CHARACTERIZATION OF SENESCENCE REGULATED GENE EXPRESSION IN ANTHURIUM

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Dedicated to all those
who listen to my aspirations,
those who have helped me reach for them,
and those in the future
who will assist me in completing them
ABSTRACT

Leaf senescence is a genetically regulated and orderly series of events signifying the final stage of leaf development prior to organ death. During senescence, the loss of photosynthesis is associated with the breakdown of chlorophyll and the disassembly of the photosynthetic apparatus. Cell structure changes, organelles are disassembled and proteins, lipids, nucleic acids and carbohydrates are hydrolyzed while the nutrients released by hydrolysis are transported and recycled to growing tissues or are stored in reserve for latter use. The factors regulating leaf senescence are complex and involve both external and internal stimuli, such as darkness, pathogens, developmental signals and hormones. These factors initiate signal transduction pathways that repress photosynthesis and activate the expression of genes involved in cellular disassembly, recycling and integral defense-related processes. The genes for mRNAs that increase markedly in abundance during senescence have been designated as "senescence-associated genes" or SAGs.

This work characterizes senescence in Anthurium andraeanum through expression analysis of the Arabidopsis senescence-upregulated promoter SAG12, isolation of a new senescence-associated gene (anth17), and expression profiling under dark induced and age dependent senescence. Analysis of Pr-SAG12 revealed a 20 fold increase in expression when transiently bombarded into senescent leaves vs. healthy leaves. This shows the senescence-dependent activation of the Arabidopsis Pr-SAG12 in Anthurium implying the existence of an orthologous senescence-regulated system with associated genes, signal
transduction pathways, and transcription factors in this plant. Two cysteine protease
cDNAs from Anthurium, \textit{anth16} and \textit{anth17}, were sequenced and found to be expressed
differently throughout development and differ structurally in comparison with known
cysteine protease genes. These cDNAs are the first to be cloned and characterized from
this plant. \textit{Anth16} is expressed in immature leaves and is structurally similar to a thiol
protease sequenced from \textit{Matricaria chamomilla}, possibly defining an alternate role in
development for these proteases. \textit{Anth17} is characterized as a senescence-associated gene
based on Northern blot analysis of developmental leaf stages in comparison with \textit{psbA}, and
\textit{cab} genes as indicators of photosynthetic gene expression. Furthermore, cytokinin
treatments decreased significantly \textit{anth17} mRNA levels during dark-induced senescence of
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LIST OF ABBREVIATIONS

$A_{260}$  Absorbance at 260 nm
$A_{280}$  Absorbance at 280 nm
ABA   Abscisic Acid
ACC   aminocyclopropane carboxylate
ADP   adenosine di-phosphate
Ala   alanine
anth16  Anthurium cysteine protease gene
anth17  Anthurium SAG cysteine protease gene
Asn   asparagine
Asp   aspartic acid
ATP   adenosine tri-phosphate
BA    benzyladenine
bp    base pair
cab   Chlorophyll A/B binding protein gene
cDNA  complementary DNA
Chl   chlorophyll
COOH  carboxyl terminus
Cys   cysteine
DLU   digital light unit
DMF   dimethyl formamide
DNA   deoxyribonucleic acid
ECM   extracellular matrix
ER    endoplasmic reticulum
EtBr  ethidium bromide
EXT   endoxylloglucan transferases
FITC  fluorescein isothiocyanate
GAL   β-galactosidase
GCG   genetics computer group
GENBANK  NIH genetic sequence database
GFP  green fluorescent protein
Gly  glycine
GPI  glycosylphosphatidylinositol
GST  glutathione S-transferase
GUS  β-glucuronidase
His  histidine
HPPD  4-hydroxyphenylpyruvate dioxygenase
IPT  isopentyl transferase
JA  jasmonic acid
KB  kilobase
Luc  luciferase
Lue  luecine
Met  methionine
MG  mature green leaf
MOPS  3-(N-Morpholino)-propanesulfonic acid
mRNA  messenger RNA
NH  amino terminus
nos  nopaline synthase
PCD  programmed cell death
PCR  polymerase chain reaction
PDS  particle delivery system
PEP  phosphoenolpyruvate
Pr-3SS  cauliflower mosaic 35S viral promoter
Pr-ANTH17  anb17 promoter
Pro  proline
Pr-SAG12  sag12 promoter
psbA  D1 protein of PSII
PVP  Polyvinylpyrrolidone
PVPP  Polyvinylpolypyrrolidone
RACE  Rapid amplification of cDNA ends
<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>REDOX</td>
<td>oxidation reduction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpPCR</td>
<td>reverse primer PCR</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription PCR</td>
</tr>
<tr>
<td>RUBISCO</td>
<td>ribulose bisphosphate carboxylase/oxygenase</td>
</tr>
<tr>
<td>S1</td>
<td>senescence stage one</td>
</tr>
<tr>
<td>S2</td>
<td>senescence stage two</td>
</tr>
<tr>
<td>S3</td>
<td>senescence stage three</td>
</tr>
<tr>
<td>SAG</td>
<td>senescence-associated gene</td>
</tr>
<tr>
<td>SARK</td>
<td>senescence associated receptor-like protein kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>T-StAR</td>
<td>Tropical &amp; Subtropical Agricultural Research</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>µE</td>
<td>micro Einstein</td>
</tr>
<tr>
<td>ubi</td>
<td>ubiquitin gene</td>
</tr>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>wtGFP</td>
<td>wild type GFP</td>
</tr>
<tr>
<td>X-gluc</td>
<td>5-bromo-4-chloro-3-indoyl glucuronide</td>
</tr>
<tr>
<td>YG</td>
<td>young green (immature)</td>
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CHAPTER I

INTRODUCTION

Senescence is a complex, highly controlled developmental phase in the final stage of the leaf life cycle that results in the coordinated degradation of macromolecules and the subsequent mobilization of components to other parts of the plant. Cells remain viable during the process and new gene expression is required. Physiological studies show that the cell structure changes, chloroplasts break down, but mitochondria remain intact until late senescence. Biochemical studies show an increase in pH, metabolic enzyme activities, the degradation of chlorophylls and the synthesis of anthocyanins and phenolics. Molecular studies show an increase in the expression of genes encoding enzymes for protein degradation (proteases), nitrogen remobilization, lipid and carbohydrate metabolism, and nucleic acid degradation (nucleases). While these genes are enhanced during senescence, the expression of photosynthesis genes that encode light harvesting components and Calvin cycle enzymes, are decreased. Analysis of gene expression during distinct developmental stages shows a concise pattern of gene regulation enabling us to predict the function of the proteins produced and their importance to the life cycle of the plant.
Current Research Involving Senescence Gene Expression

Classification of senescence associated genes

Senescence in plants can be stimulated by a variety of environmental and genetic controls, such as development, wounding, heat stress, light stress, salt stress, and pathogen attack. It is also coordinated on a molecular level by natural ripening responses (ethylene), or nutrient relocation involved in seed production, nutrient storage, flower production, changes in source-sink relationships, and the removal of leaves that no longer receive sufficient light. Natural senescence includes genes that influence protein degradation, nucleic acid breakdown, lipid remobilization, chlorophyll breakdown, and nitrogen remobilization. Several reviews of leaf senescence, (Sanchez 1973; Beevers 1976; Thimann 1980; Thompson and Platt-Aloia 1980; Biswal and Biswal 1984; Sexton and Woolhouse 1984; Kelly and Davies 1988; Matile 1992; Lohman et al. 1994; Smart 1994; Buchanan-Wollaston and Ainsworth 1997; Nooden et al. 1997; Lee et al. 2001) describe the leaf senescence of cereal, tobacco, some legumes, and a few flower model systems. Leaf senescence has been extensively studied, but its mechanisms are complex and consist of multiple pathways. By analyzing gene expression under natural and induced conditions, we can gain a better understanding of the role of each gene in leaf development.

Gene expression in leaf senescence is defined by a number of classes labeled senescence activated genes (SAGs) (Smart 1994). There are 6 separate classes of genes characterized by their pattern of expression and by the function of the proteins produced during plant leaf senescence. Four additional gene classes have been added as a result of SAG expression analysis during senescence in Brassica napus (Buchanan-Wollaston
Class 1 contains "housekeeping" genes that control essential metabolic activities of the cell and are expressed at a constant level throughout the life of the leaf. Classes 2 and 3 are genes expressed in green leaves, but their effect is seen at a later stage. Class 2 involves proteins encoded during leaf growth which are activated during senescence long after their gene is turned off. Class 3 genes are also turned off and their inactivation causes the initiation of senescence. These genes encode growth and carbon assimilation components. Class 6 genes encode proteins that remobilize storage products. These genes are also expressed during other developmental stages.

Further research on *Brassica napus* gene expression has identified 4 more classes of genes with SAG functions (Buchanan-Wollaston and Ainsworth 1997). Class 7 genes include genes that show a gradual increase in mRNA levels, from low expression in young leaves, to a high level of expression increasing throughout senescence. Class 8 genes are similar to class 7, but differ in the respect that mRNA levels are low in early stages of development, but increase dramatically at a particular stage of senescence. Class 9 genes are expressed at a specific stage in senescence, but unlike class 5 genes, their expression does not continue throughout late senescence. Class 10, the final class, is a set of genes that show strong expression in early leaf development and again during senescence.

Classes 4 and 5 genes have some involvement in senescence and their expression can be identified during a senescence inducing, or repressing event. Class 4 type genes include regulatory genes expressed for a short period of time at the initiation of senescence and control its timing and rate of progress. Class 5 genes are involved with mobilization processes specifically expressed during senescence. SAG genes include
genes involved in defense, sugar remobilization, amino acid metabolism, fatty acid metabolism, oxidation protection, sequesterization and transport of molecules and macromolecules, cell wall disassembly, and phytohormone production. It is beyond the scope of this report to list every gene involved with the coordinated process of recycling cell components for export to healthy tissues, however some genes that have been characterized through transcript analysis as being senescence-associated will be discussed.

Typical senescence up-regulated genes

Chitinases are important for the degradation of chitin, the most abundant organic nitrogen-bearing compound in nature. Chitin is most common in insects and in fungal cell walls, but is also found in many other organisms. An ethylene-induced chitin-binding protein has been purified from rose leaves (Yang and Gong 2002). It is possible that a senescent tissue synthesizes proteins to defend against pathogenic attack while in a weakened senescent state, but may also synthesis chitinases to degrade cell walls or be involved in signal transduction by acting on endogenous substrates (Hanfrey et al. 1996). Chitinases commonly induced during pathogenic attack have been found to be inducible during natural and artificial leaf senescence further implicating pathogen-related proteins role in the senescence process (Lers et al. 1998).

Pyruvate α-phosphate dikinase catalyzes the reversible conversion of ATP to AMP, pyrophosphate and phosphoenolpyruvate (PEP). Its potential role in senescence may involve converting the pyruvate produced from amino acid breakdown into PEP (Smart et al. 1995). PEP is also an intermediate in gluconeogenesis, active cycle in a
senescing leaf, turning lipids and photosynthetic products into sucrose for use in respiration or export from the senescing leaf. Many genes implicated in lipid metabolism have been characterized as SAGs, including an in-chain fatty acid hydroxylase bound to cytochrome P450 (Panavas et al. 1999). Interestingly enough, this hydroxylase is also up-regulated in the presence of certain herbicides and MnCl₂ (Cabello-Hurtado et al. 1998). Cytochrome P450 includes a class of key enzymes involved in the synthesis of a large variety of secondary plant metabolites including flavonoids, phytoalexins and lignin (Butt and Lamb 1981). The role of these enzymes in plant senescence is not understood.

Metallothioneins are small proteins that bind heavy metals such as zinc, copper, and magnesium, through clusters of thiolate bonds. During senescence it may be required to bind metal ions as either a detoxification role or a sequestering role for storage or mobilization. The metallothioneins may also protect DNA from damaging oxidative effects caused by chlorophyll and membrane breakdown and subsequent free radical production (Chubatsu and Meneghini 1993). A metallothionein gene is shown to be upregulated during senescence (Buchanan-Wollaston 1994) and also shows some sensitivity to pathogenic attack (Butt et al. 1998).

Many genes SAGs are involved in the modification and mobilization of amino acids. Genes that encode enzymes aminotransferase, branched-chain α-keto dehydrogenase, and β-methylcrotonyl-CoA carboxylase for amino acid metabolism and 4-hydroxyphenylpyruvate dioxygenase (HPPD) for aromatic amino acid degradation are upregulated at some point during senescence (Lee et al. 2001). The function of glutathione S-transferase (GST) is the conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles. GST could also have a
role in sulfur storage and mobilization released during macromolecule degradation (Smart et al. 1995). GST also retains a detoxification role with herbicides and its senescence-related expression may be related to stress. Glutamine synthase has many functions and isoforms located in the cytosolic and chloroplastic regions of the cell. A glutamine synthase like protein has been shown to be senescence upregulated in *Brassica napus* (Buchanan-Wollaston and Ainsworth 1997). This coincides with the need to transport nitrogen in the form of glutamine from senescing cells (Feller and Fischer 1994). Many genes that involve the recycling amino acids are upregulated during senescence to provide easy transport to sinks located in other parts of the plant.

Endoxyloglucan transferases (EXTs) have two distinct activities, the endo-type splitting of xyloglucans and transglycosylation. These processes are suggested to modify a major hemicellulose of the cell wall (Okazawa et al. 1993; Xu et al. 1996). Due to their expression during senescence, it is considered that the EXTs carry out hydrolysis of xyloglucan rather than transglycosylation, and is involved in cell wall degradation (Park et al. 1998).

ACC oxidase is involved with the biosynthetic pathway of a common senescence-inducing hormone, ethylene. Ethylene up-regulates its own production during senescence but also is implicating in up-regulating genes such as a calmodulin-binding protein, (Yang and Poovaiah 2000) a lipolytic acyl hydrolase, (Hong et al. 2000) and a glutathione S-transferase (Meyer et al. 1991). Most genes that are ethylene-induced during senescence were isolated from flowers such as rose, carnation, and daylily. Another phytohormone related gene induced during senescence is involved with signal transduction. A senescence associated receptor-like protein kinase (SARK) cloned from
Phaseolous vulgaris shows up-regulation slightly before senescence begins and is present throughout (Hajouj et al. 2000). This may suggest that SARK expression may regulate some pathways of the senescence program.

Genes that exhibit SAG characteristics are identified through Northern Blot analysis using cDNA clones to detect increased mRNA transcription levels in senescing leaf tissue. In order to identify the precise time during leaf senescence at which the expression of a specific gene is induced, it is essential to characterize the biochemical and physiological changes that map each stage during leaf development and senescence (Buchanan-Wollaston and Ainsworth 1997). Some SAGs are expressed in pre-senescent tissue and their expression simply increases during senescence, while other SAGs are more specific to senescence and are expressed only during senescence. This indicates the presence of multiple pathways in the regulation of senescence (Gan and Amasino 1995; Weaver et al. 2001). Based on this analysis, senescence includes pathways that are exclusively activated by developmental, environmental, or hormonal stimuli (Noh and Amasino 1999a). In order to gain a complete understanding of a particular gene and its senescence associated expression, we must expose the tissue to several experimental treatments. Through this method we are able to establish a consistent measure of SAG expression throughout the life of the plant, under hormonal control, and in response to stress conditions such as desiccation, abscission, and darkness. This enables the proper characterization of gene function and contributes to the identification of a variety of senescence-related promoters.
Genes involved with protein degradation

Senescence activated protein degradation is the most significant breakdown process that occurs in an aging leaf. The degradation of proteins to amino acids allows them to be recycled for use in other tissues. This remobilization of amino acids from senescing tissues is essential in the formation of new organs in other areas of the plant. Identification of key enzymes involved in senescence is difficult due to the high concentration already present in the vacuole of the developing leaf. Therefore, the identification of genes involved in senescence-enhanced protein degradation is particularly important and gives insight into the biochemical and physiological changes in the plant on a molecular level. Many genes encoding proteases, whose expression is enhanced in senescence, have been isolated and characterized including aspartic proteases and cysteine proteases.

The LSC760 aspartic protease gene of *B. napus* is expressed in young leaves and mature green leaves, but expression increased markedly during senescence (Buchanan-Wollaston and Ainsworth 1997). Cysteine proteases have been isolated from a variety of species and their genes vary in expression during senescence. Oryzain y protease from rice is a seed-specific protease that is expressed in germinating seeds. It functions in the remobilization of storage proteins to supply developing seedlings (Watanabe and Imasek 1982). This gene may perform a similar function in senescing leaves. The levels of other cysteine proteases are enhanced in senescing leaves. The *sag12* gene, from *Arabidopsis*, encodes a similar protein sequence to papain-like proteases and is specifically expressed during the onset of senescence in leaves (Lohman et al. 1994; Gan and Amasino 1995). A third type of cysteine protease from *B. napus* is expressed at high levels in young green
leaves, decreases in mature leaves, and increases significantly in senescing tissues (Buchanan-Wollaston and Ainsworth 1997). This gene is expressed in all developmental stages in all plant organs and has a high homology to a drought-induced cysteine protease in Arabidopsis (Koizumi et al. 1993).

Many cysteine proteases, active in both developmental and senescence stages of plant organs, have been isolated and characterized in a variety of different species. Protein degradation and remobilization during senescence involves a complex set of proteases that differ in organelle location and level of expression. This regulation may be so complex that it includes protease inhibitors that act to repress proteases until the precise stage. Chloroplast proteases are extremely important in the remobilization of stored nitrogen to other parts of the plant. The chloroplast is considered to be an important storage organelle, as 70%-80% of the nitrogen in mature leaves is located here. Roughly 90% of the nitrogen exported from senescing leaves comes from the chloroplasts (Morita 1980). Nuclear expressed genes encoding chloroplast-targeted proteases have been isolated (Mitsuhashi and Feller 1992) but senescence-specific protease activity involved with the dismantling and transport of the photosynthetic apparatus has yet to be characterized. A senescence-enhanced gene isolated from maize appears to encode a protein similar to a vacuolar enzyme present in castor bean seeds, which functions in the conversion of vacuolar proteins to their mature forms (Smart et al. 1995). This enzyme is up-regulated during senescence and may indicate that some enzymes involved in senescence are expressed during leaf development and stored in the vacuole where they await processing into a functional enzyme during the onset of senescence.
The ubiquitin pathway for targeted protein degradation is important in the elimination of abnormal cytosolic proteins and also the rapid turnover of short-lived proteins (Buchanan-Wollaston and Ainsworth 1997). Proteins destined for degradation are ‘tagged’ by ubiquitination in a series of ordered events and require ubiquitin-activating enzymes. These multi-ubiquitinated complexes are then recognized by ATP-dependent proteases which degrade the target proteins (Rechsteiner 1991). Although it appears that most of the proteases in the ubiquitin pathway are not influenced by senescence, an ubiquitin carrier protein gene has been identified showing enhanced expression in senescing leaves of the potato (Garbarino et al. 1995). The SEN3 gene of arabidopsis, a poly-ubiquitin encoding gene, is also upregulated in senescing leaves induced by age-dependent, dark detachment, light detachment, and phytohormone treatment methods (Park et al. 1998). This may indicate that the ubiquitin-dependent degradation of proteins does occur during senescence, but probably directed toward specific cytosolic proteins (Buchanan-Wollaston and Ainsworth 1997). It has also been shown that proteins that promote leaf longevity are targeted for degradation during senescence by ORE9, an F-box protein, which may form a complex with its substrate for subsequent ubiquitin tagging (Woo et al. 2001).

Although numerous research studies have been performed on isolating, sequencing, and characterizing protease genes with increased expression during senescence, little is known about the exact locations of the synthesis of protease proteins. RNA levels measured during developmental stages in plant organs are crucial in characterizing senescence-activated genes. This is seen with a Northern Blot of mRNA transcripts throughout the plants life cycle and through later stages of necrosis. However,
the exact location of where protein degradation takes place and how the encoded proteins function remain to be demonstrated.

Hormones Involved in the Regulation of Senescence

Many hormones are involved in growth, development, nutrient translocation and eventually senescence. Although senescence is a complex molecular and biochemical process, it appears to be initiated by changes in localized hormone concentrations. Numerous environmental switches have been observed to induce organ senescence including, temperature, light, wounding, pathogen attack, drought, and inadequate nutrients. Most of these factors occur with less frequency when compared with hormone-controlled age-dependent senescence. Information about the exact biochemical and molecular effects of hormones is incomplete, but current research demonstrates that hormones delay or interrupt senescence, accelerate senescence, or trigger it by a decline in concentration, or location.

*Hormones that accelerate senescence*

Ethylene, abscisic acid (ABA), and jasmonic acid can induce senescence. Ethylene is shown to increase senescence dramatically and coincides with the rapid phase of chlorophyll degradation and increased respiration in dicots (Aharoni and Richmond 1978). Ethylene also plays a key role in the progression of senescence in some species. Transgenic tomato containing an anti-sense gene leading to a reduction of polygalacturonase mRNA, showed a delayed senescence period by one week through
inhibition of ethylene biosynthesis (Picton et al. 1993). An ethylene-insensitive mutant of arabidopsis, compared with wild type, had a higher level of gene expression of photosynthetic-associated genes, while the expression of senescence-activated genes was delayed (Grbic and Bleecker 1995). The current proposal is that ethylene hastens the progress of senescence by activating senescence-associated genes while repressing photosynthesis-associated genes (Smart et al. 1995) thereby promoting membrane breakdown, loss of chlorophyll, and positive feedback responses. These responses create more ethylene that leads to a more rapid effect.

Ethylene induces the expression of genes, such as those encoding a lipase (Hong et al. 2000) and a calmodulin-binding protein, (Yang and Poovaiah 2000) which are identified as triggers of senescence. It has been demonstrated that protein phosphorylation via protein kinase up-regulates ethylene production, ethylene biosynthetic gene expression, and senescence acceleration in orchid flowers, while serine/threonine protein phosphates reverse the processes (Wang et al. 2001). The production of ethylene is primarily associated with cut flowers, fruits, and vegetables. Flowers that exhibit ethylene induced expression show increased transcription of aminocyclopropane carboxylate (ACC) and ACC oxidase genes, which encode enzymes involved in ethylene biosynthesis. Most flowers that exhibit ethylene inducible gene expression are dicots such as carnations, (Roberts et al. 1983) geranium, (Clark et al. 1997) and roses. Monocot flowers, such as anthuriums, iris, lily, and orchids, display little sensitivity to ethylene. Anthurium flowers are sensitive to lowered levels of cytokinins and daylily petals experience a rapid increase in abscisic acid content, contributing to the initiation of senescence.
Abscisic Acid (ABA) accelerates senescence in a wide variety of species but is less effective when applied to attached leaves, except at relatively high levels (50-100 mg) (El-Antably and Wareing 1967). The endogenous level of ABA increases in early stages of senescence of detached tobacco leaves and declines rapidly later into the process (Even-Chen and Atsmon 1978). The ethylene-insensitive daylily flower is very sensitive to endogenous ABA application. ABA accelerates petal death and prematurely induces many of the same biochemical changes that occur during natural senescence, such as loss of differential membrane permeability, increase in lipid peroxidation, and increase in activity of proteases and nucleases (Panavas et al. 1999). Much of ABAs role in senescence is unknown. ABA in leaves increases when plants are exposed to stresses such as drought, low temperature, and high salt concentrations.

The senescence-accelerating hormone jasmonic acid (JA), has been shown to promote senescence when applied to excised oat leaves (Ueda and Kato 1981). It promotes senescence by decreasing the expression of nuclear and chloroplast genes involved in photosynthesis (Creelman and Mullet 1997). Jasmonates appear to be derived from fatty acid biosynthesis, (Vick 1983) primarily lipoxygenase generation. The proteins stimulated by jasmonates degrade and transport vegetative storage proteins (Anderson and Spilatro 1989) and lipids. Jasmonates increase in concentration during wounding (Farmer and Ryan 1990), fungal, and bacterial pathogenic attacks (Anderson and Spilatro 1989). Although these reports suggest jasmonic acid is only involved with senescence, JA is also found in high levels in zones of cell division, young leaves, and reproductive structures (Creelman and Mullet 1997). One hypothesis is that JA works by decreasing or inhibiting the expression of photosynthetic genes. This is important in
vascular areas of the plant where the photosynthetic apparatus is not needed, and during senescence where photosynthetic nuclear and chloroplast genes are down-regulated. JA may also be induced by the energy quenching mechanisms of the chloroplasts to down-regulate chlorophyll genes. In effect, the down-regulation of chlorophyll decreases the excess energy absorbed and prevents further photochemical damage (Creelman and Mullet 1997).

Genes involved with JA biosynthesis include the plastid-derived lipoxygenase: LOX2, LOX3, LOX4, and the cytoplasmic LOX1. LOX2 plays a role in wounding and defense related senescence (Creelman and Mullet 1997) but is unlikely to be related to JA biosynthesis during senescence because its expression is turned off at the onset of leaf senescence (He et al. 2002). In contrast, LOX1 is strongly activated during leaf senescence and is likely responsible for the increased JA production in senescing leaves (He et al. 2002). There appears to be a strong evidence to support a role for JA in senescence. An exogenous application of JA induces leaf senescence and causes an increase in endogenous JA levels of senescing tissues to nearly 500% of those found in a non-senescing counterpart. It is also important to note that the JA biosynthetic pathway in senescing leaves is mediated by genes other than those involved in the wounding and defense-related JA biosynthetic pathways involving chloroplast-targeted enzymes.

**Hormones that decrease or delay senescence**

Cytokinins, and to some extent gibberellins and auxins are able to retard senescence. This section will detail the role of these hormones in senescence. Little is
known about the role of gibberellins in the plant life cycle. Gibberellins are involved in a wide range of functions that vary among species. In one case, the application of gibberellins to leaves strongly inhibited senescence where cytokinins had little effect (Fletcher and Osbourn 1966). In other species both seem to work effectively and show that senescence is coupled with a decrease in gibberellin concentration at the site. Gibberellins cause an increased of gene expression in stem elongation (Chory and Voytas 1987) and remobilization of nutrients during seed germination (Baulcombe and Buffard 1983). However, the effect of gibberellins on senescence-related gene expression has yet to be determined.

Auxins are another class of hormones that have various functions in diverse species. Although some reports indicate that they may delay senescence, there are numerous cases in which high concentrations of auxins are needed to retard senescence. Studies suggest auxins are not very powerful in suppressing senescence and high concentrations have to be used in comparison with cytokinins (Smart 1994). One complication is that auxins stimulate the production of ethylene when the concentration is only slightly above the physiological level (Thimann 1980). The production of ethylene accelerates senescence producing the opposite reaction desired. Auxin research shows a wide range of results in various species; therefore, it is extremely difficult to form universal generalizations about the role of auxins in the inhibition of senescence.

Specific roles of cytokinins in plant development

Cytokinins are very important in the healthy development and growth of young and mature plant organs. Cytokinins have been shown to prevent the natural senescence
of leaves or in some cases promote the re-greening of leaves (Van Staden 1988). During the senescence of a leaf, the tissue has no ability to generate cytokinins whereas young leaves have the ability to synthesize and accumulate cytokinins. Without the source of cytokinins, a leaf will begin to go through its natural senescence program and recycle its nutrients to supplement the generation of healthy and developing plant organs. Therefore, it has been concluded that if the proper level of cytokinins are made available to a senescing leaf, its senescence program will be interrupted indefinitely or delayed through a number of molecular and metabolic processes (Gan and Amasino 1995). Current research has shown that exogenous cytokinin application to senescing leaves delays chlorophyll breakdown, stimulates protein and mRNA synthesis, and stimulates chlorophyll synthesis. Cytokinins prevent the transient increase in respiratory rate seen in senescence (Tetley and Thimann 1974).

Cytokinins seem to operate at both the transcriptional and post-translational level. The presence of cytokinins inhibit the expression of senescence-related genes (Teramoto et al. 1995; Buchanan-Wollaston and Ainsworth 1997) and contribute to the activation of developmental genes (Flores and Tobin 1988; Chen et al. 1993). Cytokinin modulates genes and proteins by a variety of unknown signaling mechanisms. One postulated cytokinin signal transduction mechanism could be mediated by a histidine kinase analogous to two of the component regulators (Kakimoto 1996). It can also be elucidated that cytokinin regulates protein kinases/phosphatases which contribute to altering transcription factors, nucleic acid binding proteins, and mature proteins.

The tissue and organ-specific overproduction of cytokinins produced a number of morphological and physiological changes, including stunting, loss of apical dominance,
and reduction in root initiation and growth. Abnormal cytokinin concentrations can accelerate or prolong senescence in leaves depending on the growth conditions, initiate adventitious shoot formation from unwounded leaf veins and petioles, alter nutrient distribution and abnormal tissue development in stems (Li et al. 1992). While some of these morphological changes result directly from the localized overproduction of cytokinins, other changes probably result from the mobilization of plant nutrients to tissues rich in cytokinins. Therefore a system that reduces senescence using cytokinin concentrations must be engineered to regulate an effective concentration of cytokinin to a specific location. This method of regulation is crucial in the development of a senescence inhibition system.

**Anthurium Molecular Physiology**

*Anthurium andraeanum,* is one of the Hawaiian islands’ principal ornamental exports to the U.S. mainland, Canada, Japan, Italy, Germany and many other countries. The anthurium belongs to the family *Araceae,* a family chiefly of tropical plants, and is a perennial herbaceous plant usually cultivated for its attractive, long-lasting flowers (Higaki et al. 1979). The anthurium plant consists of many different species which all produce a flower that is a complex of a modified leaf (spathe) and hundreds of tiny flowers on the pencil-like protrusion (spadix) rising from the base of the spathe. The anthurium plant produces flowers that emerge from each new leaf axil, and follow a sequence of leaf-flower-leaf year-round throughout the entire life of the plant. Intervals between leaf emergences are shortened or lengthened depending on environmental conditions and usually grow more vigorously in the summer months (Higaki et al. 1979).
The anthurium flower is usually harvested when three-quarters mature and the cut flower will last from 7-10 days.

*Anthurium senescence biochemistry*

To establish a connection between physiological changes during senescence of anthurium flowers and a molecular explanation, data concerning physiological attributes of anthurium flowers (Paull et al. 1985) was analyzed and correlated with known molecular data on senescence. The first indication of senescence in anthurium flowers is marked by a 250% increase in respiration 8-12 days after harvest preceded by the first noticeable bluing of the spathe (Paull et al. 1985). A respiration increase during senescence is a molecular flag that identifies the transition from photosynthetic energy production, to sugar respiration involving the mitochondria that is functional until late senescence. Enzymes produced in early senescence include two key enzymes, isocitrate lyase and malate synthase, involved in the glyoxylic acid cycle. These enzymes drive sugar respiration to enable several processes of senescence, including nutrient remobilization (Gut and Matile 1988). This respiration increase is paralleled with a decrease in photosynthetic enzyme production including ribulose bisphosphate carboxylase (RUBISCO) and components of the light harvesting complex (Grover 1993). Basic preparations involved in chloroplast breakdown. A gradual decrease in total sugars and starch is observed during the period when respiration in the senescing anthurium flower plateaus confirming the use of anabolic substrates in the respiration process (Paull et al. 1985).
A dramatic increase of ammonium ions in senescing flowers is observed which is paralleled with spathe bluing (Paull et al. 1985). This is because the ammonium ion is a key ion in the isoenzyme of glutamate dehydrogenase, involved in packaging nitrogen for transport (Lauriere and Daussant 1983). The senescing flower also shows a small increase in alpha amino acids and proteins (Paull et al. 1985). This increase is consistent with increased levels of amides glutamine and asparagine, which are products of protein degradation and major organic forms of nitrogen able to be translocated in plants (Simpson and Dalling 1981).

All physiological and biochemical changes described in senescing anthurium flowers are consistent with known changes in the molecular physiology of senescing plants. The ammonium ion increase is believed to react by copigmentation with anthocyanins in senescing anthurium flowers, resulting in an increase in phenols and a visible bluing of the spathe (Paull et al. 1985). The blue color can also be attributed to an increase in pH caused by the activation of proteases involved in the remobilization of nutrients during senescence. These changes occur first in the spadix and are seen by the early browning. Finally, flower bluing can be related to peak respiration, ammonium ion concentration, pH elevation, and nitrogen remobilization marking the senescence of the anthurium flower, which occurs prior to the final increase in respiration as the flower begins to senesce rapidly (Paull et al. 1985).

Another important aspect involving anthurium flower production is the mechanism by which the flowers are produced. Anthurium flowers and leaves are born at a 1:1 ratio. The newly emerged immature leaf develops a negative net photosynthetic
rate, depriving the young flower bud nutrients for its cell division and growth phase. Removal of the new immature leaf 7 days after emergence results in delayed senescence of mature leaves, increased flower production, a larger flower, and an advanced rate of flower emergence (Dai and Paull 1990). It is predicted that if this practice could be followed in collaboration with the engineered auto-regulatory senescence inhibition system, that the anthurium plant could be a more hearty producer of flowers. This created by the longer lasting photosynthetically productive mature leaves supporting the plant with energy without the immediate need for fresh leaves. This allows them to be excised to allow for faster flower production increasing the economic value of each individual plant. Whether or not this remains a viable option remains to be seen.

Anthurium flower senescence control

Many attempts have been made to preserve the cut anthurium flowers life beyond the natural senescence period. These methods include temperature and fertilizer variations during a yearly growing season (Akamine and Goo 1975). Post-harvest treatments include pulsing stems with silver nitrate (Paull and Goo 1982), benzyl adenine (BA-cytokinin) drip treatments (Paull and Chantrachit 2001), commercial floral preservatives, sodium benzoate, glucose, sodium hypochlorite, and carbonated water (Akamine and Goo 1975). After many trials, the best method for floral preservation of cut anthurium, involves a benzyladenine drip treatment after cutting, and a silver nitrate pulsing after shipping of the flowers. In most cases, BA drip treatments increase flower life significantly (42%-142%) although, in one cultivar senescence is increased (Paull and Chantrachit 2001). It is concluded that an
application of exogenous cytokinin (BA dip treatment), delays the onset of molecular senescence in cut flowers, and silver nitrate pulsing, increases flower life by inhibiting bacterial growth that leads to blocking of the stem.

Through the analysis of anthurium flower senescence, it is reasonable to deduce that the flower functions much like other species during senescence and may have an advantage over other ornamentals because of having a flower comprised of a modified leaf. This includes a non-ethylene controlled flower and molecular senescent responses similar to that of a leaf. Engineering a molecular system into anthuriums that delays senescence by synthesizing endogenous cytokinin makes sense. Data showing delayed senescence due to exposure with cytokinins suggest that the main method of flower preservation is to interrupt the senescence cycle with a steady flow of regulated cytokinin production. This type of system may decrease the time between each new flower, maintain flower color, luster, and spadix life, or interrupt senescence of cut flowers indefinitely, but has to be examined for its impact on all developmental phases.

**Autoregulatory Senescence-Inhibition System**

The discovery and identification of senescence enhanced genes and promoters enable us to chart the progression of senescence and manipulate its interruption using cytokinins. The construct of this auto-regulatory system consists of the senescence-regulated SAG12 cysteine protease promoter, which is upregulated during age-dependent senescence, transcribing the ipt gene to creating the IPT enzyme. This enzyme is the rate-limiting factor of the cytokinin biosynthesis pathway and when available results in an
increase of cytokinins to the senescing tissue. This construct is crucial in providing precise amounts of cytokinin to senescing tissues to keep them healthy without exceeding normal cytokinin levels that result in altered morphology as seen in overproduction reports. This system is defined as autoregulatory because as the cytokinin levels in the leaf decline, genes are switched on to initiate senescence. The pSAG12-ipt construct is now a member of the senescence regulated genes, and is also transcribed providing the leaf with the slow production of cytokinin that will interrupt the senescence program once it reaches a threshold level. Cytokinin production will never reach abnormal levels since as the threshold level is reached, senescence related genes are switched off including the pSAG12-ipt construct. This construct will cycle in this fashion to interrupt senescence indefinitely and the leaves will exhibit a prolonged, photosynthetically active life-span.

The system has been tested in only a few plant species. To date, the effect of ipt expression in transgenic plants has been assessed mainly in a limited number of solanaceous species (Gan and Amasino 1995; Jordi et al. 2000). However, there are brief reports of the introduction of pSAG12-ipt into rice, (Fu et al. 1998) cauliflower, (Nguyen et al. 1998) and lettuce (McCabe et al. 1998). Its introduction into tobacco resulted in more green leaves, no sign of senescence, a two fold increase in flower and seed production and an increase in biomass for pSAG12-ipt plants versus wild type (Gan and Amasino 1995). When introduced into lettuce, it seemed to be regulated by a positive instead of negative feedback loop in the panicles during bolting (McCabe et al. 2001). An interaction between high concentrations of hexoses and cytokinins in these tissues may have repressed photosynthesis and stimulated senescence. Although this displays a negative impact of the system on specialized tissues, the leaves of the lettuce stayed
green for an increased period of time post-harvest, and also allowed growers to reduce
nitrogen fertilization applied to the plant prior to harvest.

When studying the impact of delayed leaf senescence on the functioning of plants
growing under conditions of nitrogen remobilization, potential consequences of modified
sink-source relations may affect plant productivity and the efficiency of light and mineral
utilization. Expression of *ipt* in older leaves causes maintenance of chlorophyll content
and PPFD absorption but is less effective in delaying other aspects of senescence such as
loss of soluble protein, and still less effective in maintaining RUBISCO and net
photosynthetic energy levels (Jordi et al. 2000). Younger developing leaves in the IPT
system showed lower contents of chlorophyll, Rubisco, and protein than those leaves in
the corresponding wild type plants. This is caused by the inhibition of remobilization of
nutrients from older leaves to younger leaves and may seriously limit potential increases
in biomass of these plants under limited Nitrogen nutrition (Jordi et al. 2000). It is
apparent, that at some capacity, nutrients are exported from older to younger leaves prior
to Pr-SAG12 initiation, or that remobilization is not completely inhibited by the auto-
regulatory senescence inhibition system.

Many genes are classified as *senescence-associated* depending only on the
increase in abundance of transcript levels during the onset and throughout senescence. A
few SAGs are characterized through expression analysis as being *senescence-
upregulated*. The expression of such genes is absent during germination, early
development and maturity but show a distinct increase at during some point during the
senescence phase. SAG12 is a cysteine protease with an expression pattern that comes
the closest to having the specificity for natural senescence. It is undetectable in younger
leaves and is not induced by any senescence-inducing treatment that does not cause visible yellowing to the leaves (Weaver et al. 1998). This makes SAG12 the best molecular marker of senescence to date and suggests a specific role for senescence-specific cysteine proteases. The successful implementation of the SAG12 promoter in

Table 1: Cysteine protease genes that are characterized as being senescence up-regulated.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Genbank Accession</th>
<th>Plant</th>
<th>Common name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAG12</td>
<td>U37336</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Arabidopsis</td>
<td>Lohman <em>et al.</em>, 1994</td>
</tr>
<tr>
<td>SAG12-1</td>
<td>AF089848</td>
<td><em>Brassica napus</em></td>
<td>Rape</td>
<td>Noh and Amasino, 1999</td>
</tr>
<tr>
<td>See1</td>
<td>X99936</td>
<td><em>Zea mays</em></td>
<td>Corn</td>
<td>Griffiths <em>et al.</em>, 1997</td>
</tr>
<tr>
<td>See1</td>
<td>AJ249847</td>
<td><em>Lolium multiflorum</em></td>
<td>Ryegrass</td>
<td>Li <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>NTH1</td>
<td>U44947</td>
<td><em>Pisum sativum</em></td>
<td>Garden Pea</td>
<td>Kardailsky and Brewin, 1996</td>
</tr>
<tr>
<td>Tpp</td>
<td>X66061</td>
<td><em>Pisum sativum</em></td>
<td>Garden Pea</td>
<td>Granell <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>NTCP-23</td>
<td>AB032168</td>
<td><em>Nicotiana tabacum</em></td>
<td>Tobacco</td>
<td>Ueda <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>SENU3</td>
<td>Z48736</td>
<td><em>Lycopersicon esculentum</em></td>
<td>Tomato</td>
<td>Drake <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>SmCP</td>
<td>AF082181</td>
<td><em>Solanum melongena</em></td>
<td>Eggplant (Brinjal)</td>
<td>Xu and Chye, 1999</td>
</tr>
<tr>
<td>SPG31</td>
<td>AF242185</td>
<td><em>Ipomoea batatas</em></td>
<td>Sweet Potato</td>
<td>Chen <em>et al.</em>, 2002</td>
</tr>
<tr>
<td>SEN102</td>
<td>X74406</td>
<td><em>Hermocallis sp.</em></td>
<td>Daylily</td>
<td>Valpuesta <em>et al.</em>, 1995</td>
</tr>
<tr>
<td>BoCP5</td>
<td>AF454960</td>
<td><em>Brassica oleracea</em></td>
<td>Broccoli</td>
<td>Coupe <em>et al.</em> (unpublished)</td>
</tr>
<tr>
<td>ALSCYP1</td>
<td>AAK63125</td>
<td><em>Astroenermia hybrid (Samora)</em></td>
<td>N/A</td>
<td>Wagstaff <em>et al.</em>, (unpublished)</td>
</tr>
<tr>
<td>PRT5</td>
<td>AF133839</td>
<td><em>Sanderosia aurantiaca</em></td>
<td>Chinese-lantern lily</td>
<td>Eason, (unpublished)</td>
</tr>
</tbody>
</table>

the autoregulatory senescence inhibition system has lead to the pursuit of orthologous SAG12 type cysteine proteases from a number of other species. Table 1 shows cysteine proteases from a variety of plants that have been characterized as senescence-upregulated through a combination of molecular techniques. Regulating cytokinin biosynthesis under
the control of a native promoter dedicated to expression under natural senescence is the most preferable from an engineering standpoint. This type of promoter is less likely to be induced by stresses, environmental pressures, and the intricacies of development thereby establishing a system that will work as desired without adverse affects.

There is promise for this system to work in the anthurium species because the flower is actually a modified leaf and should react as a leaf would respond when involved with the auto-regulatory system. It must be considered that the plant is reliant on the production of new flowers and leaves to be commercially productive and that this system may limit the production of quality flowers, especially under nutrient deprived conditions. However, with the proper nutrients and pruning techniques, the system may perform better than the results seen in tobacco.
Senescence is relatively slow and ordered, maximizes reallocation of nutrients and metabolites, and always has the potential to be reversible until the later stages. Programmed Cell Death (PCD), commonly termed apoptosis or hypersensitive response, is relatively rapid and usually reserved for responses to pathogen attack or developmental disposal of unwanted cells. These two terms are not interchangeable, however may share common pathways and gene expression. PCD is essential for normal reproductive and vegetative development, such as floral development, seed maturation, tracheary element formation, trichome development, and root development. PCD also involves responses to pathogenic attack, protecting the plant against systemic infection by minimizing the pathogens opportunity to gain a foothold. Senescence on the other hand is an extremely coordinated process that progresses in metabolically active cells.

Senescence is initiated by an environmental or developmental initiator, which induces a hormonal response and/or signal transduction events. This coordinates gene activation or inactivation that occurs on the transcriptional, translational, and post-translational level. Signal transduction also includes protein modification, mobilization, activation or inactivation. These events create a positive feedback loop, increasing the efficiency of the senescence process, by expressing genes encoding proteins involved with DNA and RNA binding, receptors, kinases, phosphatases, growth regulator synthesis, and metabolite/nutrient mobilization.
Subsequent physiological events after the onset of senescence-associated gene expression include a chlorophyll degradation pathway, accompanied by the unmasking or accumulation of carotenoids and other pigments. This process can be quantified to observe the progression of senescence through chlorophyll concentration of leaf tissue. Proteins are broken down, and the mobilized organic nitrogen and organic sulfur are exported from senescing leaves. Catabolism of nucleic acids releases inorganic phosphate. Magnesium ions are captured, chelated, and mobilized as well leaving chlorophyll inactive. Photosynthesis declines, and peroxisomes are re-differentiated into glyoxysomes, which convert lipids to sugars. Metabolic regulation during senescence involves responses to cellular REDOX conditions, compartmentalization, and enzyme specificity involving degradation. Where the great energy producing and preserving Calvin cycle of photosynthesis once predominated, gluconeogenesis spurred into action by senescence, now converts intermediates into sucrose through glycolysis.

Senescence involves plant molecular, physiological, and developmental aspects that can be examined through modern techniques. This process gives us one of the best tools to examine the complex structure of the plant cell, through its organized degradation and remobilization. Biochemistry, enzymology, proteomics, genomics, signal transduction, photosynthesis, gene expression, REDOX regulation, vacuolar compartmentalization and differentiation, and growth regulators can all be successfully studied during the slow moving senescence program. The results of this research will expand our knowledge of cellular biology paving the way for impacting successful changes to harness the power of the plant cell. Every piece of evidence we gather when studying senescence, especially in unique species, is an important contribution, not only
to our scientific understanding of plant systems, but also in increasing the quality of
genetic manipulations resulting in better agricultural performance.

The Senescence-Activated Signaling Pathway That Activates the Dicot
Promoter, SAG12, May Be Evolutionarily Conserved In Anthurium

Senescence involves a highly regulated, co-ordinated series of events
accompanied by repression and up-regulation of gene expression. Senescence-enhanced
genes encode degradative enzymes that include proteases, ribonucleases, lipases and
nucleases. Senescence-repressed genes include those that encode the photosynthetic
apparatus. Senescence can also be characterized by phenotypic changes in plant organs
including chlorophyll loss and changes in pigmentation eventually leading to necrosis.
Protein activation, modification, degradation, and mobilization are all actively changing
events during senescence. A receptor-like protein kinase induced during senescence
may have a regulatory function of some senescence pathways (Hajouj et al. 2000).
Proteins that promote leaf longevity are targeted for degradation during senescence by
ORE9, an F-box protein, which may form a complex with its substrate for subsequent
ubiquitin tagging (Woo et al. 2001). Both external and internal stimuli can trigger
changes in plant hormone concentrations thereby initiating signaling mechanisms that
modify gene expression at transcriptional and translational levels, as well as post-
translationally by modifying proteins.

Modification of gene expression involves promoters that serve as switches,
turning genes on and off in response to signals. Senescence-associated promoters are
comprised of conserved cis-acting elements and enhancer regions (Noh and Amasino 1999a) necessary for senescence-specific expression. DNA gel-shift mobility assays show these regions form different mobility complexes with DNA-binding proteins in young versus senescent leaves. The research suggests that a transcriptional repressor or inactive form of a transcriptional activator is present in the healthy state, while during senescence a new transcriptional activator is produced or the repressor/inactive transcriptional factor is modified to an active form (Noh and Amasino 1999a).

Research involving senescence mechanisms in Arabidopsis has shown a complex series of events involving gene and protein modification in an effort to recycle nutrients for mobilization to healthy portions of the plant. This process is thought to be conserved throughout all higher plant species, but a question still remains: Are the molecular signaling pathways, transcriptions factors, and promoters interchangeable in evolutionary divergent species? The successful senescence induced expression of the Arabidopsis senescence-activated promoter in Anthurium suggests that cis-acting and enhancer regions of Pr-sag12 interact with transcriptional factors present in Anthurium making an expression system using a senescence-enhanced promoter from Arabidopsis a viable option in Anthurium. This also suggests that Anthurium has senescence-activated promoters and associated transcription factors that are homologous to Arabidopsis and identification and characterization would prove to be highly useful for biotechnology.
Expression and Proper Post-Translational Modification of GFP in Anthurium, Can Provide A Necessary Reporter Gene Tool For Anthurium Molecular Studies.

Reporter genes are an important tool in evaluating promoter expression, successful transformations, and mutation analysis. They can be used in both stable transformation and transient expression analyses. In order to determine that Pr-SAG12 is regulated in anthurium, there must be a reliable reporter system. Important criteria of a reporter system includes the successful production of the protein in the host, quantifiable expression, and non-invasive analysis. The protein must not be confused with background proteins native to the host. Several reporter genes are available and examples include *uidA* (encoding β-glucuronidase ‘GUS’), *luc* (encoding Luciferase), *gal* (encoding β-galactosidase), and *gfp* (encoding green fluorescent protein). Most reporter genes encode an enzyme that is foreign to the host that can catalyze a reaction when in the presence of the correct substrate. However, for quantification analysis, the plant tissue must be destroyed by homogenation and/or exposure with an infiltration buffer. One unique protein, *gfp*, encodes a functional protein that can be visualized through excitation with a non-invasive wavelength of light. The *gfp* protein emits a different wavelength of light that is easily quantifiable in living cells (Schenk et al. 1998).

In order to conduct molecular studies in anthurium, it is necessary to identify a reporter system that is effective, and provides for non-invasive analysis. Anthurium tissues contain heavy amounts of phenolic and polysaccharide compounds that inhibit pure extraction and analysis of cellular proteins. Previous research shows that the *uidA*
or “gus” gene is effectively expressed in stably transformed anthurium tissues, but has improper post-translational modification and folding, making the reporter enzyme non-functional on the GUS substrate (Kuehnle and Chen 1994). Our research also shows that the GUS system is not an effective reporter system for transient bombardment expression analysis in anthurium (Hayden and Christopher, unpublished).

Chapter 3 will focus on determining the transient expression of Pr-SAG12 during different stages of development using GFP as a reporter gene. This work will identify the gfp4 and gfp5-ER genes as useful reporter systems for further research on promoter assays, tissue-regulated expression and protein targeting studies in anthurium.

A Senescence-Regulated Cysteine Protease Gene, Orthologous to sag12, Exists In Anthurium

Chapter 4 will focus on the identification and characterization of cysteine protease genes in anthurium. Two cysteine proteases anth16 and anth17 will be studied in detail ultimately determining the role of each gene as it applies to development. Understanding gene regulation during senescence begins with isolating and characterizing senescence-associated genes. These genes give us insight into the complex molecular process involved during senescence. The factors regulating leaf senescence are complex and involve both external and internal stimuli, such as darkness, pathogens, developmental signals and hormones. These factors initiate signal transduction pathways that repress photosynthesis gene expression and activate the expression of genes involved in cellular disassembly, recycling and integral defense-related processes (Quirino et al. 2000). The genes for mRNAs that increase markedly in abundance during senescence have been
designated as "senescence-associated genes" or SAGs (Lohman et al., 1994) and encode chitinases, cysteine proteases, lipases, aspartic proteases, and metalloproteases from plants such as rape seed (Hanfrey et al., 1996), rice (Lee et al., 2001), cucumber (Delorme et al., 2000); carnation (Hong et al., 2000), tomato (Davies and Grierson, 1989; John et al., 1997), parsley (Lers et al., 1998), arabidopsis (Gan and Amasino, 1995; Lohman et al., 1994; Yoshida et al., 2001), daylily (Valpuesta et al., 1995), ryegrass (Li et al., 2000), sweet potato Huang et al. 2001), and eggplant (Xu and Chye, 1999) and enzymes involved in phosphonate biosynthesis in carnation (Wang et al., 1993). Cysteine protease genes expressed during senescence are of particular interest since they fall under class 5 SAG expression. This stage indicates expression at the extreme initiation of senescence that is sustained throughout the later stages. There also exists many isoforms of cysteine proteases with different expression patterns suggesting unique and specific roles in the developmental cycle.

One benefit that aids in the identification of these genes is the extent of homology contained in specific regions representing enzymatic functionality. This enables the creation of degenerate primer sequences that can produce the successful cloning of small portions of DNA from cysteine protease cDNAs synthesized from unique developmental mRNA populations. Once a native anthurium fragment is isolated, it can serve as a useful tool to identify full-length cDNAs from libraries or mRNA populations. After the full-length cDNA sequence is verified, many tools are available for full identification. The deduced amino acid sequence allows the comparison of the anthurium cysteine proteases to other homologous proteases, whose sequences are available in online databases. Careful analysis of differences in deduced amino acid composition can help
predict unique features of the proteins. These include: secondary and tertiary structure prediction, signaling peptides to determine cellular location, peptide cleavage sites to predict mature protein length, and common features that characterize enzymes such as active domains and critical bonding locations.

Characterization of two full-length anthurium cysteine protease clones by Northern Blot analysis of developmental RNA populations indicates differential expression patterns confirming senescence-associated gene expression. Developmental populations include: YG (immature); MG (Mature Green); and S1, S2, S3 (Senescent leaves, progression characterized by chlorophyll content). Once the anthurium SAG is identified it can be used in correlation with the non-senescence associated anthurium cysteine protease gene, photosynthetic genes (cab and psbA), and a constitutively expressed recycling gene (ubi), to determine expression under senescence controlling conditions.

The Effect of Cytokinin and Sucrose on SAG Expression During Senescence

A comparison of expression patterns in response to stress and hormone treatments using Northern blot analysis, is an excellent indicator for characterizing senescence-associated genes (Weaver et al. 2001). Certain stresses and hormones are able to hasten or repress senescence as seen visually by leaf yellowing and detected on the molecular level by responses in gene expression. Cytokinin is a well-known senescence-delaying phytohormone (Gan and Amasino 1997; Nooden et al. 1997).
Exogenous treatments of the synthetic cytokinin benzyladenine (BA) show a repression of senescence characteristics when applied on anthurium leaves and flowers (Paull and Chantrachit 2001). Cytokinins can be used to repress many SAG transcripts, and occasionally reverse the senescence of a leaf in the later stages. Considering sugars are the main product of photosynthesis, certain sugars can serve as signaling molecules, changing sugar levels during photosynthetic decline may act as a senescing-inducing signal (Jang et al. 1997). Sucrose can effectively repress the accumulation of certain SAG transcripts when applied to detached leaves (Weaver et al. 1998).

Although these treatments are somewhat effective in reversing the effects of senescence and SAG transcripts, inducing senescence in leaves can result in a different pattern of SAG transcripts from the observed expression during developmentally-induced senescence. The most common techniques of inducing senescence include detachment and incubation of leaves in water under dark and light conditions, desiccation, and hormone treatments with ABA and ethylene. Certain SAG transcripts may not respond to this type of induced senescence even though the visual indicator (chlorophyll loss) of senescence progresses rapidly (Noh and Amasino 1999a).

Senescence is a complex process in which SAG genes encode unique transcripts having specific functions. Each of the techniques listed above can give insightful clues in characterizing a SAG gene in relationship to the many already isolated and characterized. It is necessary to include the proper controls to identify the SAG expression pattern of isolated clones, and understand that only the combination of multiple treatments in comparison to developmental populations will give a firm conclusion. This will provide valid data in the pursuit of identifying a native senescence-upregulated promoter, to
better the auto-regulatory senescence inhibition system, for future applications in engineering the *Anthurium* species for increased production and improved post-harvest floral vase life.
CHAPTER III
REGULATION OF THE SAG12 PROMOTER IN ANTHURIUM USING TRANSIENT BIOLISTIC PARTICLE BOMBARDMENT

Introduction

Modification of gene expression involves promoters that serve as switches, turning genes on and off in response to signals. Both external and internal stimuli can trigger changes in plant hormone concentrations thereby initiating signaling mechanisms that modify gene expression at transcriptional and translational levels, as well as post-translationally by modifying proteins. Senescence-associated promoters are comprised of conserved cis-acting elements and enhancer regions (Noh and Amasino 1999b) necessary for senescence-specific expression. It is proposed that a transcriptional repressor or inactive form of a transcriptional activator is present in the healthy state, while during senescence a new transcriptional activator is produced or the repressor/inactive transcriptional factor is modified to an active form. DNA gel-shift mobility assays show that regions of the Pr-SAG12 form different mobility complexes with DNA-binding proteins in young versus senescent leaves. Two orthologs of SAG12 isolated from Brassica napus show sequence similarities to SAG12 and expression analysis consistent with that of SAG genes. When the promoters of the three genes are compared, two distinct regions show over 75% identity with one another. The regions from the Brassica genes also bound to arabidopsis DNA-binding proteins as shown in a gel-shift assay using extracts from senescent arabidopsis leaves (Noh and Amasino 1999b). This
suggests these two regions are necessary for the senescence-specific regulation of these particular SAG genes. Another study compares the upstream region of a SAG gene from sweet potato. It was concluded that the promoter did not include any regions similar to those in sag12 however it did include putative ethylene-responsive elements.

Research involving senescence mechanisms in arabidopsis has shown a complex series of events involving gene and protein modification in an effort to recycle nutrients for mobilization to healthy portions of the plant. The successful senescence induced expression of the arabidopsis senescence-activated promoter in anthurium would suggest that cis-acting and enhancer regions of Pr-SAG12 interact with transcriptional factors present in anthurium making a expression system using a senescence-enhanced promoter from arabidopsis a viable option in anthurium. This would also suggest anthurium has senescence-activated promoters and associated transcription factors that are homologous to arabidopsis and identification and characterization would prove to be highly useful for biotechnology.

Understanding the regulation of a foreign promoter in a host of interest is necessary to confirming the efficacy of a gene manipulation system prior to stable transformation. In this case, Pr-SAG12 is evaluated by regulating the expression of a reporter gene, when introduced via particle bombardment in anthurium leaf tissues. Reporter genes are an important tool in evaluating promoter expression, successful transformations, and mutation analysis. They can be used in both stable transformation and transient expression analyses. The important criteria of a reporter protein include successful production in the host, quantifiable expression, and non-invasive analysis. The protein must not be confused with background proteins native to the host. Several
reporter genes are available and examples include *uidA* (encoding β-glucuronidase ‘GUS’), *luc* (encoding Luciferase), *gal* (encoding β-galactosidase), and *gfp* (encoding green fluorescent protein). Most reporter genes encode an enzyme that is foreign to the host that can catalyze a reaction when in the presence of the correct substrate. However, for quantifiable analysis, the plant tissue must be destroyed by homogenation and/or exposed to an infiltration buffer. The unique reporter, GFP, encodes a protein that can be visualized through excitation with a non-invasive wavelength of light. The GFP protein emits a different wavelength of light that is easily quantifiable in living cells (Schenk et al. 1998).

In order to conduct molecular studies in anthurium it is necessary to identify a reporter protein system that is successfully produced and also provides for non-invasive analysis. Anthurium tissues contain heavy amounts of phenolic and polysaccharide compounds that inhibit pure extraction and analysis of cellular proteins. This physiology can also interfere with substrate/enzyme interaction that is necessary in most reporter systems. This chapter will focus on the transient regulation of Pr-SAG12 in anthurium and also include data verifying the successful implementation of the GFP reporter system in anthurium tissues.

**Materials and Methods**

To determine whether the senescence-associated promoter, Pr-SAG12 (from the dicot *Arabidopsis thaliana*) is up-regulated by senescence in *Anthurium* tissues, a transient expression assay was conducted using the reporter genes of either *uidA* or *gfp*
depending on the plasmid vector introduced to the plant tissue. The following controls include: a) uncoated beads to determine the effects of particle bombardment wounding for a negative control, b) reporter gene expression with a constitutive promoter for a positive control, c) Pr-SAG12 expression in tissues of all developmental stages, d) reporter gene without a promoter for a negative control.

**Construction of Pr-SAG12 reporter vectors**

The first plasmid used to test for senescence-upregulated promoter expression was pSAG12-gus, containing, a promoter isolated from a cysteine protease and a gus reporter gene. The plasmid pSAG12-gus (provided by Rick Amasino, University of Wisconsin), was used to transform XL1-blue Ecoli cells by the standard heat shock method (Sambrook et al. 1989). After an overnight incubation at 37°C, the plasmid was isolated using a midi­prep plasmid isolation kit (Qiagen, Valencia, CA). The control plasmids, p35S-gfp4, p35S-gfp5-ER (Jim Hasselhoff, University of Cambridge, UK) and pBI221 (Clontech, Palo Alto, CA) were obtained through the UH College of Tropical Agriculture and contain the constitutive 35S CAMV promoter that drives the expression of either the green fluorescent protein (p35S-gfp) or the beta-glucuronidase enzyme (pBI426). A new plasmid, pSAG12-gfp4, was constructed from p35S-gfp4 (Fig. 3.4) and Pr-SAG12 from pSG516 (Fig. 3.3) were excised and subcloned into pBLUESCRIPT KS II (Sambrook et al. 1989) to create pSAG12-gfp4 (Fig. 3.1). Restriction enzyme cloning sites were available in both original vectors so a unique subcloning scheme was established as follows: a) gfp4-nos was excised from 35S-gfp4 and cloned into pBSII KS; b) Pr-SAG12 was excised from pSAG12-GUS; c) sag12-nos was cloned into pBSIIKS-GFP4. This formed pSAG12-
**gfp4** (Fig. 3.1). Scale-up and isolation of all plasmids were performed using identical methods as previously described for pSAG12-gus. Plasmids were concentrated or diluted to give a solution of 1 µg/µl for biolistic bombardment procedures. Plasmid pSAG12-ipt in a binary vector (Fig. 3.2) was also provided by Richard Amasino for future experiments with stable transformation.

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**Figure 3.1:** pSag12-GFP4 in pBluescript II KS. **Figure 3.2:** pSag12-ipt in a binary vector.

**Figure 3.3:** pSag12-ipt in pUC18 termed pSG516 **Figure 3.4:** pBIN 35S-mGFP4.
Particle bombardment of Anthurium leaves.

Tissue cultured leaves from anthurium varieties Marian Seefurth and Southern Blush were excised halfway down the petiole and incubated immediately on sterile deionized H₂O for 5 minutes. The leaves were then placed on agar plates, with the petiole submerged. A control was set up to bombard leaves of whole tissue culture plantlets to determine if detachment induced expression of sag12 in healthy leaves. Microprojectile bombardment of anthurium leaves was conducted with the Biolistic PDS 1000/He System (BioRad, La Jolla, CA) at 1,550 psi with 28 Hg vacuum in the chamber, macrocarrier level 3, and leaf tissue level 4. 3μg of plasmid DNA was combined with 1 mg of 1.6μm gold particles (BioRad). 50 μl of 2.5 M CaCl₂ and 20 μl of 0.1 M spermidine were added simultaneously and vortexed for 3 min. The plasmid DNA-coated gold particles were washed with 70% ethanol and suspended in 60 μl of absolute ethanol. Ten microliters of this mixture was loaded on carrier disk and used for bombardment of detached anthurium leaves (Itaya et al. 1997). Gold particles without plasmid were washed in 70% ethanol 2X, resuspended in absolute ethanol and loaded directly on the macrocarriers.

GUS determination

After bombardment, leaves were incubated for 3-5 days at room temperature under low light before visualization was detected. Staining protocols for GUS activity were performed according to Jefferson (1987). X-gluc buffer (50 mM phosphate buffer (pH 7.2) containing 0.05% [beta]-mercaptoethanol and 0.0025% Triton X-100, 0.1 mM
potassium ferricyanide and 0.1 mM potassium ferrocyanide. 0.5 mg/ml 5-bromo-4-chloro-3-indoyl glucuronide (X-gluc) was added fresh in a DMF solution prior to staining leaf tissue. The bombarded leaf tissue was incubated at 24°C for 2-3 days, then incubated in the X-gluc solution (pH 7.0) for 4 hours (arabidopsis) to 24 hours (anthurium).

**Visualization and detection of GFP**

Photo-documentation was obtained by using the Olympus BX60 UV-microscope with a “wild-type” filter set (Omega Optical cat # XF1076, #XF2040, #XF3003) that effectively excites at ~λ 400nm and detects emissions at ~λ 505-540 from wtGFP (mGFP4). The second filter set is the FITC cube filter for visual detection of GFP5 (Omega Optical cat # XF1063, #XF2054, #XF3067) excitation ~λ 488nm, emission ~λ 505-530nm. Both filter sets can detect GFP4 and GFP5. Pictures were documented with the SPOT 1.5.0 camera (Diagnostic Instruments Inc., Sterling Heights, Michigan) and SPOT software v.3.0. PCR amplification of GFP cDNA was verified via gel electrophoresis. The cDNA was constructed from mRNA fractions of pooled leaves 5 days post-bombardment. The mRNA was treated with RQ1 DNase (Promega) to avoid contamination from plasmid constructs.
Results

*Pr-SAG12 transient bombardment assays for expression of GFP*

This experiment sought to determine whether a senescence-activated promoter (Pr-SAG12) from the dicot Arabidopsis is regulated in a senescence-dependent manner in the monocot anthurium. The second purpose of this experiment was to develop a suitable reporter gene system for this plant because GUS was not expressed in anthurium under a variety of conditions (Hayden and Christopher, data not shown). Therefore, we conducted transient assays using biolistic bombardments of healthy and senescent leaves with Pr-SAG12 and the constitutive 35S-Pr fused to both green fluorescent protein genes, *gfp4* and *gfp5-ER*. The FITC filter cube, which allows orange chlorophyll autofluorescence, and the wild type GFP (wt-GFP) filter cube, which eliminates it, were used. In Figures 3.6, 3.7, 3.8A, 3.8B and Table 3.1, the positive control, 35SPr-gfp4, is robustly expressed in both healthy and senescing anthurium leaves, while Pr-SAG12-gfp4 (Fig. 3.5, 3.8C, 3.8D) was expressed 20-fold higher in senescing relative to healthy tissue. The 35SPr-gfp4 was expressed 1.4-fold higher in healthy relative to senescing tissues (Table 3.1). Both *gfp4* and *gfp5-ER* were expressed markedly (Figs. 3.8E and 3.8F), which demonstrated the suitability of these reporters for gene expression studies in anthurium. Figures 3.9A and 3.9B show the wound-induced expression of Pr-SAG12-gfp4 in healthy tissues. This also serves as a comparison of the wtGFP (Fig. 3.9B) and the FITC (Fig. 3.9A) filter sets.

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Figure 3.5: GFP in senescing leaf tissues expressed with Pr$_{rag12}$ Bar scale=100µm

Figure 3.6: GFP in senescing leaf tissues expressed with Pr$_{35S}$ Bar scale=100µm

Figure 3.7: GFP in senescing leaf tissues expressed with Pr$_{35S}$ (lower mag.) Bar scale=100µm
Figure 3.8. Representative examples of GFP expression driven by the 35S promoter or Pr-SAG12 as measured via transient assays after biolistic bombardment of healthy and senescing leaves (Table 3.1) (A,B) mgfp4 expression with the 35S promoter, properly folds and can be visualized in anthurium tissue. Visualization demonstrated with the FITC filter cube, excitation 370-420nm, emission 505-540nm. (C,D) Two focal view points of pSAG12-mgfp4 plasmid expression in senescing tissue demonstrated with the wtGFP filter cube. (E,F) A comparison of mgfp4 and mgfp5-ER transient expression in similar tissues after bombardment. (E) GFP4 localizes around nucleosomes and the cytoplasm which in epidermal leaf cells. (B) GFP5-ER seems to localize in cytoplasmic organelles including proplastids and the endoplasmic reticulum. All photographs taken with the SPOT camera system, and are at 10X magnification.
Figure 3.9: (A,B) pSAG12-mgfp4 expression in healthy tissue, small localization of areas possibly caused by wounding taken with the FITC (A) and wt-GFP (B) filter cubes. Photographs taken with the SPOT camera system, and are at 10X magnification.

Table 3.1: Quantitation of \( p_{SAG12} - gfp4 \) and \( p_{35S} - gfp4 \) expression in senescent and healthy tissues.

<table>
<thead>
<tr>
<th></th>
<th>( p_{sag12} - gfp4 )</th>
<th>( p_{sag12} - gfp4 )</th>
<th>( p_{35S} - gfp4 )</th>
<th>( p_{35S} - gfp4 )</th>
</tr>
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<tbody>
<tr>
<td>Healthy Leaf</td>
<td>6.8 (± 3)</td>
<td>135.2 (± 71.5)</td>
<td>267.6 (± 74.2)</td>
<td>180.3 (± 44.8)</td>
</tr>
<tr>
<td>Senescent Leaf</td>
<td></td>
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</tbody>
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Discussion

Plasmids \( p_{SAG12} - gfp4 \), \( p_{35S} - gfp4 \), \( p_{35S} - gfp5-ER \), \( p_{Blue} - gfp4 \) were used to test transient expression in developmental stages of leaf tissue in anthurium. Preliminary experiments focused on transiently expressing the \( uidA \) or \( \beta \)-glucuronidase (GUS) gene in both arabidopsis and anthurium Leaf tissue. These experiments show that the bombardment and staining procedures were correct as evidence of the of arabidopsis leaf tissue showing the enzymatic cleavage of \( -Bromo-4-chloro-3-indoxyl-beta-D-glucuronide \) into its colorimetric product (data not shown). It was also confirmed that the \( p_{SAG12} - gus \) vector was regulated in a senescence-associated manner, when transiently
bombarded into arabidopsis tissues. The constitutive promoter 35S also successfully expressed gus in arabidopsis leaves. Variations of incubation time and temperature, staining concentrations, and infiltration techniques were attempted for anthurium GUS assays with no success.

This is consistent with previous research determining that the gus gene is effectively expressed in stably transformed anthurium tissues, but GUS protein was not detected by Western blot analysis (Kuehnle and Chen 1994). According to Kuehnle and Chen, modifications at the gene level contribute to the inactivation of GUS. This is not entirely consistent with the data presented in the research because key experiments were missing that would effectively deduce this postulation. The authors performed PCR on genomic DNA extracted from transformed tissues and confirmed the presence of the inserted gene sequences. The second experiment showed that a Western blot of proteins extracted from the transgenic plants did not successfully respond to the anti-β-glucuronidase antibody. More research is needed to come to a firm conclusion on this data. Firstly a genomic restriction digest can be performed and run on an agarose gel, the southern of this gel can confirm the presence of multiple copies of the transgene in the genome. The presence of multiple transgene copies can lead to promoter methylation and result in gene silencing (Hobbs et al. 1990). Direct or inverted repeats can cause hairpins also noted as triggers of transgene silencing. A reverse primer PCR (rpPCR) experiment is commonly used to determine if inverted or tandem repeats of T-DNA inserts occur in the transgenic genome (Kumar and Fladung 2000). These techniques are in addition to an RT-PCR that is used to identify a mRNA transcript of the gus gene consequently determining the problem to be transcriptional or translational.
In our transient assays no study was conducted to determine if mRNA transcripts of *gus* were present. However, it was determined that there was no interaction of GUS with the substrate resulting in the colorimetric product. When both GFP and GUS were used as reporter genes, we successfully visualized GFP in parallel with the unsuccessful production of GUS. GFP mRNA transcripts were confirmed via RT-PCR, after digestion with DNase to eliminate any contaminating genomic or plasmid DNA. This may indicate that unsuccessful GUS production occurs at the translational or post-translational level. Improper folding, post-translational modification, or interaction with other cell components may be the primary cause. Anthurium tissues contain a unique ratio of phenolic compounds which could irreversible bind to a foreign protein. Thionins (small defense proteins) have also been suggested as inactivators of the GUS protein (Diaz et al. 1992). It has been determined that the GUS reporter enzyme system is an unreliable, if not unusable, system for expression analysis studies in anthurium. We report here that GFP is an effective reporter gene system for anthurium. The benefits of the GFP system include non-invasive and non-destructive analysis of transformed and transiently expressing tissues, ease of documentation, and clear differentiation from endogenous proteins. To understand GFP and how it interacts with anthurium physiology we conducted several bombardments with 35S-*gfp*4 and 35S-*gfp*5-ER. GFP4 was chosen for experiments involving the characterization of Pr-*SAG12* due to ease of sub-cloning procedures with available Pr-*SAG12* vectors.

GFP analysis was performed multiple times with healthy and senescing tissues. In this report we show that Pr-35S promotes GFP expression in anthurium leaf tissues, and Pr-*SAG12* promotes GFP in a senescence-upregulated manner. Observing GFP4 in
anthurium tissues was performed with two different confocal microscopes and two different tissues. The materials and methods section lists the primary method, tissue, and equipment. The first experiment focused on GFP visualization in senescing leaves, driven by both Pr-\textit{SAG12} and Pr-35S, from mature plants cut into 10 cm$^2$ sections. These tissues were incubated post bombardment one month to maximize expression potential and GFP accumulation. The senescing leaf squares had minimal amounts of chlorophyll left providing a source free of background fluorescence, making it easy to visualize GFP fluorescence. Figure 3.6 and 3.7 show good examples of GFP, establishing that Pr-\textit{SAG12} (Fig. 3.5.) is regulated in anthurium tissues but visually appears to be less active than the GFP regulated by the constitutive Pr-35S (Fig. 3.6). At a reduced magnification (Fig. 3.7), it is easy to see a pattern of fluorescent vs. non-fluorescent sections of the anthurium leaf square.

Further analysis was conducted on a late model confocal microscope (Olympus BX60) fitted with a digital camera for documentation. This camera allows the use of multiple filter cubes, to reduce background, and gain a different perspective on fluorescent bodies within the cell. These experiments were conducted with whole anthurium leaves (\textit{Marian Seefurth} and \textit{Southern Blush} cultivars) that were harvested from tissue culture plantlets. The size of tissue culture leaves are significantly smaller than whole leaves which results in a slightly smaller overall cell size. No significant difference was observed between leaves that were excised from tissue culture plants and placed on agar, and those leaves that were bombarded while still attached to the tissue culture plantlet.
Figure 3.8A and 3.8B demonstrate GFP4 fluorescence visualization, with a FITC cube, in anthurium leaves. The two pictures represent two different focal points and GFP intensity in the epidermal layers of the leaf tissue. GFP4 was detected in the perimeter of the cells and seemed to interact closely with the cell wall or plasma membrane creating a distinct hexagonal shaped outline. This is consistent with previous reports involving the visualization of hypocotyl epidermal cells in Arabidopsis showing cytoplasmic localized GFP pressed in a thin layer between the cell wall and vacuole (Haseloff et al. 1997). The visualization usually occurs in the nucleosome or cytoplasm with little or no association with other organelles in the cell. Epidermal cells commonly referred to as “pavement cells” commonly consist of large vacuoles, few organs, and no chloroplasts. These cells have a polygonal shape and are simply required for turgor and water retention on the surface of the leaf.

As depicted in Figures 3.8A, 3.8B, 3.8C and 3.8D the GFP4 fluorescence appears in a polygonal shape and is associated with these cells easily visualized on the surface of the leaf. The mesophyll layer is clearly present below this translucent cell layer. The mesophyll cells are filled with many chloroplasts that appear as autofluorescing, characterized by reddish/orange appearance. The FITC filter cube captured this distinct difference between fluorescence and background for healthy tissue GFP analysis. GFP5-ER has an ER retention signal. The presence of this signal peptide tends to direct it to accumulate in proplastids and other cytoplasmic organelles such as the endoplasmic reticulum, but not in the cytoplasm or nucleosome. Figure 3.8E and 3.8F demonstrate the difference between GFP4 (E) and GFP5-ER (F). These photos are consistent with the two types of patterns we see with GFP4 and GFP5-ER in our transient assays.
seems to be associated with structural organelles at or below epidermal layers and typically associated with the mesophyll cell, whereas GFP4 is easier to visualize in the cytoplasmic contents of the epidermal cells, making it more readily detectable when using the FITC filter cube, which also captures the auto-fluorescence from chlorophyll. The GFP5 is slightly difficult to visualize with the FITC filter cube because of the interfering fluorescence from chlorophyll. The Olympus microscope is limited to the photo-documentation of an area no larger than the 120 cell area we obtained at 10X. The actual image observed in the microscope optical at this magnification is nearly 5-8 times this amount. Overall, it was easier to visualize GFP4 in anthurium tissues, both healthy and senescent with the FITC filter cube. Senescent tissue had a slightly more greenish/yellow auto-fluorescence making the wtGFP cube a better choice for documentation because of it eliminated this background. Healthy tissues having GFP4 present on the epidermal portion of leaf tissue showed extremely clear results.

The main purpose of developing a reporter gene system that functions in anthurium is to evaluate the senescence-associated promoter, Pr-SAG12, from arabidopsis. Such regulation suggests that anthurium has similar developmental mechanisms involved with senescence. These include regulatory elements, transcription factors, enhancer regions, and a myriad of proteins designed to initiate, delay, or prevent the development in senescence. SAG12 is a cysteine protease isolated from arabidopsis that shows a distinct expression pattern during development. This pattern shows no expression during non-senescent conditions, but a steady increase throughout senescence, until it is one of the most abundant proteins during late senescence (Lohman et al. 1994). The SAG12 promoter was cloned from an arabidopsis genomic library and was further determined to
be regulated in a senescence-upregulated manner through reporter gene expression (Gan and Amasino 1997).

More recent research on Pr-SAG12 has identified the specific region that confers senescence specificity, and enhancer region, and a transcription factor binding site (Noh and Amasino 1999a). When these regions were compared with two SAG12 homolog promoters isolated from Brassica napus, two highly conserved regions were detected (Noh and Amasino 1999b). Both these regions are necessary for senescence specificity in both Brassica and Arabidopsis. Furthermore, transcription factors from arabidopsis senescing leaf fractions bound to both Arabidopsis and Brassica promoters when radio-labeled probes of the essential -607 to -565 promoter region were incubated in whole cell extract of arabidopsis senescent leaves. This, along with Pr-SAG12 successful regulation in tobacco, establishes that transcription factors and promoter binding locations are conserved in related species.

The research we present indicates that senescence-specific transcription factors are also present in anthurium, and may be distinctly similar to those from arabidopsis. Figures 3.6, 3.8C and 3.8D show GFP4 expression in anthurium regulated by Pr-SAG12. These pictures clearly show two unique focal points of GFP4 expression in senescing tissues with the wt-GFP filter cube. Table 3.1 shows the average expression patterns of multiple leaf bombardments using the vectors listed. Each leaf was observed post-bombardment and regions of contiguous cells were identified and individual cells that make up that region were counted and recorded. Table 3.1 shows the average number of cells within a contiguous region for each bombardment series. A distinct difference was
observed between healthy and senescing leaf tissue when bombarded with $p_{SAG12}$-gfp4. Expression was broad in senescing tissues, however it was also identified in small regions in healthy tissues. Figure 3.9A and 3.9B show the character of the localized region of GFP expression in healthy tissues. This expression might be associated with cells damaged when the biolistic particle penetrated the epidermal cells. GFP visualization was never widespread and not present outside the area of damage. Figure 3.9 also displays the difference between the FITC filter cube (A) and the wt-GFP filter cube (B) on an identical sample. Based on these results we feel confident that Pr-$SAG12$ is regulated in a senescence-associated manner in anthurium tissues, suggesting orthologous mechanisms that regulate molecular mechanisms of senescence between anthurium and Arabidopsis are present. It is also evident that the promoter may be regulated, to some extent, in response to wounding caused by the biolistic bombardment consistent with a cell autonomous view of senescence proposed by Weaver and Amasino (1998). However, this regulation is localized and $sag12$ only shows a broad overall pattern of expression in leaf senescent tissues.
CHAPTER IV

ISOLATION AND CHARACTERIZATION OF TWO CYSTEINE PROTEASE GENES FROM ANTHURIUM AND THE EXPRESSION PATTERN OF EACH DURING DEVELOPMENT AND IN RESPONSE TO HORMONE TREATMENTS

Introduction

Understanding gene regulation during senescence begins with isolating and characterizing senescence-associated genes (SAG’s). These genes give us insight into the complex molecular process involved during senescence. Cysteine protease genes expressed during senescence are of particular interest since they fall under class 5 SAG expression. This stage indicates expression at the extreme initiation of senescence that is sustained throughout the later stages. There also exists many isoforms of cysteine proteases with different expression patterns suggesting unique and specific roles in the developmental cycle.

One benefit that aids in the characterization of these genes is the extent of homology contained in specific regions representing enzymatic functionality. This enables the creation of degenerate primer sequences that can produce the successful cloning of small portions of DNA from cysteine protease cDNAs synthesized from unique developmental mRNA populations. Once a native fragment is isolated, it can serve as a useful tool to identify full-length cDNAs. This can be accomplished by using it as a probe to identify larger fragments within a cDNA library, or can simply be used to develop primers and amplify cDNA ends within any given cDNA population. The cDNA
fragment may also be useful in developing an early expression pattern using Northern Blot analysis. This analysis must be double checked for accuracy after the full-length transcript is isolated.

Analysis of SAG gene expression by Northern Blot analysis of developmental RNA populations indicates differential expression patterns confirming the senescence-associated gene expression. Once the SAG is identified it can be used in correlation with the non-senescence associated cysteine protease, photosynthetic genes (\textit{Lhcb} and \textit{psbA}), and a constitutively expressed gene (\textit{ubi}), to determine expression under senescence controlling conditions.

A comparison of expression patterns in response to stress and hormone treatments is an excellent indicator in characterizing senescence-associated genes (Weaver et al. 2001). Certain stresses and hormones are able to hasten or repress senescence as seen visually by leaf yellowing and detected on the molecular level by responses in gene expression. Cytokinin is a well-known senescence-delaying phytohormone (Gan and Amasino 1997; Nooden et al. 1997). Endogenous treatments of the synthetic cytokinin benzyladenine (BA) show a repression of senescence characteristics when applied on anthurium leaves and flowers (Paull and Chantrachit 2001). Cytokinin can be used to repress many SAG transcripts, and actually reverse the senescence of a leaf in the later stages. Considering sugars are the main product of photosynthesis and certain sugars can serve as signaling molecules, changing sugar levels during photosynthetic decline may act as a senescing-inducing signal (Jang et al. 1997). Sucrose can effectively repress the accumulation of certain SAG transcripts when applied to detached leaves (Noh and Amasino 1999a).
Although these treatments are somewhat effective in reversing the effects of senescence and SAG transcripts, inducing senescence in leaves can result in a different pattern of SAG transcripts from the observed expression during developmentally-induced senescence. The most common techniques of inducing senescence include detachment and incubation of leaves in water under dark and light conditions; desiccation; and hormone treatments with ABA and ethylene. Certain SAG transcripts may not respond to this type of induced senescence although the visual indicator (chlorophyll loss) of senescence progresses rapidly (Noh and Amasino 1999a).

This chapter presents the details of two unique cysteine proteases isolated from the anthurium species. The genes are characterized through comparison with like genes from the GENBANK data base, the deduced amino acid sequence, and expression level during development and hormone treatment.
Materials and Methods

Plant material and growth conditions

Tissue culture plantlets of *Southern Blush* (graciously provided by Agri-Starts Micro, Mt. Dora, Florida.) and *Marian Seefurth* (University of Hawaii) were grown in Magenta® GA-7 vessels (20-40 $\mu$E m$^{-2}$s$^{-1}$, 12 h light/12 h dark cycle) at 25°C and 35% Humidity. Greenhouse-grown *Marian Seefurth* was grown under 78% shade (20-28°C, 80-95% humidity) year round at the University of Hawaii, Manoa campus (Honolulu, Hawaii).

RNA isolation

Leaves from greenhouse grown anthurium plants (Marian Seefurth) were collected and developmental stages were characterized by visual inspection and chlorophyll determination. After collection or treatment of leaves, they were directly frozen in liquid nitrogen and total cellular RNA was extracted using the protocol developed by Champagne and Kuenhle (2000) with a few modifications. PVPP (sigma P-6755) was substituted in place of PVP-40 and all extractions were performed at 13°C. In addition, a low salt, low ethanol precipitation was included prior to the final ethanol precipitation step to remove polysaccharide contamination from older tissue (Asif et al. 2000). A full protocol is located in the Appendix.
Hormone treatments and chlorophyll determination

Cut leaf sections (40 mm X 40 mm) from a mature healthy leaf, were submerged in sterile H₂O for 30 seconds, then floated on either 1 μM N⁶-Benzylaminopurine (BA, Sigma B-3408) or 3% Sucrose (Sigma S-5390) solutions or sterile deionized water (controls). All samples were incubated in darkness for the duration of the experiment. Hormone treatments of whole leaves derived from greenhouse-grown plants were conducted by submerging the petioles in either sterile H₂O (controls), or 3% sucrose or 1 μM BA. For the BA treatments, a 1 mM BA solution was also sprayed on the leaves and allowed to dry. Chlorophyll was determined as μg chl / g fresh weight of tissue for all samples according to Lichtenthaler (1987).

RT-PCR, RACE, cDNA library construction, screening and cDNA characterization

Total cellular RNA isolated from greenhouse-grown leaves at various stages of development was enriched for poly(A)⁺ RNA using the PolyATtract mRNA Isolation System III (Promega Co., Madison, WI). A mixture of poly(A+) mRNA was prepared from the different developmental stages at a ratio of 4:2:2, senescent, mature and immature leaves, respectively. Reverse transcription was performed on this RNA with 100 units M-MLV Reverse Transcriptase (New England Biolabs, Beverly, MA). An additional tracer reaction with [α³²P]dCTP (ICN, Costa MESA, CA) was performed to assess the purity, quality and size of cDNA products, via alkaline agarose gel electrophoresis and autoradiography (Sambrook et al. 1989). The remaining RNA was removed with 0.2M NaOH and the single-stranded cDNA products were used directly in.
PCR. Degenerate primers with the sequences, forward 5'
TGYGGNAGYTGYTGGGCNTTY 3' and reverse 5'
TGGATHGTAARAACAGYTGGGGN 3' [Y(CT), N(ACGT), H(ACT), R(AG),
K(GT)] (IDT, Coralville, IA, USA) were constructed based on conserved regions in
known cysteine proteases from other plants. They were used in PCR to amplify two
different products of 500 bp that were cloned into the TOPO TA vector Invitrogen
(Carlsbad, CA). Sequence analysis (BMBITF, University of Hawaii at Manoa) confirmed
that two different PCR products, that shared high sequence identity with cysteine
proteases, were cloned. The inserts were used as probes to screen the cDNA library
according to Sambrook et al. (1989). The library was constructed from the mixture of
leaf poly(A)+ mRNA (ratio described above) using the ZAP-cDNA synthesis kit from
Stratagene (La Jolla, CA). Two different cysteine protease cDNAs were isolated,
sequenced, and designated, \textit{anth16} and \textit{anth17}. Rapid amplification of 5’ cDNA ends
(RACE, GeneRacer, Invitrogen, Inc.) was conducted to complete the full cDNA
sequences and verify the original cDNA clones. In addition, a cDNA encoding cab was
isolated via RACE.

All sequences were analyzed using the GCG package (Madison, WI), BLAST
(www.cbs.dtu.dk/services/SignalP), ClustalW (dot.imgen.bcm.tmc.edu:9331/multi-
align/Options/clustalw.html), Swiss EMBnet (www.ch.embnet.org/software/BOX_form)
(Boxshade) and links on the CMS MBR homepage (restools.sdsc.edu/).
Northern blot analysis, probe construction, and band quantification.

RNA concentrations were determined spectrophotometrically and verified using the Kodak 1D scientific imaging system (Kodak, New Haven, CT). Total cellular RNA (3-5 ug) was denatured and loaded on a 0.75% formaldehyde agarose gels as described (Hoffer and Christopher 1997). After electrophoresis and transfer to Genescreen plus (NEN, Boston, MA), (Sambrook et al. 1989) the blots were prehybridized for 6 hours and then hybridized to [α^{32}P] dCTP-labeled probes. The probes were assembled as follows: Anth17 full-length cDNA clone, Anth16 overlapping 5' and 3' regions from RACE, psbA 1.3 KB fragment from arabidopsis, cab overlapping 5' and 3' regions from RACE, ubi 1.3KB cDNA clone from Pineapple Library. The photosynthetic genes were used as a reference for nuclear and chloroplast photosynthetic gene activity during development (Rapp et al. 1992; Oh et al. 1996; Hajouj et al. 2000). Labeling was performed with the decaprime kit (Ambion, Austin, TX) and unincorporated radioisotope was removed with ProbeQuant G-50 Microcolumns (Amersham Pharmacia, Little Chalfont, UK). Hybridization and prehybridization were done in 50% deionized formamide, 5x SSPE and 2.5% SDS at 42°C for 24 hours. After hybridization, blots were washed 3X for 30 minutes in 0.5X SSPE/1% SDS at 42°C before exposure to Classic blue sensitive X-ray film (Molecular Technologies, St. Louis, MO). The blots were exposed to a Cyclone storage phosphor screen, and radioactive bands were quantified using the Optiquant imaging system (Packard Instrument Company, Inc. Meriden, CT). Data (digital light units, DLU•mm⁻²) was normalized to uniform probe length and by subtracting background DLU. The mean ± standard error was calculated for three replicated experiments using the highest value for each probe as 100%.
Results

Isolation and characterization of two cDNAs encoding cysteine proteases from anthurium

We sought to determine whether senescence-dependent cysteine protease genes that were homologous to arabidopsis sag12 were present in anthurium. Primers were designed, based on conserved amino acid sequences in plant cysteine proteases, and used in RT-PCR of a mixture of poly(A)+ mRNA from different tissues at a ratio of 4:2:2, senescent, mature and immature leaves, respectively. The mixture was used to increase the probability of obtaining more than one developmentally expressed gene family member. Two different PCR products were obtained that were homologous to cysteine proteases. They were cloned and used as probes to screen a cDNA library made from the same mixture of poly(A)+ mRNA. The resulting nucleotide and deduced amino acid sequences of the full-length cDNAs corresponding to the two different cysteine proteases (termed anth16 and anth17), are shown in Figures 4.1 and 4.2 respectively.

The boxshade analysis of the deduced amino acid sequences of anth17 and anth16 shows highly conserved regions with both dicot and monocot cysteine proteases (Fig. 4.3). Anth16 and Anth17 contain several characteristic regions, such as the highly conserved Cys-His-Asn catalytic triad, type II Gly residues, and several other conserved cysteine residues (Fig. 4.3) (Kamphuis et al. 1985; Cohen et al. 1986; Watanabe et al. 1991). Both proteins also contain a hydrophobic signal peptide (Fig. 4.1 and 4.2), a positively charged hydrophilic propeptide sequence (Fig. 4.9), and a C-terminal region
Figure 4.1. Nucleotide and deduced amino acid sequence of *anth16*. Computer analysis and assembly of cDNA clone sequences was performed with the GCC package. Marked features include the predicted signal peptide sequence (underline) and catalytic triad residues (Cys-His-Asn) marked with a circle.
Figure 4.2. Nucleotide and deduced amino acid sequence of *anth17*. Computer analysis and assembly of cDNA clone sequences was performed with the GCC package. Marked features include the predicted signal peptide sequence (underline) and catalytic triad residues (Cys-His-Asn) marked with a circle.
Figure 4.3. Alignment of amino acid sequences derived from anth16 and anth17 (*Anthurium andraeanum*) with those from 8 different plant proteases. Black box shade denotes complete conserved amino acids, while grey box shade denotes conservative amino acid replacements. Anth16 and anth17 are labeled along with other plant species abbreviations. The GENBANK Accession numbers, [PV] kidney bean (T12041), [ZM] corn (T01207), [OS] rice (P25776), [HV] barley (AAD10337), [HH] daylily (P43156), [AT] sag 12 (AAC49135), [MC] *Matricaria chamomilla* (AAD54424). Key features * cysteine disulfide bonds □ Type II glycine residues missing from anth16 □ amino acids that form the catalytic enzyme site * important tryptophan residues. A proline-rich segment is indicated by black bar, including the additional inserted region found in anth16 identified with a thicker black bar region. A Potential targeting signal is boxed at the C-terminus of anth16.
also predicted to be subject to post-translational cleavage (Abe et al. 1987; Watanabe et al. 1991; Yamada et al. 2000). The cysteine proteases compared (Fig. 4.3) can be divided into two groups based on the presence of an ~100 amino acid c-terminal extension in the predicted polypeptides from anthurium, kidney bean, corn, rice and chamomile. The enzymes from arabidopsis, daylily and barley lack the c-terminal extension. Of the two clones, *anth17* is most closely related to seven out of eight of the plant cysteine proteases analyzed, such as maize, rice, bean and, of special note, arabidopsis SAG12 (Table 4.1). Anth16 is more closely related to a cysteine protease from chamomile. Interestingly, the polypeptides for ChaTP and Anth16 contain a novel 10 residue proline-rich insertion beginning at positions 372 and 373, respectively.

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<tr>
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<th>Anthurium 17</th>
<th>Anthurium 16</th>
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<tr>
<td><em>Zea</em> maize</td>
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<td>58</td>
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<td><em>mir3</em> cysteine protease</td>
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<tr>
<td><em>Phaseolus vulgaris</em></td>
<td>76</td>
<td>59</td>
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<td><em>cysteine protease precursor</em></td>
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<tr>
<td><em>Oryza sativa</em></td>
<td>76</td>
<td>57</td>
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<tr>
<td>Alpha chain cysteine pro.</td>
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<td><em>Brassica napus</em></td>
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<td>56</td>
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<td><em>COT144 cysteine protease</em></td>
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<tr>
<td><em>Hordeum vulgare</em></td>
<td>62</td>
<td>58</td>
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<tr>
<td><em>cysteine protease precursor</em></td>
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<tr>
<td><em>Hemerocallis ssp.</em></td>
<td>64</td>
<td>54</td>
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<tr>
<td>Thiol protease Sen102</td>
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<td><em>Matricaria chamomilla</em></td>
<td>52</td>
<td>60</td>
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<tr>
<td>Thiol protease</td>
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<td><em>Arabidopsis thaliana</em></td>
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<td><em>SAG12 protein</em></td>
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Table 4.1. Percent identity and similarity (in parenthesis) between the deduced amino acid sequences of cysteine proteases from 8 plant species and with *anth16* and *anth17* from *Anthurium andraeanum.*
Because a senescence-regulated system was identified in the transient reporter gene expression assays (Table 3.1), we conducted northern blot hybridization analysis to determine if either \textit{anth16} or \textit{anth17} mRNA levels were regulated during senescence. As a control for photosynthesis genes that are expected to be down-regulated during senescence (Rapp et al. 1992; Oh et al. 1996; Buchanan-Wollaston and Ainsworth 1997; Hajouj et al. 2000), we also isolated a \textit{cab} cDNA, which encodes a light harvesting chlorophyll-a,b-binding protein. It was used in conjunction with a heterologous barley chloroplast \textit{psbA} gene (encodes D1 subunit of PSII) and pineapple \textit{ubi} cDNA (ubiquitin).

The samples consisted of total cellular RNAs from five different stages of leaf development, including young (YG) and mature healthy (MG) leaves and leaves from three stages of senescence (S1, S2, S3). The three stages of anthurium leaf senescence were defined based on chlorophyll levels, as shown in Figure 6 (S1: 60%-90%, S2: 30%-60%, S3: 5%-30%). In Figure 7, \textit{anth17} mRNA levels are low in healthy green leaves, while they increase during senescence, reaching peak levels at S3. In contrast, \textit{anth16} mRNA levels are undetected during senescence and are highest in young leaves. The mRNA levels for genes encoding the photosynthetic apparatus (nuclear-encoded \textit{cab} and chloroplast-encoded \textit{psbA}) are high in healthy leaves and decrease markedly during senescence. \textit{Cab} mRNA levels decrease markedly compared to \textit{psbA} mRNA levels. The \textit{psbA} mRNA levels may be maintained longer during senescence than \textit{cab} because the highly stable \textit{psbA} mRNA has a half life of 48 hrs (Kim et al. 1993). Ubiquitin gene expression increased markedly from MG to S3 and this may be associated with the numerous proteolytic processes occurring during senescence.
Figure 4.4. Expression of *anth17, anth16, cab, psbA, and ubi* mRNA in leaves at different developmental stages (A). Northern blots carrying RNA isolated from leaf tissue were hybridized with the indicated [³²P]-labeled probes. (B) Histogram displaying chlorophyll content of developmental leaves. YG refers to young leaves, fully expanded, but slightly fleshy of a brownish-green color. MG refers to mature leaves, fully ridged of a rich green color. S1, S2, and S3 indicate senescing leaves determined by chlorophyll concentration (S1:90-60%, S2 60-30%, S3 30-5%). (C) Histograms of gene expression during development for each probe set. The mean ± standard error was calculated for three replicated experiments using the highest value for each probe as 100%.
Synthetic cytokinin, BA, represses the expression of anth17

One of the defining characteristics of sag12 expression is its repression upon treatment with cytokinin. Sucrose also can affect senescence (Sheen 1990; Krapp et al. 1991; Jang et al. 1997b; Noh and Amasino 1999a). Therefore, Figure 4.5 provides the analysis of synthetic cytokinin treatment (BA, 1 μM solution/1 mM spray) and sucrose on anth17 expression relative to psbA expression in excised whole leaves placed in darkness for 10, 20, 30 and 40 days. Leaves incubated in water served as a control. Whole leaves were selected 10-15 days after full expansion. The BA-treated leaves were also sprayed with a 1 mM BA solution, similar to the treatment commercial cut anthurium flowers received prior to sale to increase shelf life (Paull and Chantrachit 2001). Anth17 mRNA levels were significantly lower in BA treated leaves during all time periods (Fig. 4.5). The levels of psbA mRNAs were higher in the BA treatments for 10 and 40 days relative to controls, but were not significantly different at 30 and 40 days. Sucrose repressed psbA expression, but did not repress anth17 expression in whole leaves. This is consistent with previous studies that showed sugars repress photosynthetic gene expression (Sheen 1990; Jang et al. 1997).

In the experiment in Figure 4.5, it was not possible to use leaves at the exact same stage of development. Thus, an additional approach was conducted using leaf disks, which were harvested from a single leaf at the mature phase in each trial. A second advantage of the leaf disks is a more uniform treatment of cells within the disk. The leaf disks were incubated by floating on a 1 μM BA, sucrose, or an H2O solution for 3, 8, 14 and 20 days. Anth17 and psbA mRNA levels were measured via northern blot hybridization analysis. In the BA treatments, anth17 mRNA levels were 50 to 75% lower
Figure 4.5. Expression of *anth17* in comparison with photosynthetic transcript *psbA* under cytokinin treatments. (A) Whole leaves were excised with petiole intact and incubated in darkness for 40 days with water serving as a control. Leaves were harvested at corresponding intervals and RNA was extracted. Expression of *anth17* and *psbA* is indicated. Northern blots carrying RNA isolated from leaf tissue were hybridized with the indicated [32P]-labeled probes. (B) Histogram analysis of *anth17* and *psbA* as expressed in specified treatments.
Figure 4.6. Leaf discs were excised from a single leaf and incubated for a time interval of 3, 8, 14, and 20 days in 1μM BA, 3% sucrose, with water serving as a control. Expression of *anth17* and *psbA* is indicated. EtBr staining is included to verify equal loading. (B) Histogram analysis of *anth17* and *psbA* as expressed in specified treatments. Histograms of expression data were calculated using the Phospholmager.
relative to water controls for 3, 8 and 14 days (Fig. 4.6). No difference was observed after 20 days, suggesting the effect of BA was breaking down. In the sucrose treatments, \textit{anth17} mRNA levels were lower in the 3, 8 and 14 day periods relative to water controls. The levels of \textit{psbA} mRNA were not significantly different in the BA compared to the water treatments, but decreased somewhat (25\%) in the sucrose treatment (Fig. 4.6). The lack of a marked effect of the treatments on \textit{psbA} mRNA levels could be related to the shorter time intervals (3-20 days, Fig. 9) relative to 10-40 days (Fig. 4.5) used to measure this highly stable mRNA (Kim et al. 1993).

Discussion

\textit{RNA Isolation and Manipulation}

Studying the molecular genetics of anthurium has been hampered by a lack of suitable protocols for the extraction and analysis of DNA, RNA, and proteins. In order to effectively research genes and promoters of any anthurium species, a suitable RNA and DNA extraction method was developed to deal with the high concentration of phenolics, polyphenolics, and polysaccharides located in all anthurium tissues. Phenolics are compartmentalized in intact cells. During homogenization for nucleic acid recovery, these compounds are oxidized and then interact irreversibly with proteins and nucleic acids (Schneiderbauer et al. 1991).

Phenolics and polyphenols are characterized as having a high oxidation potential. Phenolics most commonly oxidize to form quinones. These compounds are then covalently coupled with nucleic acids preventing their isolation and extraction. The nitrogen of the pyrimidine and the hydroxyl group of the carboxylic acid component of
RNA is highly reactive with phenolic components forming molecular aggregates. These interactions can also occur with "face to face" and "edge to face" interaction of aromatic surfaces, protonation of amides, hydrogen bonding, dipole-dipole, and dipole-induced dipole interactions leading to the crystallization of the structures. These structures can exist alone preventing separation or can form large molecular aggregates that sequester proteins, carbohydrates, phenolics and nucleic acids (Sanenger 1984; Haslam 1998). The resultant solution we observed in our attempts to extract RNA appeared in many forms. It has resembled a dark brown mixture that becomes extremely fluffy during re-precipitation with salts, extremely viscous solutions containing many polysaccharides, and a solution containing no RNA at all due to its removal during purification. These types of solutions contained little or no recoverable nucleic acids available for analysis.

Many attempts were conducted to isolate total RNA from anthurium using traditional extraction methods (Sambrook et al. 1989), guanidinium isothiocyanate-phenol-chloroform (Chomczynski and Sacchi 1987), high phenolics extractions (Schneiderbauer et al. 1991; Levi et al. 1992), removal of polysaccharides, additional precipitation steps, and commercially available RNA extraction kits (Qiagen, Valencia CA; Ambion, Austin TX; Promega, Madison WI). None of these methods was suitable for isolating high quality DNA/RNA. It was determined that a broad variety of secondary compounds, especially phenolics found in anthurium leaves, reacted with the RNA and bound irreversibly, resulting in low 260/280 ratios. The RNA was also unsuitable for any further manipulation such a template for cDNA synthesis and subsequent polymerase chain reactions. This is a typical result presented other attempts which
extract RNA or DNA from recalcitrant tissues (Schneiderbauer et al. 1991; Levi et al. 1992).

To extract high quality RNA from anthurium tissues of all developmental stages, we assembled a variety of methods to create a reproducible technique. The core extraction is based on a method published from researchers at the University of Hawaii (Champagne and Kuehnle 2000). It is suggested that introducing phenol during the early part of the extraction procedure may itself be responsible for an adverse reaction (Levi et al. 1992). The extraction does not introduce phenol until after the cellular material and non-nucleic acids have been removed by replacing early phenol/chloroforms steps with pure chloroform extractions. Chloroform extractions are sufficient enough in removing proteins and denaturizing ribonucleases (Murray and Thompson 1980). \(\beta\)-mercaptoethanol and PVPP are two components present in the extraction buffer that make a significant difference in preventing the oxidation of phenolic compounds.

Polyvinylpolypyrrolidone (PVPP) and Polyvinylpyrrolidone (PVP) have been used in multiple RNA isolation protocols to reduce oxidation, to bind phenolics, and to reduce contaminating substances. In an aqueous solution, PVP has a loose, random-coil type of conformation analogous to that of proline rich proteins and displays a strong affinity towards dissolved aromatic compounds including phenols (Molyneaux and Frank 1961). PVPP is an insoluble cross-linked polymer of PVP and resembles similar properties to PVP, but differing in solubility. PVP is used in a many extractions to bind contaminating phenolics by forming a complex by hydrogen bonding thereby protecting nucleic acids. This generally allows the phenolics to be separated from the RNA when combined with a phenol extraction of acidic pH. Insoluble PVP is preferred in extractions
since soluble PVP might obstruct or co-precipitate with the RNA. PVP may also be incompatible with phenol and require an ethanol precipitation prior to the introduction of phenol (Salzman et al. 1999). β-mercaptoethanol is a well known and well used strong reducing agent. In the absence of PVP, it has been shown to directly affect the quality of extracted RNA when present in high enough concentrations (Lal et al. 2001). Both compounds were used to facilitate the rapid removal of phenolic compounds during extraction, inhibiting oxidation reactions, which cause irreversible damage to the RNA.

Three versions of PVP/PVPP were used, a high molecular weight PVP (360,00 kDa), a low molecular weight PVP (40,000 kDa) and PVPP. β-mercaptoethanol was added at a concentration of 550mM and the extraction was conducted in 15 ml to maximize the dilution of polyphenolic compounds. Both PVP extractions seemed to produce RNA at a mediocre quality, contaminated with polysaccharides and genomic DNA but free of phenolics. Occasionally interfaces became unclear and a high loss of RNA occurred during phenol/chloroform extractions. The substitution of PVP with PVPP resulted in cleaner extractions and higher quality RNA. This method was also reproducible. It appeared most PVPP was transferred to the organic phase in the first few steps but was highly effective in eliminating phenolic contamination. PVPP also did not interact with phenol when it was introduced. A white pellet may form following the first precipitation, (2-propanol and Sodium Acetate) depending upon the preciseness of the phase transfers during the extraction, and contains insoluble material and RNA. However, if the pellet is exposed to gentle agitation at room temperature for a few hours, high quality RNA is obtained after the insoluble fraction is centrifuged and removed. This insoluble precipitate may be residual PVPP carried over in the precipitation. If in
fact this precipitate is PVPP it is able to be effectively removed from the RNA when water is added. This is most likely due to its reversible binding to RNA which enables PVPP to release the RNA when in a lower ionic solution. RNA yields are reasonable (50-100μg per gram of tissue), pure, and reproducible. Figure 4.7 shows 10μg of developmental RNA fractions extracted from anthurium leaf tissue run on a 1.5% Formaldehyde/MOPS gel and stained with ethidium bromide. It clearly shows the quality of RNA does not diminish until the leaf is severely senescent and the low presence of genomic DNA contamination. Figure 4.8 shows 14 separate leaf of hormone treated RNA. This RNA was loaded directly on the gel (5μl) to show the reproducibility of the extraction. This extraction is not efficient, fast, or cost effective but it has worked on all tissues of anthurium and Pineapple plants.

Figure 4.7. 10μg of Total RNA loaded on a 1.5% Formaldehyde MOPS gel and stained with Ethidium Bromide. RNA samples are from Immature, Healthy, S1, S2, and S3 leaves (left to right).
Analysis of Anth16 and Anth17 protein sequences

Two genes were sequenced from cDNA created using mRNA isolated from anthurium leaves. Comparisons of the deduced amino acid sequences with others that have been reported indicate high homology with monocot cysteine proteases (Table 3.1). The deduced polypeptide sequence of both cysteine proteases contain several regions characteristic of cysteine proteases including a highly hydrophobic signal peptide, a positively charged hydrophilic propeptide sequence, a mature protein, and a C-terminal region also predicted to be subject to post-translational cleavage. In addition both proteins contain the highly conserved Cys-His-Asn catalytic triad, type II Gly residues, and cysteine residues. Figure 7 and 8 show the nucleotide and deduced amino acid sequence of \textit{anth17} and \textit{anth16}, respectively and also shows marked features that will be discussed. Figure 4.3 shows boxshade analysis with other closely related cysteine proteases including \textit{sag12} a known senescence-associated cysteine protease from arabadopsis. Both clones were sequenced from cDNA created from mRNA extracted from all developmental stages of anthurium leaf tissue. Based on the deduced amino acid sequence, several attributes about the mature proteins can be predicted.

Anth17 and Anth16 have hydrophobic signal peptides located at Met$^1$ – Ala$^{27}$ and Met$^1$-Ser$^{25}$ respectively, which indicates the encoded polypeptides enter the secretory
pathway. The algorithms of (Nielsen et al. 1997; Emanuelsson et al. 2000) predict Anth17 to be involved in the secretory pathway with a final destination outside the cell. The same algorithm predicts Anth16 to also enter the secretory pathway and may associate with the plasma membrane to some degree. The prediction for Anth16 is at a lower level of confidence than Anth17. Several papers indicate common regions that are unique identifiers of cysteine proteases. Anth17 and Anth16 contain the conserved catalytic triad (17: Cys^{154}_5-His^{290}_5-Asn^{310}_5)(16: Cys^{170}_6-His^{308}_6-Asn^{328}_6) considered to be integral to the active site of cysteine proteases. 6 Cys residues integral for the formation of disulfide bridges are also present in their respective locations (17: 151,185,284,336) (16: 167,201,299,353). A conserved glutamine (17: 148)(16: 164) has been suggested to be the proton donor necessary for cleavage of the peptide bond. When the hydropathy profiles (Fig. 4.9) of both proteins are compared, the longer Anth16 seems to have increased regions of hydrophobicity over Anth17.

Kamphuis, Drenth and Baker (1985) performed comparative studies based on high-resolution structures of several thiol proteases and defined several key structural attributes necessary for proper activity. Cysteine proteases contain specific glycine residues that are usually associated with conformational flexibility of the protein that are involved mainly in secondary structure turns. Type I residues provide conformational flexibility and facilitate insertions and deletions in the sequence while type II are less flexible. Serious alterations of the main-chain conformation through steric interactions caused by the C^8 of amino acids other than glycine.
Figure 4.9. Hydropathy profile of the deduced amino acid sequences of Anth16 and Anth17 predicted using the Kyle and Doolittle method. Plot was derived to compare the two cysteine protease sequences with each other, substituting gaps in areas to increase alignment. Gaps are indicated below the region of introduction with the line color corresponding to the protein with the missing segment.

Position 284 may not be serious because of the presence of glycine residues upstream of 284 and directly following a conserved Glu-Pro-Val-Ser-Val segment. Position 300 is of particular interest because it is directly following an important Cys residue, and includes a 3 amino acid insertion increasing the distance between the Cys residue and the integral His involved in the catalytic triad. This substitution increases the distance between this highly conserved region from 5 to 8 amino acids. There is a small number of single amino acid insertions/deletions in the mature protein region of various cysteine proteases, however generally the interior portion of the polypeptide is extremely conserved. Anth16 and a thiol protease isolated from cultured shoot primordia of Matricaria chamomilla (termed ChaTP for identification purposes in this report) both exhibit this 3 amino acid insertion as well as the missing glycine residues.
Mature cysteine proteases are translated into long polypeptides and then cleaved, generally at both the NH and COOH termini, to produce a functionally active enzyme. Oryzain, a family of rice seed cysteine proteases, follows this methodology, while some cysteine proteases lack an extended COOH terminus tail (Abe et al. 1987; Watanabe et al. 1991; Yamada et al. 2000). Anth17 exhibits similar properties to these types of cysteine protease and it is most likely that the two termini tails are processed to yield a fully functional protein. The cleavage in the NH termini typically occurs before the first conserved Leucine (17: Lue\textsuperscript{136}) and near the Asp-Pro-Pro (17: Asp\textsuperscript{351}) sequence at the COOH termini. This cleavage would produce a mature fully functional cysteine protease of about 23.5 kilodaltons (Abe et al. 1987).

Anth16 and ChaTP exhibit slightly different properties at these two cleavage sites. At the NH termini cleavage site, they are missing the characteristic leucine which is replaced by a Cys-Asp-Ala motif before the highly conserved region begins at Pro\textsuperscript{147}. The COOH terminus includes a 10 amino acid, proline-rich, addition that includes the absence of the Asn-Pro-Pro conserved motif. No conserved or special sequence has been identified that regulates cleavage at these locations, however the Asn-Pro-Pro sequence of long tailed COOH cysteine proteases seems to be followed by a proline-rich segment that includes as many as 10 proline residues within a 15 amino acid segment. Anth16 and ChaTP have 17 and 19 proline residues, respectively, within a 26 amino acid segment.

Anth16 is unique in that the inserted 10 amino acid proline-rich segment contains a possible palindrome-like sequence that includes a Ser-Pro\textsubscript{4} segment. The Ser-Pro\textsubscript{4}, generally referred as Ser-Hyp\textsubscript{4} due to the post-translational hydroxylation of proline
residues in specific amino acid combinations, is present in almost all types of extensin peptides in repeats of three or more (Kieliszewski 94 for review). Extensin modules can also be found in chimeric proteins where the protein is homologous to a certain family, and a module, such as a tail region, is homologous to extensin. Such examples include cysteine-rich lectins (Kieliszewski and Lamport 1994), stylar transmitting tissue proteins (Baldwin et al. 1992), and chitinases (Sticher et al. 1992) as well as specialist proteins involved in defense (Kurata et al. 1993), wounding (Sheng et al. 1991), and lateral root initiation (Keller and Lamb 1989). Anth16 contains a proline-rich region that is longer slightly more organized than most long-tailed cysteine protease propeptides. It is unclear whether this region is of appropriate size or configuration to be considered a proline-rich region, however TARGETP analysis of the deduced amino acid sequence suggests that Anth16 may be specifically targeted towards the plasma membrane or cell wall, in comparison with the more conserved Anth17 which is predicted to go outside or to vacuole locations. Both proteins are predicted to be secreted.

Anth16 contains a COOH terminus tail sequence that includes a di-leucine (LL) and the Tyr-based sorting motif, YXXΦ (where Y refers to tyrosine, X refers to any amino acid residues, and Φ refers to hydrophobic residues with a bulky side-chain). Both motifs regulate specific sorting of proteins through intracellular vesicle compartments by associating with coat complexes such as clathrin (Sandoval and Bakke 1994; Mellman 1996). Although Anth16 contains both the YXXΦ and the LL signals, its is not known whether these motifs or the proline-rich sequence serve as anything that is functionally significant for differences in targeting, location, or unique function from other cysteine proteases.
It is interesting to note the inclusion of three potential signal or structural motifs in Anth16 and characterize it as having a potential function unique in comparison with typical cysteine proteases. Several proteins with similar signaling motifs have been identified or suggested to exhibit protease like functions. A class of group I allergens from grass pollen has the characteristic catalytic triad and exhibits some protease activity after the propeptide sequence is cleaved (Grobe et al. 2002). An arabidopsis drought-inducible cysteine protease-like protein was identified as putatively GPI (glycosylphosphatidylinositol) anchored (Borner et al. 2002). GPI anchors provide a potential mechanism for targeting the protein to the plant plasma membrane and cell wall (Morita et al. 1996; Youl et al. 1998; Sherrier et al. 1999). Extracellular proteases could be involved in the remodeling and degradation of plant extracellular matrix (ECM) proteins and are also involved in pathogen or wound response such as a matrix metalloproteinase that is activated in response to pathogen infection in soybean (Liu et al. 2001).

Both Anthurium andraeanum and Matricaria chamomilla are plant species that exhibit unique secondary metabolites. Anthurium includes a diverse combination of phenolics, polysaccharides, and cell wall components. This type of cysteine protease may play a role in negotiating interactions between these compounds during development as shown by the Northern analysis. Anth16 is expressed at low levels in immature tissues and not later in development. Analysis of the mature protein size, post-translational modification, and sub-cellular location would help further characterize this type of cysteine protease in anthurium.
Expression analysis of cysteine proteases during development

Gene expression throughout plant development tends to fluctuate and is greatly influenced by environmental factors. Any gene that plays a role in senescence can be considered as *Senescence-Associated Gene* or SAG. These genes can be present during other developmental stages, be induced by pathogen attack, or extremely sensitive to hormone interactions. A gene only needs to be regulated to some extent during senescence to be classified as a SAG. Other genes are senescence-enhanced, or senescence-upregulated, due to increased mRNA accumulation during senescence. However, only a select few show increasing expression from the precise onset of senescence through its conclusion, without being present during development. These genes are extremely important to any program which uses very specifically regulated promoters to manipulate senescence. In general these genes are not induced by pathogen attack, wounding, or desiccation. Overall a SAG gene is characterized by observing enhanced transcript abundance coupled with chlorophyll loss. Many SAG mRNAs appear to increase only after decreases in the nuclear-encoded *cab* mRNA, an important protein involved with photosynthesis (Rapp et al. 1992; Oh et al. 1996; Hajouj et al. 2000). Many factors can classify genes as senescence-associated, nevertheless a combination of factors needs to be analyzed to determine to what extent a gene is age-dependently *senescence-upregulated* or regulated by other senescing inducing pressures.

It can be reasonably concluded that the anthurium cysteine protease gene, *anth17*, has an expression pattern consistent with that of a senescence-associated gene. It is also determined that *anth17* is senescence upregulated and shows little or no expression during early development and maturity. *Anth17* expression increases during
developmental senescence and is present in increasing quantities even as total RNA levels decline 90% as senescence progresses (Fig. 4.4A). S3 stage RNA, ~10%-30% chlorophyll content compared to healthy leaves, show this expression pattern. A histogram of chlorophyll levels throughout development is provided (Fig. 4.4B). Anth16 shows relatively light expression and is present only in the early development of leaves. Photosynthetic gene expression is a good indicator of senescence initiation (Hensel et al. 1993). We show that the expression of the nuclear encoded gene for light-harvesting chlorophyll a/b binding protein (cab) is down-regulated rapidly in correlation to chlorophyll levels in senescing tissues (S1, S2, S3, Fig. 4.4). In comparison, expression of the chloroplast encoded gene psbA shows a more progressive decline during developmental phases S1, S2 and S3. This is consistent with the longevity of chloroplast transcripts and a potential delayed response to signaling events involved with senescence. The psbA mRNA levels may be maintained longer during senescence than cab because the highly stable psbA mRNA has a half life of 48 hrs (Kim et al. 1993).

The ubiquitin gene shows baseline expression during early development and increases expression throughout senescence. Thought to be constitutive, ubiquitin pathway genes have been shown to increase during senescence, since the massive degradation of proteins during leaf senescence likely involves ubiquitin tagging and proteolysis (Park, Oh et al 1998). Northern analysis of developmental leaf RNA was conducted several times with consistent results. The combination of many trials is displayed in a histogram of gene expression (Fig. 4.4C). The average percent is calculated as a function of the highest expression within each gene transcript listed. Some radiolabeled probes were stronger than others and certain mRNA levels
represented stronger than others in each developmental fraction. Figure 4.4C is presented to give an indication of overall change of expression throughout development for each individual transcript, rather than show exact expression intensity in comparison with the entire group. The histogram accurately reflects by providing the highest level for each transcript sample as 100%.

This research has concluded some rules and exceptions for SAG characterization compared to other SAG research. Detaching leaves from the plant and incubating them in total darkness is a popular technique first reported by Thimann (1980). This technique has been extensively researched (Becker 1993; Oh et al. 1996; Weaver et al. 1998) and concluded to be a controlled method to induce senescence as determined by chlorophyll concentrations, but includes mixed results. One result shows an increase in SAGs transcripts of detached leaves incubated in darkness but not observed in natural senescence. (Becker 1993). Another shows that 9 out of 10 SAGs are induced in older leaves, however 2 out of 10 SAGs were induced when younger leaves were detached and placed in darkness (Weaver et al. 1998). Most studies that use the dark detached leaf treatment conclude that senescence-associated genes are differentially regulated when leaf senescence is induced by different factors. Factors which induce chlorophyll loss are most likely to mimic gene expression of natural age-induced leaf senescence.

Expression analysis of anth17 mRNA under dark induced senescence and hormone treatment.

Cytokinins have long been associated as a senescence-retarding hormone that delays the loss of chlorophyll and proteins (Gan and Amasino 1997; Nooden et al. 1997).
Cytokinin has also been shown to reestablish leaves to a pre-senescent state after they have begun to exhibit senescent characteristics. It is considered that tissues high in cytokinins compete more effectively as nutrient and sugar sinks. This is apparent in developing tissues and those with apical dominance. Tissues rich in nutrients and sugars have a longer lifespan in addition to cytokinins concentrations that regulate molecular and signal transduction events to keep leaves actively developing and metabolizing, preventing senescence. Cytokinins are more likely to keep pre-senescent leaves in a pre-senescent state than to prevent SAG induction in older leaves. Cytokinins are able to frequently block visible senescence but do not necessarily block expression of all SAGs (Oh et al. 1996). The common idea is that cytokinins may only block a portion of senescence progression. This is reflected by a loss in photochemical efficiency detected in dark detached BA treated leaves (Oh et al. 1996). Although only a portion of the senescence program is blocked, most SAG transcripts are retarded by endogenously applied cytokinins. Most importantly for this work, exogenous treatments of the synthetic cytokinin benzyladenine show a repression of senescence characteristics when applied on anthurium leaves and flowers (Paull and Chantrachit 2001).

True senescence, induced by age, is a finely tuned process involving delicate regulation of gene expression. It is clear that many different pathways may be involved in inducing senescence. Commonly suggested methods include: drought or desiccation; dark incubated whole plants; detached leaves in light or darkness; abscisic acid treatment; ethylene treatment; cold treatment; wounding; and pathogen attack. A particularly interesting report describes the induction of senescence through the placement of “mittens” on individual leaves still attached to the plant. This type of senescence actually
increases if re-exposed to light. Interestingly, if a hole is punched in the mittens, yellowing occurs in all covered areas of the leaf but not the punched portion that is exposed to light (Weaver et al. 1998).

This result is consistent with that of Rousseaux et al. (1997) that showing far-red induced chlorophyll loss is extremely localized. This may suggest that the senescence response is highly localized, and possibly cell autonomous (Weaver et al. 2001). This type of example solidifies the suggestion that a phytochrome may be directly or indirectly involved with the light-mediated inhibition of senescence. Both continuous white light and pulses of red light are able to inhibit senescence in detached leaves and that the red-light effect is reversible by far-red light (Tucker 1981; Biswal et al. 1983; Biswal and Biswal 1984). More recently this result was reconfirmed in soybean (Guiamet et al. 1989), sunflower (Rousseaux et al. 1996), and tobacco (Rousseaux et al. 1997) when the plants senesced more rapidly when the red: far-red ratio was decreased. These results suggest that phytochrome involvement may be an important inducer of the senescence pathway which has many different significant branches. Weaver and Amasino (1998) present a model stating that senescence induced by age occurs in a “leaf autonomous” fashion, while senescence induced by darkness occurs in a “cell autonomous” fashion. If this is correct it may be possible for senescence to occur in a cell autonomous fashion in response to wounding, stress or even pathogen attack. This remains to be elucidated but it also suggests that phytochrome involvement may be a cellular or subcellular inducer of senescence in all plant tissues.

Considering sugars are the main product of photosynthesis and certain sugars can serve as signaling molecules, changing sugar levels during photosynthetic decline may
act as a senescing-inducing signal (Jang et al. 1997). Sucrose can effectively repress the accumulation of certain SAG transcripts when applied to detached leaves (Noh and Amasino 1999a). However, in non-senescent leaves, sugar accumulation can lead to a decline in chlorophyll and photosynthetic proteins (Stitt et al. 1990; Von Schaewen et al. 1990; Krapp et al. 1991; Krapp and Stitt 1994). Sugars can repress the transcription of photosynthetic genes (Sheen 1990) and tend to increase during leaf senescence (Crafts-Brandner et al. 1984). The accumulation of sugars can both accelerate and delay senescence. This is as a result of other factors in the leaf including Nitrogen and Carbon ratios (Paul and Driscoll 1997), light (Dijkwel et al. 1997), and plant growth regulators (Koch 1996).

In an effort to further characterize *anth17* as a true senescence-upregulated gene, a few previously described methods were attempted. Anthurium leaves 10 days after full expansion were cut at the base of the stem and incubated in 12 hours light and 12 hours of darkness. The leaves incubated in light seemed to senesce at variable rates lasting from 15 to 40 days. Leaves incubated in darkness senesced in a more controlled fashion. Leaves covered with a “mitten” type covering failed to induce senescence even after 30 days. A whole healthy anthurium leaf was cut into sections and incubated in darkness floating on appropriate solutions. This experiment was successful but the leaf discs showed no chlorophyll loss over 28 days. Leaves were also desiccated, wounded, and treated with cytokinin (Benzyladenine). It was determined that dark incubation of detached whole leaves provided the best method for inducing senescence similar to age-dependent senescence. Extreme care was exercised in choosing anthurium leaves of an identical developmental stage even though anthurium plants have many variable...
attributes. Leaf discs may have been more reliable because all of the treated tissue was
provided from a single leaf, assuring a controlled developmental phase as a starting point.
Whole leaves might have suffered from “stem plugging” (Paull and Goo 1982) resulting
in an accelerated senescence state and also differ in developmental attributes caused by
differences in age of the anthurium plant, time of harvest, and environmental pressures
during leaf maturity.

Leaf disc experiments show that \textit{anthi17} expression was moderately inhibited by
cytokinin and sucrose treatments for 14 days in comparison with the water control.
Visible markers of senescence such as a sharp decline in chlorophyll levels were not
detected during the experiment. The leaves were incubated in the dark for 20 days
without any appreciable difference in chlorophyll concentration. The expression of
\textit{anthi17} was inhibited by cytokinins and sucrose in these treatments for up to 18 days.
Leaves floated only on water and incubated in the dark showed a consistently high level
of \textit{anthi17} mRNA throughout the experiment. Baseline levels of \textit{anthi17} mRNA in all
samples may suggest a type of wound response. Northern analysis of \textit{cab} expression in
leaf disc samples showed no transcripts. This is consistent with reports that show \textit{cab}
mRNA transcripts and proteins nearly disappear after 2 days in dark incubated leaves (Oh
et al. 1996; Weaver et al. 1998; Hajouj et al. 2000; Weaver et al. 2001). Northern
analysis of \textit{psbA} mRNA was also inconclusive due to the stable nature of the transcript.
Whole leaf treatments show a similar pattern with developmental populations when
whole leaves are excised and incubated in darkness for 40 days. Leaves incubated in a
cytokinin solution, in combination with a cytokinin spray treatment, show delayed
expression of \textit{anthi17} and prolonged \textit{cab} and \textit{psbA} expression. These leaves also showed
a delay in chlorophyll loss compared with the control. Leaves incubated in a sucrose solution senesced extremely rapidly as indicated in the histogram of chlorophyll levels. Both treatments confirm that cytokinin inhibits the expression of *anth17*. Whole leaf treatments may be a more reliable indicator of inhibition due to chlorophyll loss, and comparable to developmental expression of leaves from plants. It is not surprising that the addition of a sucrose solution to incubated leaves in the dark increased the rate of senescence. Many studies show sugar accumulation in non-senescent leaves can lead to a decline in chlorophyll, photosynthetic proteins (Krapp et al. 1991), and photosynthetic gene transcripts (Sheen 1990). Both results are consistent with research that shows cytokinins are frequently able to block visible senescence and tend to retard the expression of some SAGs depending on developmental stage and treatment parameters (Becker 1993; Oh et al. 1996; Weaver et al. 1998).

The results presented here are consistent with previous studies on SAG type genes. *Anth17* is a SAG cysteine protease from the *Anthurium* species. This protease is most likely *senescence-upregulated*. *Anth17* occasionally shows very slight expression in healthy leaves, but this may be as a result of cell autonomous expression, localized wounding, or curling of the leaf perimeter. An anthurium leaf can still be productive and green even if wounded, torn, or under pathogenic attack, making it plausible that senescence conditions in anthurium leaves can both be cell and leaf autonomous. *Anth17* increases dramatically throughout senescence and the transcript is at its highest levels as the leaf nears necrosis. This result is consistent with that of *sag12* expression from *arabidopsis*. 

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CHAPTER V

CONCLUSION AND PROSPECTIVE

The research contained in this dissertation was funded by a special grant of the USDA termed Tropical & Subtropical Agriculture Research (T-STAR) awarded to Dr. David A. Christopher. Some of the program goals include providing research that maintains and enhances production of established tropical and subtropical agriculture products; enhancing the role of value-added agriculture in tropical island ecosystems; developing agricultural practices in the tropics and subtropics that are environmentally acceptable through an agro-ecosystems approach; expanding tropical and subtropical agricultures linkages to related industries and economic sectors. This research provides a foundation for enhancement in post-harvest quality of Anthurium flowers and a more productive crop. We also harvest molecular resources from anthurium in an effort to design an auto-regulatory senescence inhibition system from anthurium components. The research also contributes to some fundamental questions of molecular biology.

Establishing Molecular Techniques to Deal with Recalcitrant Tropical Species

Plant Molecular Biology and Physiology are advancing fields of science that require a thorough understanding of molecular methods unique to each individual species being researched. Molecular methods involved with researching tropical species are
more difficult because of the recalcitrant nature of the plant materials and the uniqueness of the evolutionary divergent molecular physiology. This research defines techniques that are new developments in the field concerning the anthurium species and can serve as a reference for future studies involving anthurium molecular biology. These techniques include RNA and DNA extraction; successful reporter gene introduction and analysis; transient expression; cDNA library construction; and foreign promoter evaluation, SAG characterization, and hormone and stress treatments. These molecular tools can also be used to successfully evaluate other economically valuable tropical ornamentals for increased yield and post-harvest quality. The research has characterized the SAG gene \textit{anth17} and establishes the effective regulation of Pr-SAF12 in anthurium tissues confirming that the molecular events of senescence do occur supporting the claim that orthologous mechanisms exist between monocots and dicots.

Our understanding of \textit{anth17}, its role during development, and regulation by cytokinins, will lead to the isolation of a SAG promoter and the eventual development of \textit{pANT17-ipt}, an auto-regulatory senescence inhibition system that is regulated by an anthurium SAG promoter. This system is considered a more ideal approach to molecular engineering since the regulatory element is native to the plant. The research enables the isolation of Pr-\textit{ANT17} and its comparison to other SAG promoters to determine its relative homology and conserved regions. This type of analysis helps to further our understanding of the regulatory elements, transcription factors and enhancer regions involved with SAG expression.
Contribution to the Fields of Post-Harvest Physiology and Molecular Biology of Leaf Senescence

Post-harvest physiology is an important science concerning many agricultural crops. These concerns range from preserving the quality and life of a commodity from grower to consumer, increasing the commodities resistance to the stresses of shipping and storage, and manipulation to increase or produce beneficial compounds upon harvest. Senescence is an extensively investigated field that includes the study of plant growth regulators, gene expression, proteins, and metabolites contributing to a better understanding of plant molecular physiology. Many of these processes contribute to the overall knowledge of all plant cell and molecular systems. This research provides additional resources to the overall goal of understanding leaf senescence by providing information on two novel Anthurium cysteine proteases, their role in developmental, hormonal, and stress induced senescence, and the comparison and addition of them to the GENBANK database. This research also defines the senescence-upregulated performance of a dicot promoter in a monocot system suggesting similarities between dicot and monocot senescence transcription machinery. Publishing the information obtained through this research contributes to the molecular analysis of senescence and post-harvest physiology of tropical ornamentals. Finally, research on senescence-regulated gene expression in a tropical species may be valuable for studies on other tropical species involved with food, medicine, and ecosystem management fulfilling many of the T-STAR program goals and improving the future of tropical plant research.
Hydrolases which cleave peptide bonds are generally known as protease, peptidases, proteinases or proteolytic enzymes. Enzymatic studies on such enzymes usually focus on those directly involved in the digestion of dietary protein, such as trypsin and chymotrypsin, which are completely characterised. Proteases are amongst the most studied proteins. It is through detailed characterization of the structure and function of several proteases that they are also used as models in explaining the basics of enzyme function. Cysteine proteases are actively researched for expression profiling during senescence because some of the most specific age-dependent SAG genes are cysteine proteases. In this sense, gene expression and promoter regulation of cysteine proteases is very important for models explaining senescence.

Other proteases with varying functions, especially those pertaining to the regulation of physiological processes, have also been studied. Proteolytic activity involved in the processing and regulation of enzymes and hormones, cascade reactions in the blood coagulating process, fertilization, embryo development and industrial processes for food and feed production.

Proteases are found in all forms of organisms regardless of kingdom. Some examples include plant proteases include papain of papaya, commonly used as a meat tenderizer, and bromalein a mixture of several proteases of pineapple used in meat tenderizers, chill-proofing beer, manufacturing precooked cereals, certain cosmetics, and in preparations to treat edema and inflammation. Bromelain is also nematicidal. Animal proteases include rennin, used in dairy industry to produce a stable curd with good flavor,
chymotrypsin, used extensively in deallergenizing of milk protein hydrolysates, and trypsin, used for the biocontrol of insects and in the preparation of bacteriological media. Proteases of bacteria, fungi and viruses are of interest due to the importance and subsequent application of those enzymes in industry and biotechnology. Examples of these include application of bacterial neutral and alkaline proteases from the Bacillus sp. in fermentation and detergent industry; acid protease of Aspergillus sp. in food industry namely, the production of cheese. Commercial application of microbial proteases is attractive due to the relative ease of large scale production as compared to proteases from plants and animals. However, some proteases from plants have specific functions, substrates, and inhibitors, making them more attractive for use in unique applications.

Microbial proteases have been studied for their role in the development and manifestation of diseases such as AIDS, cancer and acute and chronic systemic infections. Characterization of these proteases, as with other virulence factors, provides vital information to design new approaches in treatment, therapy and control of infectious diseases, which include designing and production of vaccines, synthetic protease inhibitors and pharmaceutical research. One such success story is of the HIV protease. Structural studies of HIV protease have enabled the successful development of synthetic HIV protease inhibitors for the treatment of AIDS patients. Similar studies are being undertaken to develop synthetic inhibitors/2nd generation antibiotics for bacterial proteases.

For each application there is a different protease that performs the work, or is stable in the environment. Unique proteases may increase the efficiency or lower the cost of production of a particular product. By performing knockout experiments with recently
isolated proteases, we can better understand its role in development and subcellular metabolism. Characterization of two cysteine protease genes, *anth16* and *anth17* from *Anthurium andraeanum* contributes to this knowledge.


