expression level of AtGAAP-YFP fusion protein in transgenic Arabidopsis plants to subcellular localisation of the fusion protein. Furthermore, we sought to correlate our knowledge of the subcellular localisation of AtGAAPs to the observed phenotypes in the plant. AtGAAP5-YFP overexpression caused a striking phenotype at relatively low levels of expression. Subcellular localisation of the fusion protein was confirmed to be in the Golgi. Higher expression levels are presumably lethal for the plant. A similar level of expression of AtGAAP1-YFP (based on fluorescence levels) also resulted in localisation to the Golgi. The phenotype of these plants, however, was indistinguishable from WT. Therefore it appears that overaccumulation of AtGAAP1 and possibly AtGAAP2 in the Golgi does not lead to developmental defects, unlike for AtGAAP5. At higher expression levels, both AtGAAP1 and AtGAAP2 localised to the tonoplast. Developmental phenotypes caused by AtGAAP1-YFP and AtGAAP2-YFP overexpression were therefore associated with localisation of these proteins to the tonoplast. High
expression levels may allow the protein to accumulate at the proper location, but after saturation can also lead to ectopic accumulation (Dixit et al., 2006; Sparkes et al., 2006). It is therefore possible that localisation of AtGAAP1 and AtGAAP2-YFP to the tonoplast was an artefact of overexpression. If this is the case, the phenotypes observed for AtGAAP1 and atGAAP2, although interesting, may not reflect the true function of AtGAAPs, but instead be caused by ectopic localisation of these proteins to the tonoplast.

Overexpression of AtGAAP2-YFP fusion protein leads to widened angles of cauline branches and siliques relative to the stem axis. This phenotype resembles that of mutants that are unable to sense, or respond to gravity. Mutations in SGR2, SGR3, ZIG/SGR4, GRV2 are thought to primarily affect the tonoplast, causing irregularities in the morphology and dynamics of the vacuole or vesicle trafficking to the vacuole (Kato et al., 2002; Morita et al., 2002; Silady et al., 2004; Silady et al., 2008; Yano et al., 2003). These alterations disrupt the positioning and movement of the amyloplasts, resulting in gravitropic defects. Under normal conditions amyloplasts sediment under gravity in statocytes (gravity perceiving cells) but are unable to do so in these mutants. AtGAAP2 localised to the tonoplast of the central vacuole and highly mobile bulbs (also part of the tonoplast) under medium expression levels. Under high expression levels AtGAAP2 localised to static single membraneous structures that were attached to the tonoplast. We propose these structures to be an early form of bulbs, which are unable to detach from the membrane of the central vacuole due to accumulation of AtGAAP-YFP. This prediction should be confirmed by ultrastructural analysis. However, it appears that AtGAAP2-YFP overexpression caused alterations in the vacuolar dynamics. The severity of the phenotype was proportional to the AtGAAP2-YFP expression levels. Therefore, the phenotype observed upon AtGAAP2-YFP overexpression may be caused by perturbations in vacuolar dynamics, possibly affecting gravitropic responses. If AtGAAP2-YFP localisation to the tonoplast is due to ectopic expression caused by overexpression, then the observed phenotype may not reflect the true function of AtGAAP2.

In summary, our data strongly suggest that AtGAAPs represent a gene family with a high level of redundancy in Arabidopsis. We have studied their intracellular localisation and tissue specific expression patterns in detail. We have generated a set
of transgenic lines, including multiple KO mutant combinations and promoter-GUS fusions, with which to further study the function of AtGAAPs in plants. In addition, analysis of plants overexpressing individual members of this gene family has been shown to constitute a powerful method to study the role of these proteins in plant development and stress responses.
References


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Acknowledgements

First of all, I’d like to thank my PhD supervisor, Bart Feys, for his guidance and support throughout my PhD.

I’d also like to thank my progress review panel, Colin Turnbull and Peter Nixon. I’m very grateful to Marc Knight for invaluable scientific discussions and a chance to carry out a part of this work at Durham University.

I’d also like to acknowledge the financial support of BBSRC, Ella and Georg Ehrnrooth Foundation and the Gregory Fund.

Many people have inspired my interest in biology and served as scientific mentors. I’d like to especially thank Leena Silvola, John Thain, Anne Osbourn, John Rathjen, Kosmas Haralampidis and Manuel Montero for guiding me during my early years of scientific pursuit. I initially discovered how much fun life in the lab can be during the years in the Sainsbury lab (this is before I started my PhD, the fun part has mostly been replaced by stress since then). Thanks to my SL and JIC friends, it really was fun!

I’d like to thank my fellow PhD student in the Feys lab, Natalia, for your help and friendship throughout the past four years. I’m particularly grateful for your help during the last few days (and minutes!) before submission of this thesis: your figure-making expertise, knowledge and speed with all the thesis-related procedures and your calmness throughout the stressful times. I’d also like to thank previous members of the Feys lab, Belinda and Calin, for helping me settle into the lab initially.

I’d like to thank all my friends in the SAF building: Ellen- for arty and other forms of entertainment, Tim- I’ll start talking to you again tomorrow, Nick and Mike- your bickering kept me entertained in the lab, Jasmine-the smell of that Vietnamese fish will stay with me forever, Thorsten- see you in Eppendorf challenge, Volker-for the backrub and the template, Lucy- you convinced us it was possible in the given time. Dom – for reminding me how comfy hoodies are. No space to name everyone, you know who you are, thank you!

Elinalle ja Kirsille, huh huh, KIITOS! Maija, Liisa, Susanna, Anne, Matu, Hale, Gigi, Frances. For giving me what I most needed at times, i.e. something else to think about, and for keeping me (quite) sane.

Most of all I’d like to thank my family, mum, dad and my brothers Antto and Olli. You’ve always loved me, guided me, encouraged me without pushing me, supported me, listened to me and advised me. I’m forever grateful for your love and support. Mum and dad, this thesis is dedicated to you.

Finally, thank you Lee for making me laugh every day, for all the tasty victuals, for our adventures, for your mum, and for your limitless love.