2004-01-01

The Effect of an Altered Cytokinin Metabolism on Plant Development and C ell Aging in Crop Plants

Ian McEvoy
Technological University Dublin

2004-01-01

The effect of an altered cytokinin metabolism on plant development and cell aging in crop plants

Ian McEvoy (Thesis)
Dublin Institute of Technology

The effect of an altered cytokinin metabolism on plant development and cell aging in crop plants

Ian McEvoy

MPhil

September 2004

Supervisors:

Dr. Renee Malone & Dr. Gary Henehan
School of Food Science and Environmental Health
Dublin Institute of Technology

Professor Philip J. Dix
Plant Cell Biology
Institute of Bioengineering and Agroecology
National University of Ireland, Maynooth

Declaration

I certify that this thesis which I now submit for examination for the award of MPhil, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute or University. The Institute has permission to keep, to lend or to copy this thesis in whole or in part, on condition that any such use of the material of the thesis be duly acknowledged.

Signature Ian McEvoy

Date 20/09/04

Acknowledgements

Firstly, I would like to thank Professor Phil Dix and Dr Renee Malone for the speedy corrections and their assistance in the completion of this project.

I would also like to thank my parents and my sister for helping me through my education and for putting up with me! Thanks also to Ed, Annemarie and Miranda for the constant support and to my colleagues at PCB for their help and friendship.

And finally, but most importantly, I am extremely grateful to Dr. Karen Sheil who gave up so much of her free time in teaching me how to clone. I am sure I would never have gotten this far without it.

Abstract

Isopentenyl transferase (IPT) is an enzyme encoded by a bacterial gene that catalyzes the rate-limiting step in cytokinin biosynthesis. Under the control of senescence-associated promoter ($pSAG_{12}$), isolated from *Arabidopsis thaliana*, it has been shown to delay senescence in several herbaceous species. Cytokinin synthesis begins at the onset of senescence and is then stopped when senescence ceases. This auto regulatory control of the transgene ensures normal development and morphology of the transformed plant, which may be affected in plants constitutively expressing *ipt*.

In this study, it was investigated if an altered cytokinin metabolism, as a result of transformation by $pSAG_{12}$: *ipt,* will display a delayed ageing phenotype in a deciduous tree species as has been demonstrated in herbaceous species. This was carried out using various physiological assays and several transgenic lines were shown to display the symptoms of a plant exhibiting delayed senescence. A parallel study was carried out, investigating recombinant protein leakage during senescence (in detached leaves from tobacco plants containing the $pSAG_{12}$: *ipt* cassette in the nucleus and *gfp* in the chloroplast). $pSAG_{12}$: *ipt* was found to be efficient in maintaining the integrity of the choloplast and thus preventing leakage and degradation of the recombinant chloroplast protein.

Contents

1.1 Senescence

1.1.1 Recycling of nutrients

Senescence is the final stage in the life of a plant or a plant part and involves the breakdown of macromolecules and the transportation of the products to other parts of the plant (Gan and Amasino, 1997). In herbaceous plants, many of the nutrients stored in the leaf are transferred to developing parts of the plant during senescence. In deciduous trees however, the nutrients are stored in specialized cells in the trunk until the next growing season (Buchanan-Wollaston *et al.,* 2003). Senescence is a highly adaptive feature of plants and since plants are immobile, it proves extremely useful when parts of a plant are placed in an unfavourable environment. It can be seen as a way of reshaping a plant by removing unwanted or damaged parts while recycling nutrients.

The senescence process involves the change from net carbon and nitrogen assimilation to a period of catabolism involving macromolecule breakdown and transportation to developing sinks (Morris *et al.*, 2000). The process is under the direction of certain genes, known as Senescence Associated Genes (SAG's), as has been demonstrated in experiments where senescence was inhibited by RNA and protein inhibitors (He and Gan, 2002). At the onset of senescence, cell degradation is initially only partial and compartmentation is maintained, even in photosynthetic cells (Kunkel *et al.,* 1999). Later on, membrane lipids are hydrolysed by lipid degrading enzymes during leaf senescence. There is also an increase in membrane permeability, which reduces photosynthetic capacity (He and Gan, 2002). As senescence progresses, the chlorophyll, lipid, protein

and RNA content of the cell then decrease as they are being broken down and mobilized to storage areas or developing parts of the plant (Quirino *et al.*1999).

1.1.2 Senescence Associated Genes

The vast majority of genes are down-regulated during senescence but Senescence Associated Genes (SAG's) are genes that are up-regulated during senescence. These genes are responsible for the disassembly of the cellular components (Yoshida, 2003). To date there have been 30 SAG's identified in different plant species. SAG's can be divided into two classes, one being senescence specific with mRNA detectable only during senescence and the other class having a basal level of expression before, which increases during senescence (Gan and Amasino, 1997). Since the SAG's are responsible for the dismantling of the cellular components, it is not surprising that they include RNAases, lipases and proteases (Quirino *et al.*, 2000).

Total RNA levels drop rapidly during senescence but nuclear DNA is maintained to allow gene expression of SAGs to continue (Gan and Amasino, 1997). Mitochondria are important in providing energy, in the form of ATP, for the disassembly of the cell and this is why they are one of the final organelles to broken down during senescence (Kunkel *et al.*, 1999). The necessity for functional ribosomes during senescence has been demonstrated in experiments using cycloheximide, a protein synthesis inhibitor, which results in leaf senescence being retarded (Quirino *et al.*1999).

1.1.3 Senescence, aging and programmed cell death

Senescence is not the same as aging. Aging can be defined as 'the age-related deterioration of physiological functions necessary for the survival and fertility of an organism' (Pandi-Perumal *et al.,* 2002). Aging refers to events occurring with time, without death as a result (Smart *et al.*, 1991). Senescence is only part of this process and is reversible up to a point (Buchanan- Wollaston *et al.,* 2003).

It is generally accepted that senescence is a form of programmed cell death (PCD: the selective elimination of unwanted cells (Pennell and Lamb, 1997)). What distinguishes senescence from other types of programmed cell death is the transportation of nutrients out of the senescing cell (Quirino *et al.*1999). This is illustrated in the maintenance of tissue around the vascular system, required for transport of nutrients, until late in senescence (Gan and Amasino, 1997).

1.1.4 Chlorophyll catabolism

Chlorophyll degradation is generally accepted to be one of the first visible symptoms of senescence (van Staden *et al.,* 1988). It is an energetically expensive process and serves to detoxify the cell of the end products of chlorophyll catabolism rather than recycling nutrients (Buchanan- Wollaston *et al.,* 2003). When autumn leaves appear yellow or orange, this is due to the unmasking of already present carotenoid pigments (Field *et al.,* 2001). Autumn senescence involves the liberation of the entire pool of chlorophyll and if this is not catabolized or protected from light the uncontrolled generation of singlet oxygen could jeopardize the efficiency of nutrient recovery through photo-oxidative

damage (Field *et al.*, 2001). The key enzyme involved in the catabolism is pheophorbide *a* oxygenase. This cleaves the tetrapyrrole ring to produce red chlorophyll catabolite. The end products of the catabolism are known as non-fluorescent chlorophyll catabolites and are stored in vacuole and not recycled (Buchanan- Wollaston *et al.*, 2003). Chlorophyll degradation during senescence shares the detoxification pathway for xenobiotic compounds (Field *et al.*, 2001). Chelators of iron are effective in retaining the green colour of leaves, since they target an iron dependant step in chlorophyll degradation (Thomas and Howarth, 1999).

1.1.5 Cues for senescence

Senescence is an active process and is regulated by exogenous and endogenous factors (Weaver and Amasino, 2001). There are both environmental and autonomous cues for plant senescence. Environmental cues include drought, pathogen infection, extremes of temperatures, shading and nutrient deficiency. Autonomous cues include age, reproductive development and hormone levels (van Staden *et al.*, 1988).

1.1.6 The role of light and darkness in senescence

Darkness is generally considered to be a promoter of senescence but it was shown to delay senescence when whole *Arabidopsis thaliana* plants were placed in darkness before being returned to a normal photoperiod. This effect was not observed with detached leaves, suggesting that the delaying effect is dependent on communication between plant parts (Weaver and Amasino, 2001).

Light is generally an inhibitor of senescence. Phytochrome may be involved in this inhibition. Continuous white light and pulses of red light have been shown to inhibit senescence in detached tobacco leaves (Weaver and Amasino, 2001). Also, tobacco plants over-expressing phytochrome A exhibit delayed senescence. High light levels have also been shown to induce senescence. (Weaver and Amasino, 2001). Plants possess a number of mechanisms in coping with excess light levels. It has been suggested that anthocyanins act as an optical protection screen in some plants, reducing the light capture of senescing chloroplasts. It is possible that species not producing anthocyanins depend on alternative methods (Field *et al.*, 2001).

1.1.7 Senescence and stress

Although senescence will occur with age in the absence of stress (He and Gan, 2002), plants are subject to many biotic and abiotic stresses that may result in the premature onset of senescence. It was suggested that the decrease in antioxidants and the increase in lipid peroxidation as a result of stress are possible causes for stress-induced senescence (Prochazkova *et al.*, 2001). There are likely to be common gene expression pathways in normal senescence and response to stress since numerous genes, which are expressed in response to stress, are also expressed during senescence (Puddephat *et al.*, 1999). It is possible that these defence genes may be reacting to common conditions present in a stress and senescence situation (Quirino *et al.*,1999).

1.1.8 Oxidative stress

Earth was once an anaerobic planet until the evolution of the water-splitting reaction of photosynthesis resulted in accumulation of oxygen in the atmosphere. This dramatic change in the environment placed a huge selection pressure on all organisms to develop mechanisms to defend themselves from the toxic consequences of atmospheric oxygen. This toxicity is a result of so-called reactive oxygen species, (ROS) that are formed primarily through the reduction of O_2 to $2H_2O$ (Froidovich, 1998).

During senescence, accompanying changes in cell structure, metabolism and gene expression, is a sharp increase in the generation of ROS. This increase is thought to be the result of macromolecule degradation (Buchanan-Wollaston *et al.*, 2003). ROS include H_2O_2, superoxide O^{2-} radicals, hydroxyl radicals, and singlet oxygen are produced in the chloroplasts, mitochondria and peroxisomes. ROS are known to have a detrimental effect on cellular structure and function (He and Gan, 2002).

1.1.9 Ascorbate Peroxidase

Peroxidases are a class of enzymes that catalyse the oxidation of a substrate with the reduction of H_2O_2 to $2H_2O$ (Froidovich, 1998). Ascorbate peroxidase (APX) is a H_2O_2 scavenger that works directly at the site of H_2O_2 generation. For photosynthesis to continue uninterrupted, direct scavenging of H_2O_2 at the site of production, in the chloroplast, is essential. Most APX in leaf cells is localized in the chloroplast and it mainly uses ascorbate as the electron donor, hence the high concentration of ascorbate in

plants compared to other organisms, but cytosolic isoforms can also use aromatic phenols (Asada, 1997).

There are several isoforms of APX, two soluble forms in the cytosol and two present in the chloroplast (one stromal and the other thylakoid bound). APX activity increases during periods of stress to the plant coinciding with the increase of ROS (Shi *et al.*, 2001). Catalase is another scavenger of H_2O_2. An advantage is that it can work without an electron donor but is only efficient in high concentrations, peroxidases being effective at very low concentrations (Asada, 1997). Another scavenger in aerobic cells, this time of the superoxide O^{2-} radical, are the superoxide dismutases (SOD's). SOD's keep the level of O^{2-} in the 10^{-10} mol l^{-1} range (Froidovich, 1998).

1.1.10 The chloroplast

The chloroplast is the organelle where photosynthesis occurs in higher plants. Light energy is captured and converted into biologically useful energy (Bogorad, 2000). It is believed that the evolution of the green plant began by the engulfment of the cyanobacterial progenitor of the chloroplast by a eukaryotic host. The ancestral genome is believed to have had around 3,200 genes. 1,700 genes have been lost to redundancy and 1,400 of these lost genes have migrated to the nuclear genome, leaving only about 87 plastid-encoded genes (Bogorad, 200). The majority of polypeptides present in the chloroplast are encoded by the nucleus (Hibberd *et al.*, 1998). Chloroplasts carry 500-10,000 copies of their circular genome (Svab and Malaga, 1992). Non-photosynthetic

plastids possess an identical genome to that of the chloroplast but the genes are generally expressed at much lower rates (Hibberd *et al.*, 1998).

1.1.11 The chloroplast during senescence

Chloroplasts are believed to be the first organelle to be affected during senescence, while the nucleus and mitochondria remain intact until later on in the process, in order to provide energy and control gene expression of the SAG's (Quirino *et al.*1999; Noh and Amasino, 1999). Despite this, a study of changes in the chloroplast of *Brassica napus* during senescence revealed that the outer membrane of the chloroplast was one of the last components to be dismantled. This may be due to studying chloroplasts in differing species and tissue (Ghosh *et al,*. 2001).

A minimal level of thylakoid damage is usually observed before chlorophyll loss during senescence (Ghosh *et al.*, 2001). As senescence progresses, the degradation of chloroplast components leads to a reduction in photosynthetic activity (Ghosh *et al,*. 2001). In plastids, as much as 90% of nitrogen recycled from senescing leaves come from the degradation of stroma proteins and thylakoid membranes. Chlorophyll molecules must be unbound from their associated proteins and degraded before this event (Field *et al.*, 2001).

1.2 Cytokinin

1.2.1 Plant hormones and senescence

Of the phytohormones: auxin, cytokinins and gibberellins typically inhibit senescence while abscisic acid and ethane generally promote it (Yoshida, 2003). Exogenous application and inhibitors of ethane production have both been shown to promote senescence symptoms in many species (Grbic and Bleecker, 1995). The inhibition of ethane production, through antisense transformation, has been shown to delay ripening in climacteric fruits (Laties, 1993). Antisense transformation is when the transformed gene produces molecules of RNA complementary to the sequence of endogenous ones, reducing protein production (Botella, 2000).

1.2.2 Cytokinin and senescence

The phytohormone most associated with senescence is cytokinin. However, the effect of cytokinin on senescence is still not completely understood, possibly due to the action of cytokinin being masked by its interaction with other plant hormones (Mok and Mok, 2001). Skoog and Miller first discovered and coined the term cytokinin after observing a substance promoting cell division, or cytokinesis, and shoot differentiation in a tobacco cell culture (Skoog and Miller, 1955). Since then, cytokinins have been shown to play an important role in many other plant functions.

Cytokinins are present in plant tissue at very low concentrations as free bases, their ribosides or conjugates with sugars or amino acids (Vlasakova *et al,*. 1997). Zeatin, iso-pentenyl adenine and dihydrozeatin are the three main families of naturally occurring

cytokinins. All three are N^6 –substituted adenine derivatives differing only in the side chain, which is responsible for biological activity (Smart *et al.*, 1991). The natural cytokinins are all adenine derivatives with isoprenoid or aromatic N^6- side chains. The biosynthetic pathways of cytokinins with aromatic side chains still remain unknown but it is likely that the biosynthesis of other cytokinins is similar to that of isopentenyl adenine, as elucidated in *Agrobacterium,* although the possibility of cytokinins with different side chains having separate origins must not be ignored. It is thought that the addition of iso-pentenyl side chain to 5–AMP is the initial step in biosynthesis and this reaction is catalysed by an iso-pentenyl transferase (encoded by a gene called *ipt* in *Agrobacterium tumafaciens*) (Smart *et al.*, 1991).

Chromatographic analyses of cytokininin activity bioassays were initially used in the isolation of cytokinins. This led to the identification of those members of the group present in larger quantities (Mok and Mok, 2001). Since then, many techniques have become available for measuring cytokinin, including high performance liquid chromatography (HPLC), RIA, ELISA, GC-MS and LC-MS, each having its specific merits and shortcomings (Vlasakova *et al.*, 1997).

1.2.3 Cytokinin degradation

The key enzyme involved in cytokinin degradation is cytokinin oxidase. The process involves the oxidation of cytokinin with unsaturated isoprenoid side chains, using molecular oxygen as the oxidant (Motyka *et al.,* 1996). Cytokinin oxidases selectively degrade unsaturated N^6-isoprenoid side chains, the most prevalent form, converting

active cytokinins to adenine. The properties of cytokinin oxidases are so diverse, varying in mass from 25 kD to 94 kD and pH optima from 6 to 9, that it is possible that there may be more than one class of this cytokinin degradation enzyme (Mok and Mok, 2001).

1.2.4 Cytokinin receptors

For over 30 years scientists have been searching for cytokinin receptors. In order to qualify as a receptor, the hormone must first bind to the receptor protein resulting in a change in conformation, triggering the beginning of the signal transduction cascade and eventually leading to changes in transcription of certain genes. There have been several canditate receptor/signal transduction genes identified, such as CKI1, ARR and GCR1, but the function for most of these genes is unknown and the lag time before changes in transcription is too long to be considered as early response genes (Mok and Mok, 2001).

1.2.5 Studying the effect of cytokinin on senescence

There have been three main approaches to studying the effect of cytokinin on senescence. Firstly, there is the exogenous application of synthetic cytokinin to plant tissue. A delay in senescence has been observed using this method in the laboratory. However for practical application, on an agricultural scale, this procedure would require extra handling, and adverse effects have been observed (Chen *et al.*, 2001). Secondly, the levels of the hormone can be monitored during senescence, and finally levels of cytokinin can be modified using recombinant DNA approaches (Smart *et al.*, 1991). It is important to consider the efficiency of uptake and transport when studying the effect of increased levels, exogenous or endogenous, of cytokinin on the plant (Smart *et al.*, 1991).

1.3 Genetic transformation

1.3.1 Introduction to genetic modification

With the invention of agriculture, some 10,000-12, 000 years ago, mankind was able to produce food surpluses for the first time (Borlaug, 1997). Early farmers achieved better crops through saving seeds of more desirable plants, and the discovery of plant breeding led the way to a higher quality crop yield (Gasser and Fraley, 1992). In today's world we rely heavily on the end products of agriculture for our survival and since the world's population is expected to reach 8.3 billion by 2025, there will be a huge increase in the demand for food (Borlaug, 1997).

Recombinant DNA techniques now allow us to transfer segments of genetic information from one organism to another. Since the dawn of this technology we have witnessed the generation of plants which are resistant to stress, pests and herbicides (Gasser and Fraley, 1992). This process is faster and more efficient than conventional breeding since you can introduce a new trait to an already elite crop cultivar. Today, most harvested crops must be transported immediately to intermediate destinations before being transported again to their final destination. It is therefore logical to expect a lot of scientific research into genetically modified plants with improved storage and "shelf-life" characteristics (Botella, 2000).

1.3.2 *Agrobacterium*-mediated transformation

There are various methods of plant transformation, including biolistic and *Agrobacterium*-mediated transformation. The latter involves manipulating an existing

natural form of gene transfer. *Agrobacterium tumefaciens* is a gram-negative bacterium, which causes crown gall disease by transferring native genes, which induce irregular phytohormone production, resulting in tumour formation (Hedden and Phillips, 2000). Pathogenic strains of *A tumefaciens* contain a large plasmid termed the Ti (tumour-inducing) plasmid, which includes a region known as the T-DNA. The T-DNA is a transferable segment of DNA, encoding enzymes for producing plant hormones, which becomes randomly integrated into the plant genome during infection. This results in the formation of a mass of undifferentiated cells on a plants stem at the crown (the soil line) (Bogorad, 2000). T-DNA can be modified by removing the *onc* genes (the genes that result in tumour production) which are located between two terminal repeat sequences and replacing them with the desired gene(s) (Hedden and Phillips, 2000). This T-DNA is then placed into a small plasmid and introduced into a pathogenic strain of *Agrobacterium* lacking T-DNA. The resulting plasmid is then put into contact with plant cells and infection occurs resulting in transformation (Bogorad, 2000).

Today, most scientists use the binary vector system in *Agrobacterium*-mediated transformations. This entails the *vir* genes, those responsible for the virulence of the Ti plasmid, being situated on a different plasmid to the recombinant DNA. These *vir* genes direct the transfer of the genetic code situated between the right and left border sequence, known as the T-DNA, into the genome of the organism being transformed (Armitage *et al.*, 1988)

1.3.3 Biolistic transformation

Foreign genes can also be introduced into the plant cell via microprojectile bombardment. This procedure, known as Particle bombardment or Biolistics involves DNA being bound to gold or tungsten particles and shot into the cells under a vacuum (Finer *et al.*, 1999). The efficiency of this procedure depends on ensuring penetration of the tissue while minimising tissue damage during the bombardment (Puddephat *et al.*, 1999), these considerations dictating the particle size, and various bombardment parameters. The main advantage of this approach over *Agrobacterium*-mediated transformation is the absence of biological incompatibility (*Agrobacterium* transformation has been less effective on monocotyledon plants (Bogorad, 2000)). Biolistic transformation also appears to be genotype and tissue independent (Finer *et al.*, 1999).

Microparticle size, target distance and acceleration can be optimised according to the plant species, and plant tissue being transformed. Location of plant tissue on plates, plasmid DNA concentration and gap distance also play an important role in successful biolistic transformation. Young plant tissue is preferable, with moderate concentrations of DNA. Too high a plasmid DNA concentration however, can cause agglutination of microparticles, which can cause greater tissue damage during bombardment (Puddephat *et al.*, 1999).

Recently, a transformation procedure, referred to as "Agrolistics", involving both *Agrobacterium* cells and biolistics has been developed. The *Agrobacterium* cells were used as microprojectile coating, as opposed to just the plasmid containing the gene of

interest, and stable transformation was achieved in strawberry plants. Since plant wound response is such an important factor in *Agrobacterium*- mediated plant transformation, the shooting may be an efficient means to wound the plant directly before gene transferral (de Mesa *et al.*, 2000).

1.3.4 Reporter genes

Reporter genes have been essential tools in confirming the genetic transformation of plants and also for rapid analysis of features of the vector (eg. promoter efficacy). They are usually fused to the gene of interest and have screenable qualities, which more or less confirm the presence of the transgene of interest. The most commonly used reporter gene is the β-glucuronidase (GUS, or more accurately, *uid*A) gene. Expression of the gene, leading to β-glucuronidase activity, allows breakdown of a colourless substrate to a product that is visible to the eye, but destructive to the cell (Blumenthal *et al.*, 1998). An alternative and equally common reporter gene is *gfp* (green flourescence protein). This jellyfish gene, isolated from *Aequorea vitoria*, expresses a green fluorescent protein which can be detected using a long-wave UV light source of fluorescence microscopy (Casper and Holt, 1995). This has provided the attractive opportunity of using a non-destructive reporter gene in transgenic organisms.

1.3.5 Transgenic plants with altered cytokinin metabolism

Transformed plants with altered cytokinin metabolism have been shown to display delayed senescence in several herbaceous plants. These plants were transformed with a bacterial gene, *ipt,* encoding isopentenyl transferase, an enzyme that catalyzes the rate-limiting step in cytokinin biosynthesis, and exhibit this delayed senescence phenotype

(Gan and Amasino, 1995). Since cytokinin is such a multifunctional hormone, plants displayed an altered and sometimes undesirable morphology when *ipt* is constitutively expressed (Smart *et al.,* 1991). For example, when *ipt* is introduced under the control of the constitutive cauliflower mosaic virus promoter, CaMV 35S, stunted growth was observed. These plants exhibited poor internode elongation, altered leaf morphology and delayed leaf senescence. Root growth was often delayed or absent and plants were often sterile (Kunkel *et al.,* 1999).

In *Arabidopsis thaliana,* SAG12 is a cysteine protease and is involved in the breakdown and mobilizing of nutrients during senescence (Grbic and Bleecker, 1995). Its promoter, $pSAG_{12}$, is strongly regulated by senescence. When *ipt* is fused to $pSAG_{12}$, and introduced into several plant species including tobacco (Gan and Amasino 1995) and lettuce (McCabe *et al.,* 2001), the expression of *ipt* is under autoregulatory control. The promoter of this gene initiates expression of *ipt* at the onset of senescence. Cytokinin synthesis begins, halting or reversing the senescence process, and the transgene is switched off when senescence ceases. Normal development of transgenic plants with the $pSAG_{12}$: *ipt* cassette showed that the autoregulatory system only operated in senescing parts without modifying the development of other parts of the plant (Gan and Amasino, 1995). Transgenic plants were also shown to have prolonged photosynthetic rates. Premature senescence can have a detrimental effect on yield and these stay-green plants display a longer shelf life and may also exhibit enhanced stress resistance (Gan and Amasino, 1997).

The autoregulatory nature of $pSAG_{12}$: *ipt* was observed by GUS fusion to the SAG12 promoter resulting in visible detection of the transgene (Ludewig and Sonnewald, 2000).

It has been shown that SAG12 is under the control of developmental pathways and its expression is age related and not triggered by stress factors that can also induce senescence. It is important to bear this in mind when designing senescence experiments on plants containing the $pSAG_{12}$: *ipt* cassette. However, SAG12 can be induced by detachment, ABA and ethane (Noh and Amasino, 1999).

Plants have also been generated with reduced cytokinin levels. This was achieved through transforming *Arabidobsis thaliana* with the gene encoding a catabolic enzyme CKX, a cytokinin oxidase. This experiment demonstrated the role of cytokinin in the transition from undifferentiated stem cells to differentiation. The reduced number of leaf cells also highlighted the necessity for cytokinin in leaf formation (Werner *et al.*, 2001). Cytokinin oxidase contributes to keeping cytokinin levels below a lethal level in plants constitutively expressing *ipt* (Motyka *et al.*, 1996)

1.3.6 Possible drawbacks of pSAG12:*ipt* plants

Studies by Jordi et al. in 2000 on tobacco plants containing the $pSAG_{12}$:*ipt* cassette demonstrated that the delayed ageing of the older leaves was found to have a detrimental effect on the younger leaves. In wild type plants, the younger leaves benefit from the senescence of older leaves without older leaves competing for nutrients. In $pSAG_{12}$:*ipt* plants, when senescence is delayed in older leaves, nutrients are not being transported to

younger leaves as would happen if senescence progressed as normal. This may lead to a premature senescence of younger leaves as the cost for the delay in the senescence of older leaves. It is also hypothesized that the elevated cytokinin levels in younger leaves of $pSAG_{12}$: *ipt* plants can be accounted as a response to a nitrogen limitation caused by the nutrient trapping of the older leaves. This is based on the fact that there is little difference in cytokinin levels when both transgenic and wild type plants are provided with an abundance of nutrients. Carbon trapping is also likely to occur and this may result in a reduction in carbon transported to the roots. This may be the reason why $pSAG_{12}$: *ipt* plants were shown to have lower dry root weight than the wild type, resulting in lower nitrogen uptake. The expression of *ipt* in these plants also resulted in the effective retention of chlorophyll and light absorption but not in soluble protein or Rubisco content (Jordi *et al.*, 2000).

1.3.7 Tree Transformation

Genetically transformed trees have always been considered an unattractive study tool due to long breeding cycles, difficulty in studying in a laboratory setting, large genome sizes, difficulty in plant regeneration from *in vitro* cultures, and since trees generally have a long juvenile period, the necessity for long term stable expression of the transgene over many vegetation periods can also be problematic (Herschbach and Kopriva, 2002). *Populus* has been the pioneer species in transformation studies and has more data published on genetic engineering than all other forest tree species together, due to its rapid growth and relative ease of tissue culture (Pena and Seguin, 2001). Recently there has been an increase in protocols for *in vitro* propagation and genetic transformation of

trees with transformants being generated in species such as apple, citrus, radiata pine, spruce and walnut (Herschbach and Kopriva, 2002).

1.3.8 Apple

Apple (*Malus* x domestica) is an extremely important species for the fruit industry. It is currently experiencing intense competition worldwide and producers are sometimes forced to dump last years fruit at the onset of a new harvest season (Harker *et al.*, 2003). Since consumers associate taste with different cultivars, quality is extremely important in the sale of fruit (Harker *et al.*, 2003). Apples exhibiting a lengthened shelf life may eventually become a consumer favourite and these cultivars may be transported over long distances if they age more slowly. Almost all of the commercial apple cultivars today are susceptible to fungal and bacterial diseases, apple scab and powdery mildew being two examples, so a successful transformation procedure is as much needed in this species as other economically important crops (Puite and Schaart, 1996).

Up to this point, leaf senescence in perennial trees had not been extensively studied using modern molecular genetic tools (Kunkel *et al.*, 1999). Autumnal senescence however, is an attractive system to study since senescence can be induced by shortening the photoperiod (Kunkel *et al.*, 1999). It also provides the opportunity to investigate underlying similarities and differences between the control of, and metabolic events in, simultaneous senescence and the much more extensively investigated sequential senescence. In the current investigation, apple plants cv. *Greensleeves*, transformed using the plasmid pVDH394 (Fig. 1.1) containing the $pSAG_{12}$:*ipt* cassette (Williams 2000)

were characterised in order to investigate the effect of an altered cytokinin metabolism in a deciduous species.

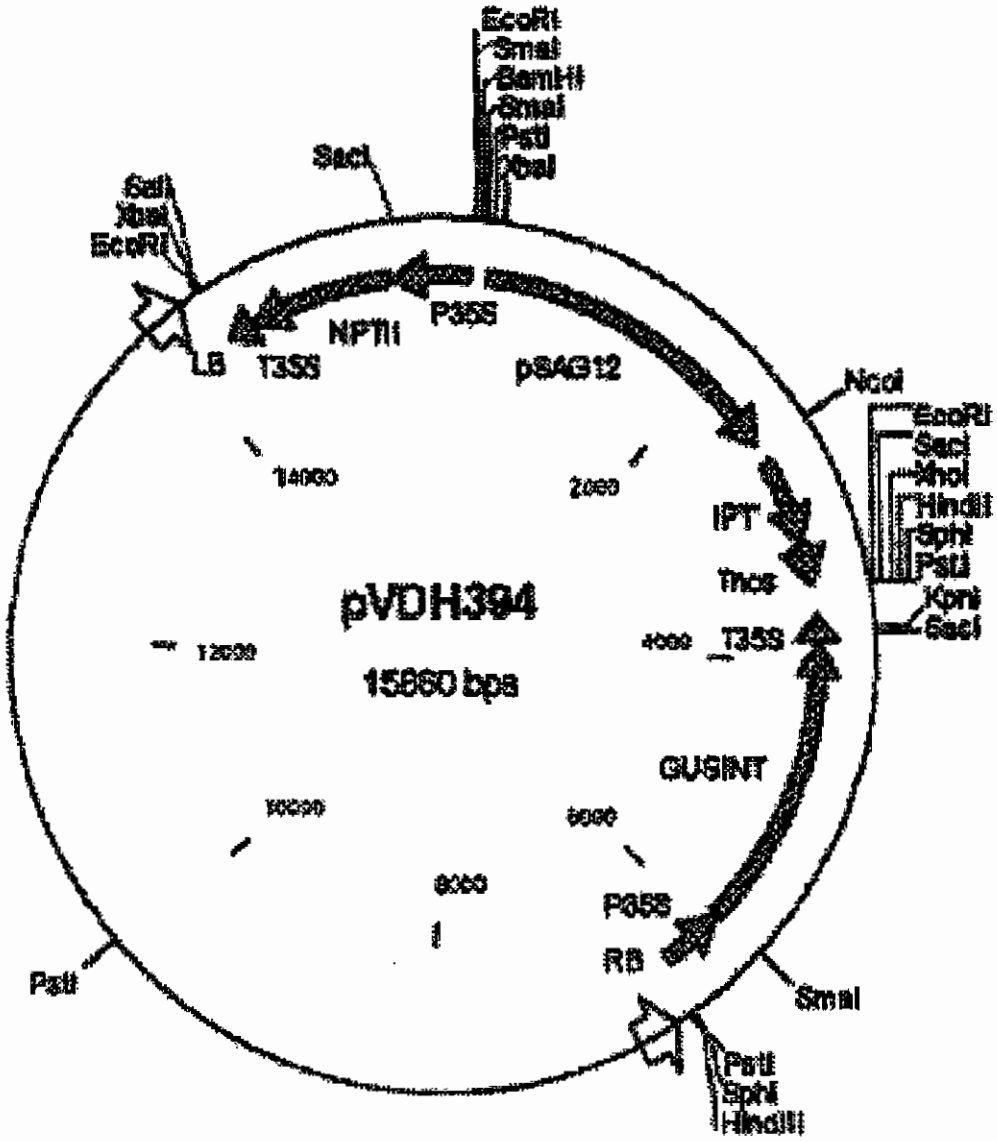

Fig. 1.1. Plasmid pVDH394, used in the transformation of $pSAG_{12}$:*ipt of Malus domestica cv. Greensleeves*. The plasmid contains a kanamycin selectable marker (NPTII) and the GUS reporter gene (GUSINT driven by the 35S (CaMV) promoter)). *ipt* is driven by the senescence specific SAG_{12} promoter.

Materials and Methods

2.1 Tissue Culture

2.1.1 Plant material and growth conditions

2.1.1.1 Apple

All $pSAG_{12}$:*ipt* apple lines (*Malus* x *domestica* cv. Greensleeves) were transformed by Linda Williams in 2000 (Williams, 2000). 23 plants (comprised of 8 transgenic lines and wild type) were maintained in the greenhouse and 23 lines *in vitro*. Apple plants *in vitro* were grown under a 16/8 hr light/dark photoperiod regime at 22°C and were subcultured to fresh medium every 6 weeks. Wild type plants were maintained on A17 medium (MS (Murashige and Skoog, 1955) including vitamins 4.6 gl^{-1}, Sucrose 30 gl^{-1}, BAP 1 mgl^{-1}, IBA 0.1 mgl^{-1}, Agar 7.5 $gl^{-1}$and GA_3 0.1 mgl^{-1} [filter sterilised after autoclaving]) and transgenic plants were maintained on A8 media (MS 4.6 gl^{-1}, Sucrose 30 gl^{-1}, BAP 2 mgl^{-1}, IBA 0.1 mgl^{-1}, Agar 7.5 gl^{-1} and GA_3 1 mgl^{-1} [filter sterilised after autoclaving]).

2.1.1.2 Tobacco

Nicotiana tabacum cv. Petit Havana (wild type and chloroplast *gfp* transformants) and cv. Wisconsin (wild type and $pSAG_{12}$:*ipt* nuclear transformants [homozygous and hemizygous]) plants were maintained *in vitro* on Gamborg B5+vitamins medium (Duchefa) 3.2 gl^{-1}, Sucrose 30 gl^{-1}, MES 0.5 gl^{-1}, pH 5.8 and Agar 7 gl^{-1} under a 16/8 hr light/dark photoperiod regime at 25°C. cv. Petit Havana *gfp* plants were obtained from John Gray (Cambridge) and Wisconsin $pSAG_{12}$:*ipt* plants from Gan and Amasino (Untiversity of Wisconsin).

2.1.2 Root induction

Apple shoots were rooted by placing shoots on induction media R13 (MS 4.6 g/L, sucrose 30 gl^{-1}, IBA 3 mgl^{-1} and Agar 7.5 gl^{-1} pH 5.4) for 3-4 days and then transferring to R37 media (MS 2.3 gl^{-1}, Sucrose 15 gl^{-1}, Agar 7.5 gl^{-1} pH 5.4). Plants were potted up 4-6 weeks later.

2.1.3 Sterilization of tobacco seeds

250µl of 70% ethanol was added to a microfuge tube containing seeds and shaken for 20 seconds. The ethanol was the poured off and 250µl of $HClO_3$ was added and the tube was placed on a shaker at 220rpm for 10 minutes. After decanting the $HClO_3$, 250µl of sterile dH_2O was added and the tube was placed on the shaker for another 10 minutes. The dH_2O step was repeated twice more and then 2-3 seeds were placed in each tub of MS. Seeds were germinated in darkness for 7 days. They were then transferred to the culture room at 25°C, 16hr light/ 8hr dark.

2.2 Molecular analysis

2.2.1 Agarose gel electrophoresis

All DNA samples were run on 0.8% agarose gels. 0.8g agarose was made up in 100ml 0.5X TBE buffer (Tris-borate 45 mM, EDTA 1 mM, pH 8). After reaching a temperature of about 50 C, ethidium bromide was added to the agarose to a final concentration of 0.5 µg/ml. Samples were prepared for the electrophoresis by adding 6X loading buffer. Samples were run in 0.5X TBE buffer at 100 V for 70 minutes. The gel was analysed under UV light and photographed using the Eagle Eye Stratagene system.

2.2.2 Isolation of genomic apple DNA for molecular characterisation

Whole genomic DNA was extracted using 2 methods (see below). The DNA concentration was determined at OD_{280}.

1. The DNeasy mini kit for plant DNA extraction from QIAGEN was used according to the manufacturer's instructions.

2. A modified version of a protocol by Frey, 1999:

0.1g of leaf tissue was frozen in liquid nitrogen and ground in 500 µl of lysis buffer (20 mM tris pH 8, 20 mM EDTA and 2 M NaCl) in an Eppendorf tube. The Eppendorf was heated three times for 5 minutes at 85°C with 5 minutes on ice between treatments. The sample was then centrifuged at 13,000 rpm for 10 minutes after vortexing. The supernatant was decanted into a new tube and RNase was added (10µl of RNase A (10 $mgml^{-1}$), 37°C for 30 minutes). 3M sodium acetate (1:10 v/v) and isopropanol were then added and the sample was placed at -20°C for 30 minutes. The sample was then

centrifuged at 13,000 rpm for 10 minutes and the supernatant was removed. The pellet was washed with 70% ethanol and centrifuged at 13,000 rpm for 10 minutes. The supernatant was removed; the pellet was dried and resuspended in 50µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8).

2.2.3 Polymerase cChain rReaction

2.2.3.1 Determination of presence of *ipt* gene

PCR for *ipt* was carried out on putative tobacco transformants and on all of the transformed apple $pSAG_{12}$:*ipt* lines available. The following internal primers and cycle were used (Williams, 2000):

ipt forward primer: TCAACCGGAAGCGGACGACC

ipt reverse primer: GCCATGTTGTTTGCTAGCCAG

PCR programme

Cycle 1	94°C, 1 minute
Cycle 2 (x35)	94°C, 1 minute
	55°C, 1 minute
	72°C, 2 minutes
Cycle 3	72°C, 10 minutes

PCR master mix

Sterile H_2O	17 µl
10X reaction buffer	2.5 µl
$MgCl_2$ (25mM)	1 µl
ipt f primer (5µM)	1 µl
ipt r primer (5µM)	1 µl
Taq polymerase	0.5 µl
dNTP's (5mM)	1 µl
Template DNA	1 µl

2.2.3.2 Determination of presence of the *gfp* gene

Primers were designed, using Cybergene AB, for amplifying the *gfp* gene in the pMSK18 plasmid (Fig 2.1). The forward primer was designed to include a ribosomal binding site.

gfp AscI forward:	ATGGCGCGCCAGTTGTAGGGAGGGATTTATGAGTAAAG AGAAGAACTTTTC
gfp PacI reverse:	CGTTAATTAATTATTTGTATAGTTCATCCAT

PCR programme

Cycle 1	94°C, 1 minute
Cycle 2 (x24)	94°C, 1 minute
	55°C, 1 minute
	72°C, 2 minutes
Cycle 3	72°C, 7 minutes

PCR master mix

Sterile H_2O	34 µl
10X reaction buffer	5 µl
$MgCl_2$ (25 mM)	2.5 µl
gfp f primer (5 µM)	1 µl
gfp r primer (5 µM)	1 µl
Taq polymerase	0.5 µl
dNTP's (5 mM)	5 µl
Template DNA	1 µl

Other *gfp* primers were also used in later PCR reactions and are as follows:

*gfp*2 forward primer: TGGAGAGGGTGAAGGTGATG

*gfp*2 reverse primer: TGGAGAGGGTGAAGGTGATG

PCR programme

Cycle 1	94°C, 1 minute
Cycle 2 (x30)	94°C, 30 seconds
	58°C, 1 minute
	72°C, 2 minutes
Cycle 3	72°C, 7 minutes

PCR master mix

Sterile H_2O	12.3 µl
10X reaction buffer	2.5 µl
$MgCl_2$ (25 mM)	1.5 µl
gfp f primer (5 µM)	3 µl
gfp r primer (5 µM)	3 µl
Taq polymerase	0.5 µl
dNTP's (5 mM)	1.2 µl
Template DNA	1 µl

2.3 Transformation

2.3.1 *Agrobacterium*-mediated transformation of tobacco cv. Petit Havana *gfp* plants with pVDH396.

Agrobacterium tumefaciens strain LBA4404 containing the plasmid pVDH396 (Fig. 2.1) was grown on LB plates (Tryptone 10 gl^{-1}, Yeast extract 5 gl^{-1}, NaCl 10 gl^{-1}, agar 15 gl^{-1}, pH 7.2) with 50 mgl^{-1}kanamycin for 2 days at 28°C. A colony was inoculated in 5 ml

YEP (Bacto peptone 10 gl^{-1}, Yeast extract 10 gl^{-1}, NaCl 5 gl^{-1}, pH 7.2) containing 50 mgl^{-1} kanamycin. 500µl of 0.1M acetosyringone (AS) stock and 1M betaine stock were added to 500 ml MS20 (MS complete 4.6 gl^{-1}, Sucrose 20 gl^{-1}) and the pH was adjusted to 5.2 using sterile NaOH and HCl. 100 µl of cells were added to 900 µl of YEP and the absorption read at 420nm. The result was multiplied by 10 (dilution factor) and divided by 0.5 to give X. The remaining cells were centrifuged at 3000 rpm for 15 minutes and the YEP poured off. The cells were resuspended in X*10 ml of MS20 (containing AS and betaine), divided into 10 ml cultures and shaken at 200 rpm at 20°C for 5 hours.

Ten ml of the inducted bacteria was added to each RMOP plate (30 leaf squares per plate), the plate was swirled and left for 20 minutes. Leaf discs were then blotted on sterile filter paper and transferred to sterile filter paper (dampened with 200 µl of MS20 + AS + betaine) on RMOP plates containing 50 mg/l kanamycin and 200 mg/l cefotaxim. After 3 days explants were washed in bacterial wash (dH_2O, 200 mg/l cefotaxime, pH 5.2), blotted on sterile filter paper and transferred to RMOP plates containing 200mg/ml cefotaxime and 5mgl^{-1} hygromycin.

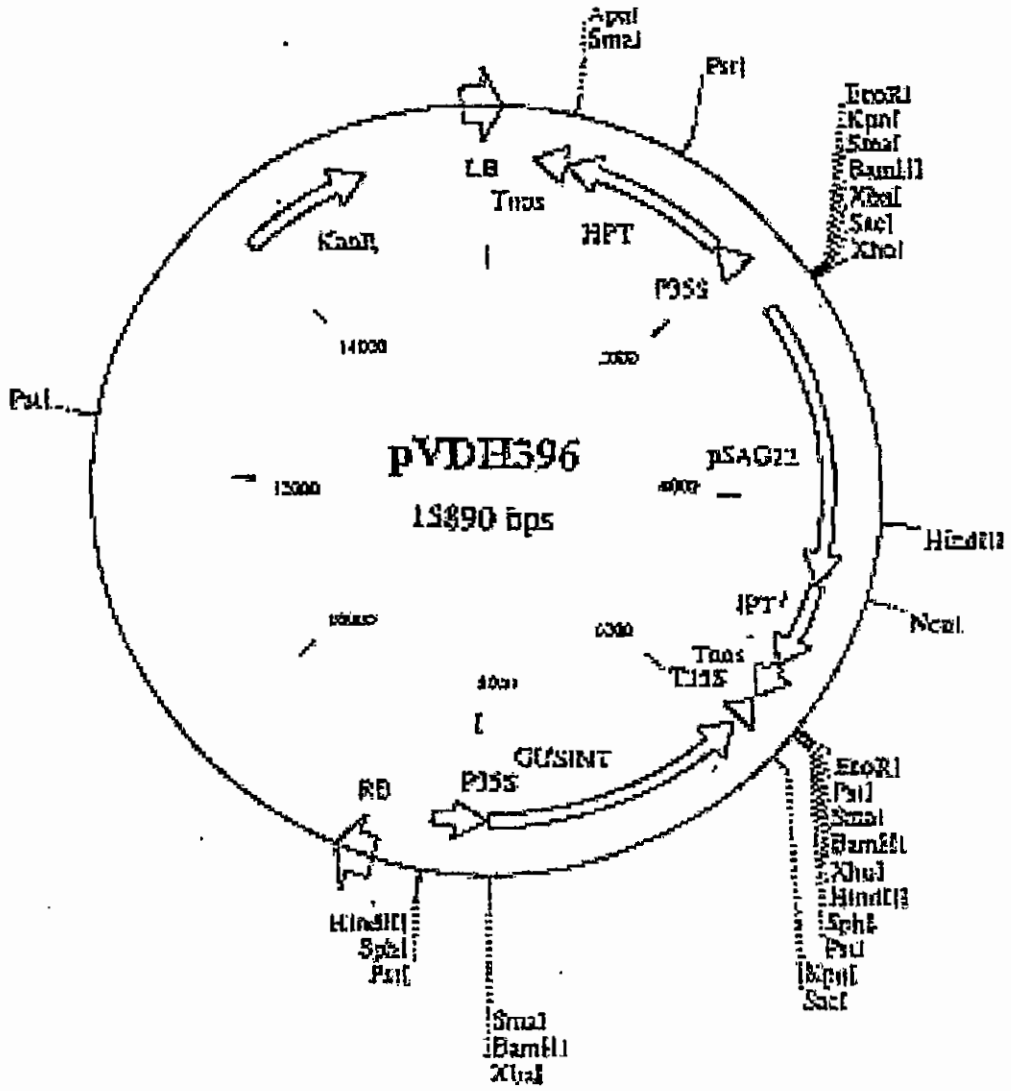

Figure 2.1. Plasmid pVDH396, used in the transformation of pSAG12:*ipt* tobacco lines. The plasmid contains a hygromycin selectable marker (NPTII) and the GUS reporter gene (GUSINT driven by the promoter of the cauliflower mosaic virus (P35S)). *ipt* is driven by the senescence specific SAG_{12} promoter.

3 weeks later the calli were transferred to RMOP plates containing 10 mgl^{-1} hygromycin. After concentration response experiments, the putative transformants were placed on RMOP (and shoots on MS) containing 35 mgl^{-1}hygromycin and 200 mgl^{-1} cefotaxime.

2.3.2 Biolistic transformation of tobacco cv. Wisconsin pSAG12:*ipt* plants with pZS:*gfp* and pMSK18.

2.3.2.1 Cloning *gfp* into pZS197

pZS197 (Fig. 2.2) and the *gfp* PCR product were digested using AscI and PacI restriction enzymes.

Vector	Insert	
3 µl	15 µl	DNA
2.5 µl	2.5 µl	AscI
3 µl	3 µl	Buffer 4
2.5 µl	2.5 µl	PacI
3 µl	3 µl	Buffer 1
3 µl	3 µl	10X BSA
3 µl	1 µl	dH2O

The tubes were left for 3 hours at 37°C. After running the samples on a gel, the backbone of the pZS197 was cut out using a QIAGEN gel extraction kit and treated with Shrimp alkaline phosphotase to prevent the backbone closing in on itself. 1 µl of SAP and 1.5 µl

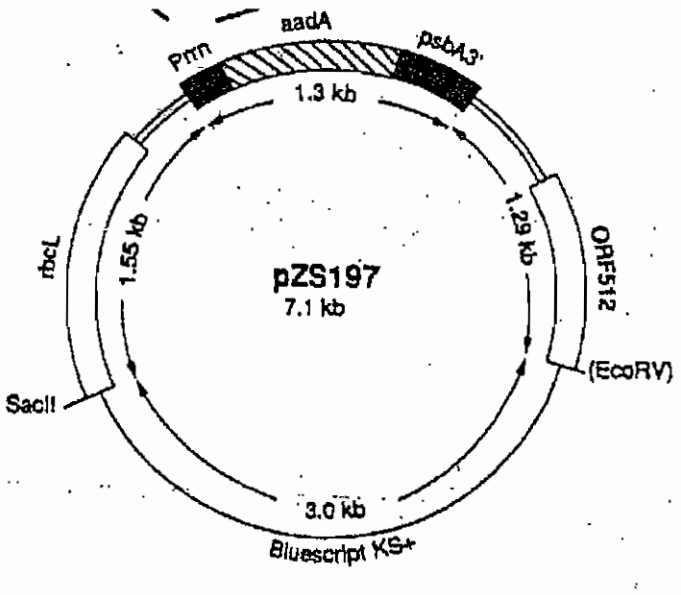

Fig. 2.2. The original pZS197 vector. *aadA* confers resistance to spectinomycin and streptomycin and is controlled by a modified plastid ribosomal RNA operon promoter.

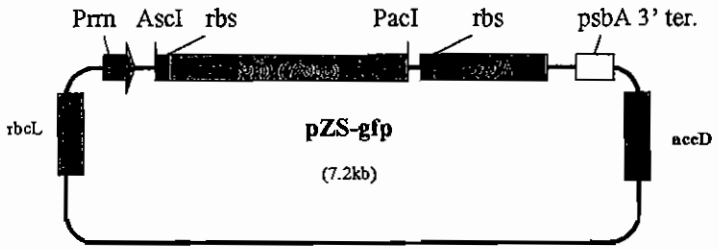

Fig. 2.3. The pZS: *gfp* vector. The pZS197 vector was modified by cloning *gfp* into the vector.

of 10X buffer were added to 15 µl of the vector and the tube was incubated at 37 °C for 15 minutes. The SAP was inactivated by a 15 minute incubation in a 65°C water bath. The vector was then ligated with the *gfp* PCR product overnight at 15°C (Fig. 2.3). The ligation reaction was as follows:

pZS197 (mod)	3 µl
gfp insert	3 µl
10X ligation buffer	1 µl
dH_2O	2 µl
ligase	1 µl

2.3.2.2 Transformation of *E.coli*

Top10F' competent *E.coli* cells were thawed on ice for 5 minutes. Ligated DNA was added to the tubes and after gentle swirling, they were left on ice for 30 minutes. The cells were then heat shocked at 42°C for 90 seconds and 800 ml of LB was then added to each sample and the cells were incubated at 37°C for 1 hour with shaking at 220 rpm. The tubes were centrifuged for 30 seconds and 600 ml of the supernatant was removed and the bacterial pellet was resuspended in the remaining LB. Various volumes of the solution were spread on LB plates (containing 100 mgl^{-1} of ampicillin) and grown overnight at 37°C. Colonies were selected and grown in 2 ml liquid LB (amp 100 mgl^{-1}) overnight. Plasmid isolation was carried out using a QIAGEN miniprep kit. The samples were then digested with AscI/PacI and PCR was carried out for the selection of *gfp* transformants.

2.3.2.3 Biolistic transformation

Both plasmids were isolated using the QIAGEN Plasmid Maxi Kit.

2.3.2.3.1 Preparation of leaves for bombardment

Small leaves were joined together on medium (pH 5.4) containing MS (4.6 gl^{-1}), Sucrose (30 gl^{-1}), BAP (5 mgl^{-1}), NAA (1 mgl^{-1}), TDZ (1 mgl^{-1}) and Agar (7.5 gl^{-1}). After shooting the plates were incubated in darkness and 3 days later the leaves were transferred to Selection medium, which is the same as above with the inclusion of antibiotic (hygromycin 3 mgl^{-1}) for selection.

2.3.2.3.2 Preparation of solutions

Before bombardment, solutions of $CaCl_2$ (2.5 M), spermidine (0.1 M) and gold (40 mg/ml) were made up. 50 µl of the gold stock was sonicated for 1 minute. 10 µl of plasmid DNA was placed in the tube. 50 µl $CaCl_2$ and 20 µl spermidine was added to the tube and it was mixed well in the lid of the tube. The lid was closed and the contents were not mixed. The tube was inverted and tapped on a bench top before being vortexed for 5 seconds and then spun at 13,000 for 10 seconds. The supernatant was removed and 150 µl EtOH was added. The pellet was broken up with a pipette tip, vortexed for 5 seconds and then spun for 5 seconds. The supernatant was removed and 85 µl of EtOH was added. After the pellet was broken up again, the tube was left on ice. 5 µl of this solution was used on each membrane.

2.3.2.3.3 Preparation for bombardment

The microcarrier and microcarrier holder were sterilized with 100% and 70% ethanol respectively. The stopping screens were also sterilized in 100% ethanol, as were the 1100 rupture discs, which were left to air dry in a laminar flow hood after 5 minutes of soaking. 5 ml of the gold/DNA mixture was pipetted onto the centre of the microcarrier and it was placed in a Petri dish to dry.

2.3.2.3.4 Bombardment procedure

The chamber was sterilized with 70% ethanol. The helium tank was turned on to 1100 psi and the vacuum was turned on. A rupture disc was placed in the retaining cap, which was then screwed tightly before a flying disc was put in place. The Petri dish containing the leaves were placed in the chamber at 6 cm on the sample holder and the door was closed. The vacuum valve was opened and when it reached 26-28 inches of Hg and the fire button was pressed until the noise of the gas breaking the rupture disc was heard. The vacuum was released and the sample was then removed.

2.4 Assays

2.4.1 GUS analysis

GUS analysis was carried out on all $pSAG_{12}$: *ipt* apple lines.

Solution 1: Substrate

X-Glu (10.44 $mgml^{-1}$) in N, N'-dimethylformamide.

Solution 2: Oxidation catalyst

$K_3Fe(CN)_6$ (0.329 $mgml^{-1}$) and $K_4Fe(CN)_6$ (0.422 mg/ml^{-1}) in 0.1M $NaPO_4$ buffer (pH 7).

Solution 3:

EDTA (8.27 $mgml^{-1}$) and Triton X-100 (1 $mgml^{-1}$) in 0.1M $NaPO_4$ buffer (pH 7).

6 ml of incubation solution (300 µl of solution 1, 3,000 µl of solution 2 and 2,700 µl of solution 3) was made up and 200 µl was aliquotted to each tube containing a small amount of leaf tissue. The samples were left overnight at 37°C. The incubation solution was then removed and 200 µl of destaining solution (3:1 ethanol: glacial acetic acid) was added and the samples were incubated at 65°C for 1 hour. The solution was then replaced with 70% ethanol and the tubes were visually inspected for GUS activity.

2.4.2 Chlorophyll analysis

Chlorophyll content was extracted by soaking leaf discs in methanol overnight and then measuring the absorbance at 650 nm and 665 nm. The total chlorophyll estimation in µg/ml was calculated using the following formula:

$$25.8A_{650}+4.0A_{665}$$

A= Absorbance

2.4.3 Ascorbate peroxidase assay

0.3 g of leaf tissue was ground in liquid nitrogen and 10ml of extraction buffer (50 mM sodium phosphate buffer pH7, 1 mM ascorbic acid, 1 mM EDTA, 1% Triton X-100 and 1% PVP) was added. The tube was vortexed and centrifuged at 2,500 rpm for 15 minutes at 4°C. The supernatant was then filtered through miracloth. 3 µl of the supernatant was added to 297 µl of reaction mixture (50 mM sodium phosphate buffer pH7, 100 mM

H_2O_2 and 1% enzyme extract) and the absorbance was read at 290 nm. 10 µl of 50 mM ascorbic acid was added and the increase in absorbance was read over 3 minutes. Ascorbate peroxidase activity was then calculated in mM per minute using the following formula:

$$c=dA/bE$$

c= concentration (mM)

dA= change in absorbance per minute

b= light path (cm) (1 cm)

E= extinction coefficient (mM^{-1} cm^{-1}) (2.8 for ascorbate peroxidase at 290 nm)

2.4.4 Total soluble protein extraction

0.3 g of leaf tissue was ground in liquid nitrogen and 10 ml of extraction buffer (50 mM phosphate buffer (pH 7), 10 mM ascorbic acid and 1% PVP) was added. The mixture was centrifuged at 3,500 rpm for 20 minutes. 2ml of the supernatant was then added to 2ml H_2O. 0.1g of BSA was added to 100ml of the above extraction buffer to give a 1 mg/ml solution and 6 standards were then made up as follows:

Tube	Extraction Buffer (ml)	$mgml^{-1}$ BSA
1	1	0
2	0.8	0.2
3	0.6	0.4
4	0.4	0.6
5	0.2	0.8
6	0	1

0.1 ml of either the protein standard or the diluted sample was then added to 4ml H_2O and 1ml of Bradford reagent. The samples were left for 5 minutes and then the absorbance was read at 590 nm. The weight of protein was plotted against the corresponding absorbance resulting in a standard curve, which was used to determine the total soluble protein content in the unknown samples.

2.5 GFP analysis

2.5.1 Protoplast isolation

The solutions used in this procedure were as follows:

K3: Stock A 100 $ml l^{-1}$, Stock B 5 $ml l^{-1}$, Stock C 2 $ml l^{-1}$, Stock D 10 $ml l^{-1}$, Stock E 10 $ml l^{-1}$, 2,4 D 0.1 $ml l^{-1}$, BAP 0.2 $ml l^{-1}$ and NAA 1$ml l^{-1}$

Stock A: NH_4NO_3 2.5 $g l^{-1}$, KNO_3 25 $g l^{-1}$, $CaCl_2.2H_2O$ 9 $g l^{-1}$, $(NH_4)_2.SO_4$ 1.3 $g l^{-1}$, $NaH_2PO_4.2H_2O$ 1.5 $g l^{-1}$

Stock B: $FeSO_4.7H_2O$ 5.57 $g l^{-1}$, Na_2EDTA 7.45 $g l^{-1}$

Stock C: $CuSO_4.7H_2O$ 12.5 $mg l^{-1}$, $CoCl_2.6H_2O$ 12.5 $mg l^{-1}$

Stock D: Thiamine HCl 1 $g l^{-1}$, Nicotinic acid 100 $mg l^{-1}$, Pyrdoxine HCl 100 $mg l^{-1}$, Myo- inisotol 10 $g l^{-1}$, Xylose 25 $g l^{-1}$

Stock E: $MnSO_4.4H_2O$ 1 $g l^{-1}$, H_3BO_3 300 $mg l^{-1}$, $ZnSO_4.7H_20$ 200 $mg l^{-1}$, KI 75 $mg l^{-1}$, $Na_2MoO_4.2H_2O$ 25 $mg l^{-1}$

0.2 g of cellulase and 0.06 g of macrozyme were dissolved in 20 ml of K3 solution containing 0.4M sucrose. The sample was spun at 4000 rpm for 10 minutes and the supernatant was filter sterilized into a conical flask. 1 g of a healthy tobacco leaves were cut into narrow strips and incubated for 16 hours, at room temperature, in the enzyme solution.

The solution was shaken vigorously for a few seconds to release the protoplasts and then filtered through a nylon mesh. The solution was spun at 1,500 rpm for 3 minutes and the intact protoplasts were carefully transferred from the liquids meniscus to fresh tubes. W5 solution (NaCl 9 gl^{-1}, $CaCl_2.2H_2O$ 18.4 gl^{-1}, KCl 0.4 gl^{-1}, glucose 1 gl^{-1}) was added and carefully mixed.

2.5.2 Quantitative analysis of GFP

GFP protein was isolated by grinding 3 leaf discs (diameter of 18 mm) in 10 ml extraction buffer (0.1 M Na_2CO_3, pH 9) at 4°C. After centrifugation at 13,000 rpm for 20 minutes at 4°C, the supernatant was used to make up GFP standards in untransformed total soluble protein extract. Dilution series ranging from 0.1-0.4 mg/ml GFP in 400 mg/ml of untransformed protein extract). Protein samples were isolated as described in 2.4.4 and were equilibrated to the lowest concentration recorded. A VersaFluor fluorometer was used to determine GFP concentration, with a 490 nm excitation filter and a 510 nm emmision filter. All samples were measured at 20, 40 and 60 seconds and the mean value was used as a result figure.

Results

3.1 Characterisation of pSAG$_{12}$:*ipt* apple plants

3.1.1 Morphological characterization of older transgenic apple plants

Measurements were made during the growing season of 2002 and 2004, on nine pSAG$_{12}$:*ipt* transgenic apple lines grown in the greenhouse. Three clonally propagated plants of all lines except two (6r and 6F were represented by single plants) were compared to two control cv. Greensleeves plants. 6G(1), 6G(2) and 6G(3) is an example of their naming, these are all derivatives of the same line, just separately grown plants. Height, number of nodes, internodal distance and overall condition of leaves on greenhouse grown apple plants were measured and assessed throughout the summer and part of these results are presented in Table 3.1. Recordings were also made on the browning of the leaves, and are summarized in Table 3.2.

In late November 2002, when the wild type plants had lost all of their leaves, leaves on some of the transgenic lines had not begun senescing. 6A(3) is an example of such a plant and this is illustrated in Fig. 3.1. This was also the case in late November 2003, Fig. 3.2 shows the plants 6G(3) and 4B2(3) which retained their green leaves when the wild type plant had lost all of its leaves. In the case of both years, the better plants at retaining their leaves kept their leaves up to 4 weeks after all of the leaves had been lost on the wild type plants.

	23/09/2002		05/08/2004	
Line	Height	# nodes	Height	# nodes
w/t (1)	185	24	197.5	27
w/t (2)	157.5	20	160	20
2A3(1)	185	19	207.5	22
2A3(2)	175	16	190	18
2A3(3)	205	30	200	30
4B2(1)	145	29	145	28
4B2(2)	157.5	26	162.5	28
4B2(3)	200	12	205	12
6A(1)	152.5	16	150	20
6A(2)	185	11	192.5	13
6A(3)	155	21	162.5	24
6B(1)	192.5	41	197.5	36
6B(2)	242.5	18	240	22
6B(3)	170	15	165	16
6D(1)	150	14	160	15
6D(2)	187.5	35	190	30
6D(3)	180	16	185	16
6F	182.5	20	187.5	21
6G(1)	192.5	26	117.5	9
6G(2)	200	16	197.5	16
6G(3)	112.5	9	192.5	20
6r	200	22	207.5	22
15B(1)	170	21	172.5	21
15B(2)	155	13	145	13
15B(3)	195	21	197.5	23

Table 3.1. Height (cm) and number of nodes on greenhouse grown apple plants in the summer of 2002 and 2004.

Line		# Brown Leaves				Colour of Leaves
		22/07/02	27/08/02	24/09/02		21/11/02
W/T	1	(+)	(+/-)	(+/-)		(-)
	2	(+)	(+)	(+/-)		(+/-) Y/B
2A3	1	(++)	(++)	(+)		(++) G/Y
	2	(++)	(+++)	(++)		(++) G
	3	(++)	(+)	(+/-)		(++) Y
4B2	1	(++)	(++)	(+)		(++) Y
	2	(+)	(++)	(+)		(++) Y
	3	(+)	(+)	(+)		(+++) G/Y
6A	1	(++)	(+)	(+)		(++) Y/B
	2	(+)	(++)	(+)		(+++) G/Y
	3	(+++)	(++)	(++)		(+++) G
6B	1	(++)	(++)	(++)		(++) B
	2	(+)	(+)	(+)		(++) Y
	3	(+)	(++)	(+)		(++) B
6D	1	(+)	(++)	(+)		(-)
	2	(++)	(++)	(+)		(++) G/Y
	3	(+)	(+)	(+)		(++) G/Y
6G	1	(+)	(+)	(+)		(+) Y
	2	(+)	(+)	(+)		(++) G/Y/B
	3	(+)	(+)	(+)		(+) B
6r	1	(+)	(+)	(+)		(++) G/Y
15B	1	(+)	(+)	(+/-)		(++) Y
	2	(+)	(+)	(+)		(+) Y
	3	(+)	(+)	(+/-)		(+/-) Y
6F	1	(+)	(+)	(+)		(+) Y

Table 3.2. The occurrence of brown leaves on transgenic apple plants in 2002. Measurements were made at monthly intervals through the summer, and the presence and colour of the leaves also recorded during November. The colours of the leaves are abbreviated as follows: G= Green, B= Brown and Y= Yellow. The number of leaves are denoted by the following symbols: (-)= 0, (-/+)= <5, (+)= <10, (++)= <50 and (+++)= >50. (w/t= wild type)

Fig. 3.1. Line 6A(3) (left) had retained many of its leaves in November 2002 when the wild type plant (right) had lost all of its leaves

Fig. 3.2. Left side: Wild type plant (left) and 4B2(3) line (right). Right side: Wild type plant (left) and 6G(2) line (right). Both transgenic lines retained their leaves in November 2003 when the wild type plants had lost all of theirs.

3.1.2 Molecular analysis of transgenic apple trees

The presence of the *ipt* genes was investigated in representative plants of the total 27 transgenic apple lines. PCR was carried out to confirm the presence of the *ipt* gene in all of the 8 greenhouse lines that were to be used in assays. 18 of the 27 lines tested positive for *ipt* (All 8 greenhouse plants were included in this number). Fig. 3.3 shows representative PCR results for the lines L, L(II), 14B, 37C and 47A. The 27 lines were also tested for GUS activity as described in 2.4.1, and 19 of the 27 lines tested positive for the presence of this reporter gene (Fig. 3.4)

Fig. 3.3. PCR analysis of apple plants transformed with pVDH396. Internal *ipt* primers were used and a band of 355bp was detected. This is a representative sample of plants. Lane 1 is a 100bp ladder. Lanes 2 and 8 are the negative controls and contain the wild type DNA and water, respeetively. Lane 3, 4, 5, 6 and 7 contain the lines L, L(II), 14B, 37C and 47A. The latter 3 tested positive for the presenee of *ipt*.

Fig. 3.4. Representative GUS assay. Left is the wild type sample and right is the transgenic line L. The GUS enzyme cleaves the X-gluc substrate resulting in the production of an insoluble blue coloured product

3.1.3 Chlorophyll analysis

In August 2002, leaf discs, of a 5mm radius, punched from leaves at nodal positions 1,5 and 9, from each apple plant were floated on 10 ml sterile H_2O at 25°C and left in the light. Three discs were used per sample. The chlorophyll was extracted as described in 2.4.2. Measurements were taken at day 0 and day 20. The results are presented in Fig. 3.5 and 3.6. This procedure was repeated in May 2004, except that young leaves were removed randomly from the plant and the leaf discs were left in the darkness. These results are presented in Fig. 3.7 and 3.9. Measurements were taken at day 0, day 14 and day 34 after detachment from the plant.

Table 3.3 summarizes the percentage retention of chlorophyll in leaf discs for both assays. In 2002, wild type leaves lost 67-79% of their chlorophyll content. 13 out of 23 transgenic plants lost less chlorophyll but not all were significantly different from the wild type. 4B2(1)(52% loss), 6A(3)(46% loss), 6D(1)(49% loss), 6D(3)(26.3%) and 6r (26%) were the strongest lines in chlorophyll retention, post detachment. 6A(3) was also the best plant, in terms of keeping its leaves, during the morphological studies in November 2002. In the 2004 assay, the two wild type plants lost 44% and 26% of their chlorophyll 34 days after detachment. 9 out of 23 transgenic plants lost less than 26% and another 9 of the remaining plants lost less than 44%. The better plants, in terms of chlorophyll retention, were 6B(1), 6G(2), 6G(3), 15B(1), 4B2(3) and 2A3(3).

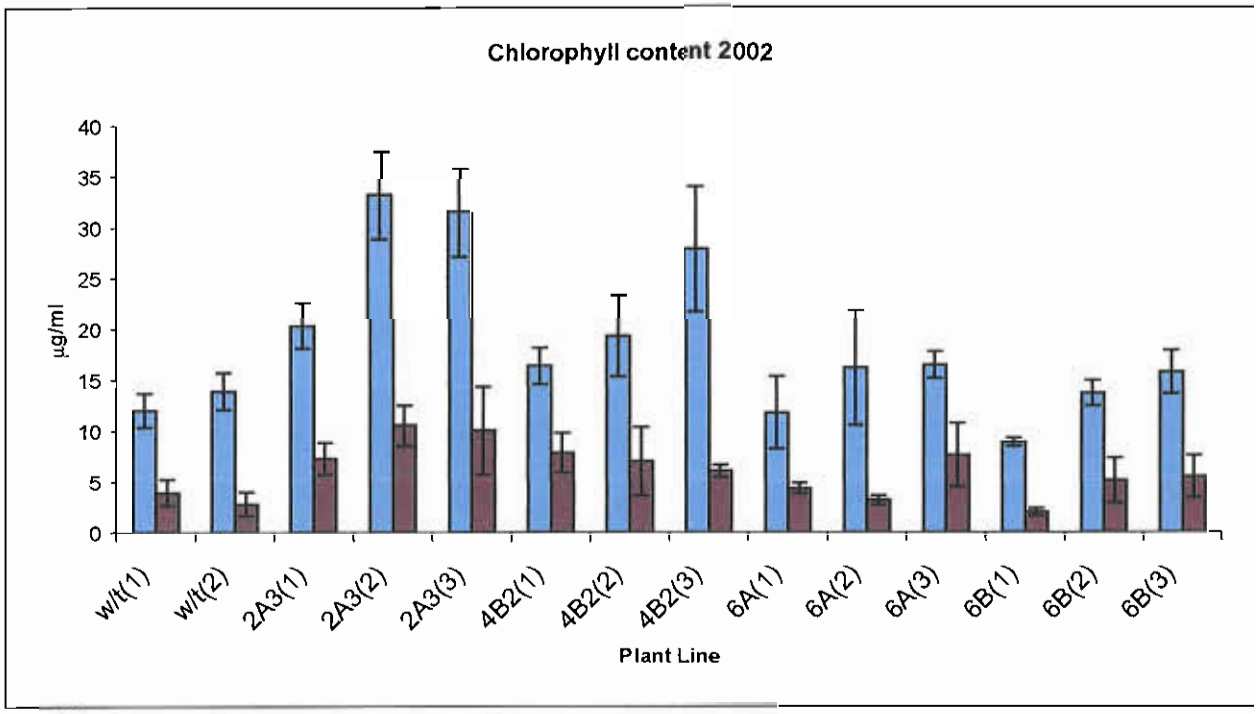

Fig. 3.5

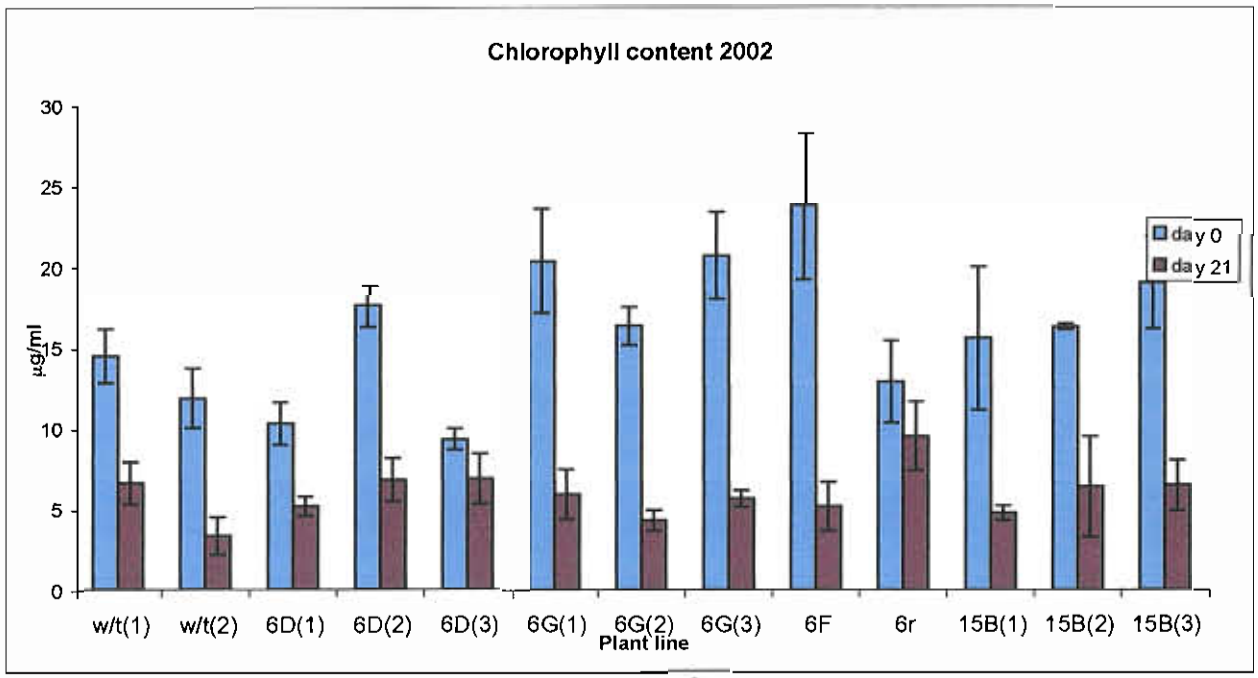

Fig. 3.6 (and Fig. 3.5).Chlorophyll content (µg/ml) in leaf discs from all greenhouse grown plants. Leaves were detached from apple plants in August 2002, at day 0. Discs, of radius 5 mm were floated on H_2O in the light and chlorophyll content was measured 20 days after detachment.

w/t= wild type control

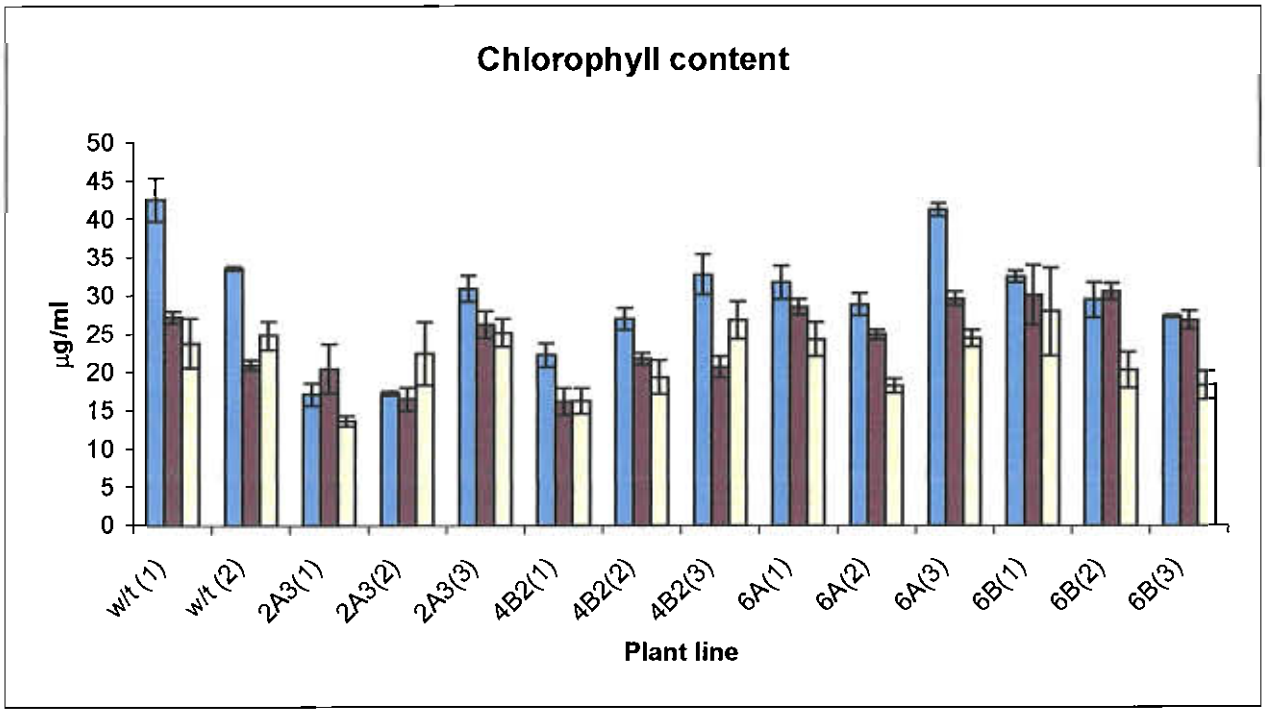

Fig. 3.7

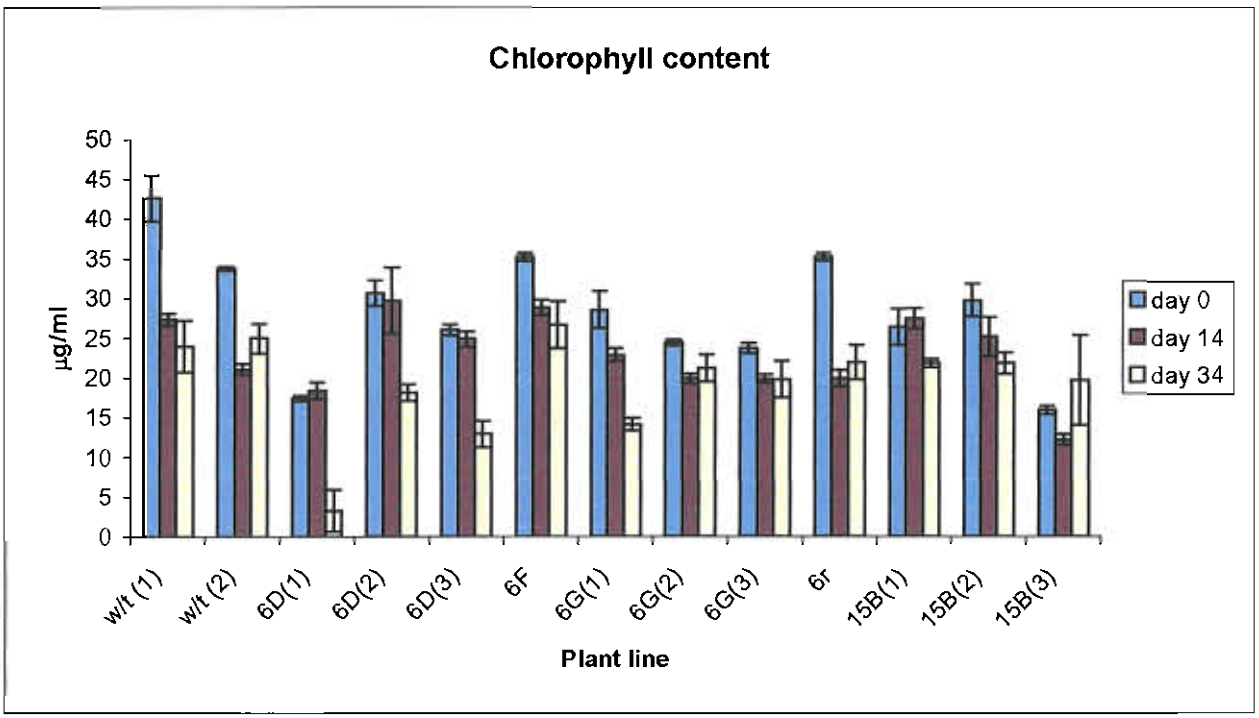

Fig. 3.8 (and Fig. 3.7). Chlorophyll content (µg/ml) in leaf discs from all greenhouse grown plants. Leaves were detached from apple plants in May 2004, at day 0. Discs, of radius 5 mm were floated on H_2O in the dark and chlorophyll content was measured 14 and 34 days after detachment.

Line	2002	2004
w/t (1)	32.7	55.7
w/t (2)	20.4	73.9
2A3(1)	35.8	80.3
2A3(2)	31.9	(N/A)
2A3(3)	31.9	81.9
4B2(1)	47.9	73.1
4B2(2)	36.2	71.6
4B2(3)	21.7	81.9
6A(1)	36.8	77.8
6A(2)	19.4	63.6
6A(3)	53.7	59.3
6B(1)	22.5	85.3
6B(2)	37.5	70.7
6B(3)	35.0	67.1
6D(1)	50.2	19.1
6D(2)	38.5	59.4
6D(3)	73.7	49.3
6F	21.7	75.7
6G(1)	29.2	50.1
6G(2)	26.3	86.8
6G(3)	27.3	84.2
6r	73.9	62.3
15B(1)	30.6	83.7
15B(2)	39.2	73.7
15B(3)	34.0	(N/A)

Table 3.3. Pcreentage of chlorophyll lcft after detaehment in 2002 and 2004. N/A indieates when more chlorophyll was present after detachment.

3.1.4 Total soluble protein measurement

In August 2003, detached leaves from older apple plants were kept on H_2O in darkness at 25°C and total soluble protein levels were measured as described in 2.4.4., 6D(3), 6G(1) and 6r and a wild type plant were tested (Fig. 3.9.). To determine if there is a notable difference in protein retention of these samples, data points beyond day 24 were necessary. This was carried out in May 2004, on younger leaves of all 25 of the greenhouse grown apple trees (comprised of 8 transgenic lines and wild type). These early summer leaves exhibited a much faster loss in total soluble protein levels (Table 3.4). At day 15, the two wild type plants had lost 44% and 66% of their original total soluble protein content compared to that of 2A3(3), 6D(2) and 6D(3) which were 1%, 5% and 20% respectively.

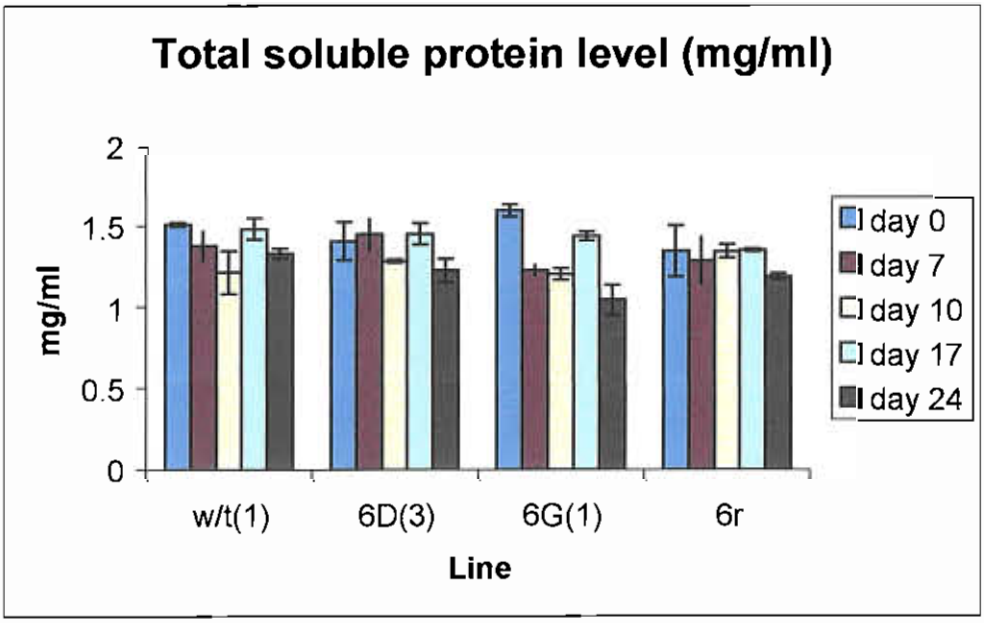

Fig. 3.9. Total soluble protein analysis of 0.3g apple leaf sections. Leaves were detached from apple plants in August 2003, at day 0, were floated on H_2O in the dark and protein content was measured 7, 10, 17 and 24 days after detachment.

Line	Day 0	Day 15	% Protein retained
w/t (1)	0.48 (+/- 0.09)	0.27 (+/- 0.01)	56.0
w/t (2)	0.65 (+/- 0.09)	0.22 (+/- 0.02)	34.7
2A3(1)	0.33 (+/- 0.05)	0.09 (+/- 0.02)	26.3
2A3(2)	0.34 (+/- 0.13)	0.13 (+/- 0.01)	38.4
2A3(3)	0.24 (+/- 0.06)	0.24 (+/- 0.08)	99.2
4B2(1)	0.29 (+/- 0.07)	0.13 (+/- 0.03)	45.9
4B2(2)	0.43 (+/- 0.1)	0.18 (+/- 0.06)	41.5
4B2(3)	0.28 (+/- 0.09)	0.07 (+/- 0.03)	25.6
6A(1)	0.39 (+/- 0.13)	0.18 (+/- 0.04)	46.2
6A(2)	0.61 (+/- 0.08)	0.15 (+/- 0.01)	23.9
6A(3)	0.63 (+/- 0.09)	0.17 (+/- 0.01)	26.4
6B(1)	0.7 (+/- 0.15)	0.22 (+/- 0.06)	31.5
6B(2)	0.52 (+/- 0.09)	0.12 (+/- 0.01)	23.9
6B(3)	0.28 (+/- 0.12)	0.17 (+/- 0.02)	59.2
6D(1)	0.29 (+/- 0.04)	0.06 (+/- n.a)	19.2
6D(2)	0.27 (+/- 0.02)	0.25 (+/- 0.04)	95.1
6D(3)	0.34 (+/- 0.05)	0.27 (+/- 0.03)	80.4
6F	0.52 (+/- 0.12)	0.14 (+/- 0.01)	25.7
6G(1)	0.45 (+/- 0.03)	0.19 (+/- 0.01)	42.8
6G(2)	0.35 (+/- 0.03)	0.18 (+/- 0.04)	51.5
6G(3)	0.28 (+/- 0.04)	0.17 (+/- 0.03)	59.6
6r	0.38 (+/- 0.08)	0.1 (+/- 0.02)	26.6
15B(1)	0.48 (+/- 0.03)	0.21 (+/- 0.01)	43.0
15B(2)	0.42 (+/- 0.05)	0.22 (+/- 0.04)	52.1
15B(3)	0.54 (+/- 0.07)	0.09 (+/- 0.03)	17.5

Table. 3.4. Total soluble protein levels (mg/ml) of 0.3g apple leaf sections. Leaves were detached from apple plants in May 2004, at day 0, were floated on H_2O in the dark and protein content was measured 15 days after detachment.

3.1.5 Ascorbate peroxidase activity

This assay was carried out on 0.3 g sections of detached leaves from greenhouse grown apple trees in August 2003, as described in 2.4.3. These leaves were kept on H_2O in the darkness at 25°C and 0.3g sections were used per reading and readings were made at day 0, 7, 10, 17 and 24 (Fig. 3.10) Ascorbate peroxidase levels were fairly constant during this period. 6G(1) is the only plant in which there was a significant decrease at day 24. The assay was repeated in May 2004 on all of the transgenic apple lines. Again there were no dramatic changes in ascorbate peroxidase activity within the time frame of the experiment (Table 3.16). There were much lower levels of APX detected in the latter assay, possibly due to the fact that the leaves were removed very early in the growing season. A detached leaf from 4B2(3) was still green 60 days after detachment while the wild type leaves had rotted long before that (Fig. 3.11).

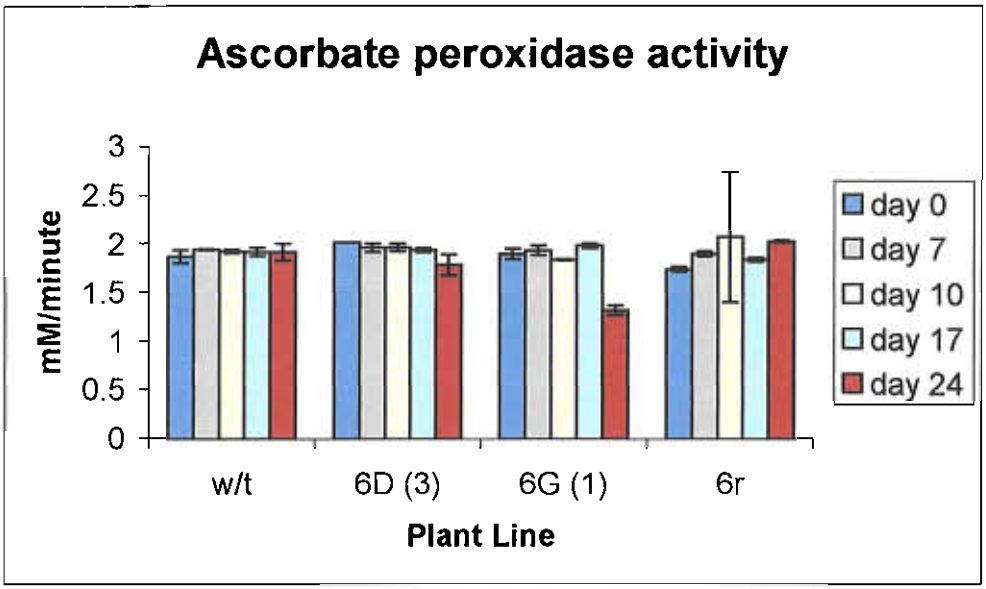

Fig. 3.10. Ascorbate peroxidase activity of 0.3g apple leaf sections. Leaves were detached from apple plants in August 2003, at day 0, were floated on H_2O in the dark and APX activity was measured 7, 10, 17 and 24 days after detachment.

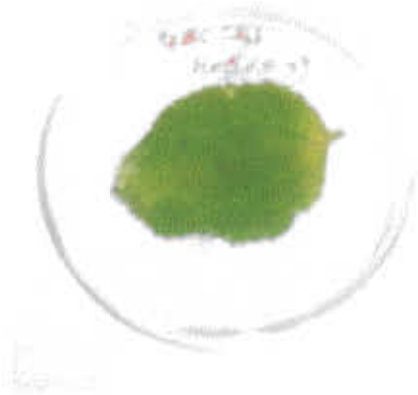

Fig. 3.11. A detached leaf from the plant 4B2(3). This leaf was detached for an ascorbate peroxidase assay on 11th May 2004 and was still green on 11th August, over 90 days after detachment from the plant (the wild type leaves had rotted 30 days after detaehment).

Line	Day 0	Day 15
w/t (1)	196.43 (+/- 16.55)	266.27 (+/- 7.97)
w/t (2)	248.01 (+/- 8.55)	96.03 (+/- 156.64)
2A3(1)	249.60 (+/- 30.44)	213.49 (+/- 58.68)
2A3(2)	229.36 (+/- 9.84)	204.37 (+/- 136.10)
2A3(3)	176.98 (+/- 29.22)	308.33 (+/- 14.21)
4B2(1)	346.03 (+/- 26.65)	209.12 (+/- 20.32)
4B2(2)	340.87 (+/- 32.68)	112.30 (+/- 58.2)
4B2(3)	227.78 (+/- 26.44)	205.55 (+/- 28.68)
6A(1)	367.06 (+/- 15.9)	272.22 (+/- 31.69)
6A(2)	401.19 (+/- 8.45)	167.86 (+/- 57.14)
6A(3)	357.94 (+/- 36.46)	267.86 (+/- 33.63)
6B(1)	325.8 (+/- 19.12)	302.78 (+/- 52.20)
6B(2)	352.78 (+/- 31.34)	325.39 (+/- 47.89)
6B(3)	327.38 (+/- 68.28)	348.81 (+/- 9.10)
6D(1)	240.08 (+/- 9.08)	75.0 (+/- n.a)
6D(2)	276.19 (+/- 12.74)	175.79 (+/- 46.99)
6D(3)	273.41 (+/- 26.7)	368.25 (+/- 106.01)
6F	248.41 (+/- 28.94)	326.98 (+/- 34.46)
6G(1)	331.35 (+/- 30.53)	200.39 (+/- 79.29)
6G(2)	382.14 (+/- 38.51)	184.13 (+/- 35.89)
6G(3)	316.27 (+/- 59.44)	179.37 (+/- 50.47)
6r	378.17 (+/- 29.85)	195.24 (+/- 77.51)
15B(1)	334.52 (+/- 17.92)	210.71 (+/- 14.93)
15B(2)	386.90 (+/- 57.35)	256.74 (+/- 24.09)
15B(3)	344.44 (+/- 28.11)	257.54 (+/- 34.15)

Table 3.5. Ascorbate peroxidase activity (μM/m) of 0.3g apple leaf sections. Leaves were detached from apple plants in May 2003, at Day 0, were floated on H_2O in the dark and APX activity was measured 15 days after detachment.

3.2 Investigating GFP leakage in *gfp* transformed plant cells exhibiting delayed senescence

A parallel investigation was carried out using the model species, tobacco, to further probe the cellular events taking place during the senescence process. Transgenic plants containing autoregulated *ipt* in the nucleus and *gfp* in the chloroplast were generated in order to study GFP leakage from the chloroplast during senescence.

3.2.1 Generation of tobacco plants containing autoregulated *ipt* in the nucleus and *gfp* in the chloroplast

Initial materials were *Nicotiana tabacum* cv. Petit Havana *gfp* plants (chloroplast transformants), which were obtained from John Gray (Cambridge) and cv. Wisconsin $pSAG_{12}$:*ipt* plants (nuclear transformants), obtained from Gan and Amasino (University of Wisconsin). Since both of these plants were already characterised seperately for *gfp* and *ipt*, it was decided to transform the cv. Petit Havana *gfp* plants with *ipt* and the cv. Wisconsin $pSAG_{12}$:*ipt* plants with *gfp* . The *gfp* gene was amplified in the pMSK18 vector (Newell *et al.*, 2003) and ligated into a modified version of the pZS197 vector (Fig. 2.2 and 2.3). Young leaves of cv. Wisconsin $pSAG_{12}$:*ipt* plants were bombarded with a 1 μg/μl solution of pZS: *gfp* as described in 2.3.2.3. cv. Petit Havana *gfp* plants were subjected to agrobacterium mediated transformation (using the vector pVDH396 [Fig. 2.1]) as described in 2.3.1.

3.2.1.1 Amplification of the *gfp* gene and cloning into the pZS197 vector

The *gfp* gene was amplified from the pMSK18 vector (Fig. 3.12) by PCR using the internal *gfp* primers as described in 2.2.3.2. A modified form of the pZS197 vector was used in the cloning of *gfp*. The insert between the ribosomal binding site and the *aadA* gene was removed and the vector was treated with Shrimp alkaline phosphotase to prevent it from closing in on itself (Fig. 3.13). After a 1:3 vector: insert (pZS197: *gfp*) ligation reaction, competent *E.coli* cells were transformed with the resulting pZS: *gfp* vector. Putative transformants were those which grew on ampicillin. Plasmid isolation was carried out as described in 2.3.2.3. *E. coli* transfomants were confirmed by the presence of a 720 bp band after PCR with *gfp* primers (Fig. 3.14) and an AscI/PacI digestion (Fig. 3.15).

Fig 3.12. Amplification of *gfp* from pMSK18 by PCR. A band of 720 bp was produced. Lane 1 is a 1KB ladder. Lanes 2,3,4 and 5 are pMSK18 samples, all of which gave a 720bp product.

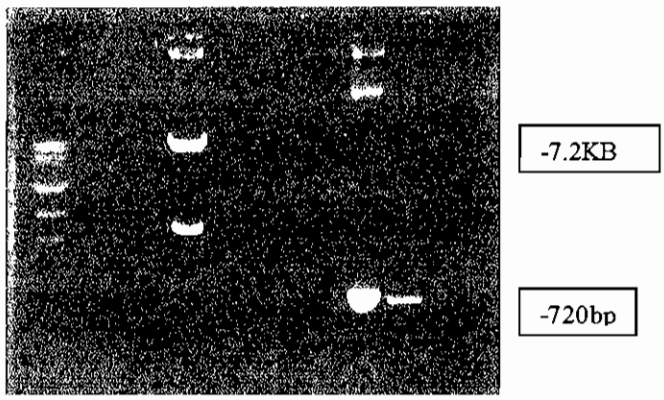

Fig 3.13. AscI/PacI digestion of pZS197 and the *gfp* PCR product. The backbone of the modified pZS197 vector produced a band of 7.2 KB after the HA gene was cut out and the *gfp* produced a 720 bp band. Lane 1 is a 1KB ladder. Lane 4 is the digested pZS197 vector and Lanes 8 and 9 contain the digested *gfp* PCR product from above.

Fig 3.14. PCR of plasmid DNA from *E.coli* growing on ampicillin (putative pZS:*gfp* transformants) with *gfp*2 primers. A band of 510bp was detected. Lanes 1 and 6 are 100bp ladder. Lanes 2 and 3 are DNA isolated from separate colonies growing on LB containing amp, lane 3 is the negative control (H_2O) and lane 4 is the pMSK18 vector.

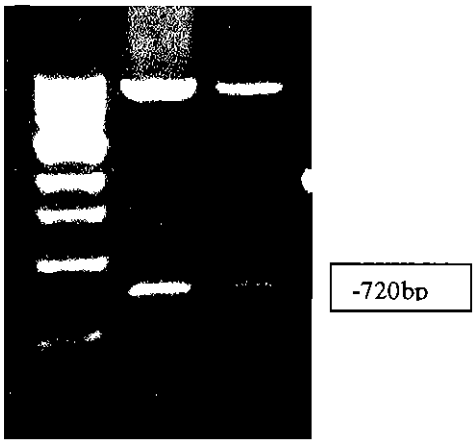

Fig 3.15. AscI/PacI digestion of plasmid DNA of putative pZS: *gfp E. coli* colonies. A band of 510 bp was detected. Lane 1 is a 1KB ladder. Lanes 2 and 3 are the digested samples.

3.2.1.2 Biolistic Transformation of Tobacco plants

20 *N. tabacum* cv. Wisconsin $pSAG_{12}$: *ipt* and 5 wild type cv. Wisconsin leaves were bombarded with microprojectiles coated with a 1 μg/μl solution of pZS: *gfp*. The leaves were cut into small squares after two days and transferred to RMOP containing spectinomycin (500 mg/L). Two independant chloroplast transformants were obtained (Fig. 3.16).

3.2.1.3 *Agrobacterium*- mediated transformation of Petit Havana *gfp* plants

Agrobacterium tumefaciens strain LBA4404, containing the plasmid pVDH396, was used in the transformation of *N. tabacum* cv. Petite Havana containing the *gfp* gene in the chloroplast. The *Agrobacterium* was grown on LB medium containing kanamycin (50 mg/L). Transformed shoots were selected on MS medium containing hygromycin (35 mg/L) and cefotaxime (200 mg/L). PCR for the *ipt* gene was carried out on the 10 shoots obtained and all tested positive for the *ipt* gene (Representative PCR is shown in Fig. 3.17). These lines were named I1- I8, I1 and I2 were tested for *gfp* by PCR (Fig. 3.18) to ensure its presence and after confirmation of the presence of *ipt* (Fig.3.17), these *in vitro* shoots, along with cv. Petit Havana wild type and just *gfp* plants were potted up. The older plants were used in later assays.

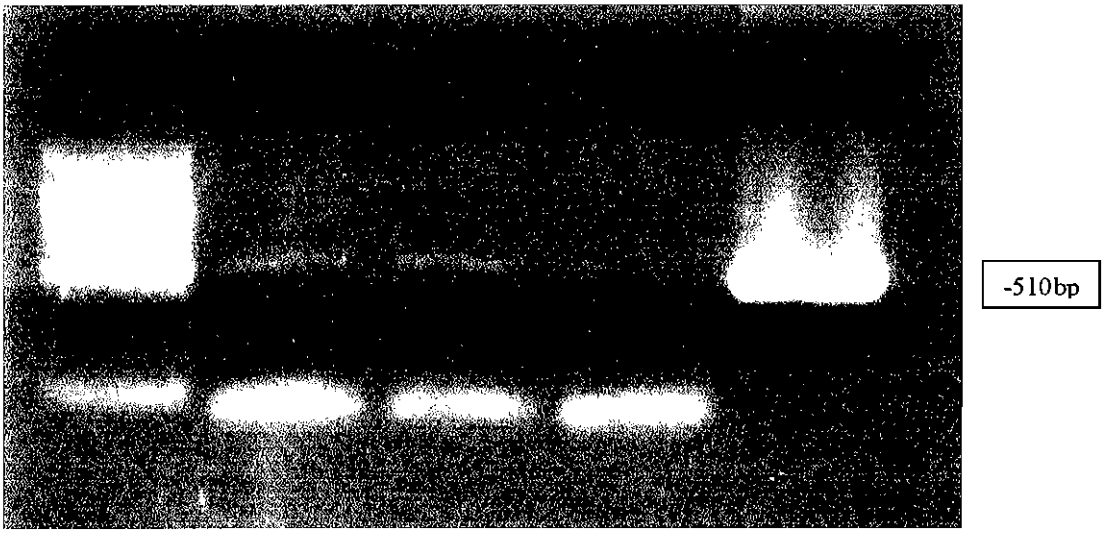

Fig 3.16. PCR analysis on putative pZS: *gfp* plants. A 510 bp band was detected. Lane 1 contains a 100bp ladder respectively. Lane 2 and 3 are the lines pGFP1 and 2, lane 4 is the negative control (H2O) and Lane 5 is the positive control (the pMSK18 vector).

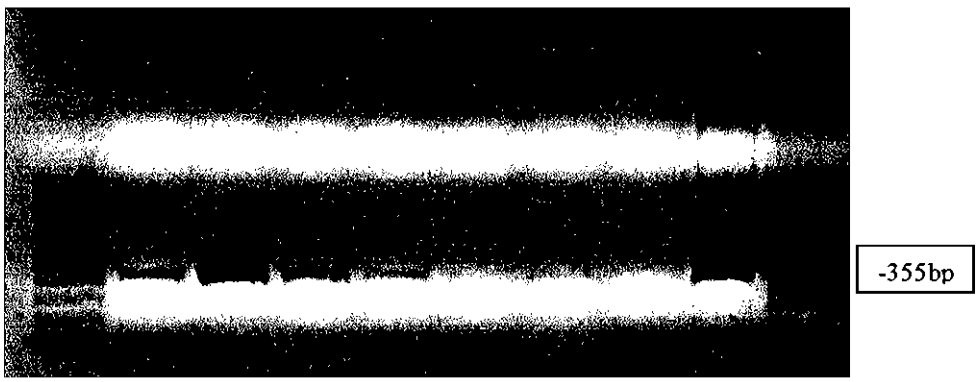

Fig 3.17. PCR analysis on *gfp* plants transformed with *ipt* (colour inverted) Internal *ipt* primers were used and a band of 355bp was expected. Lanes 1 and 10 contain a 100bp ladder. Lane 2, 3, 4, 5, 6 and 7 are the lines I3. I4, I5, I6, I7 and I8 respectively. Lane 8 is the negative control (H2O) and lane 9 is the positive control (the pVDH396 vector).

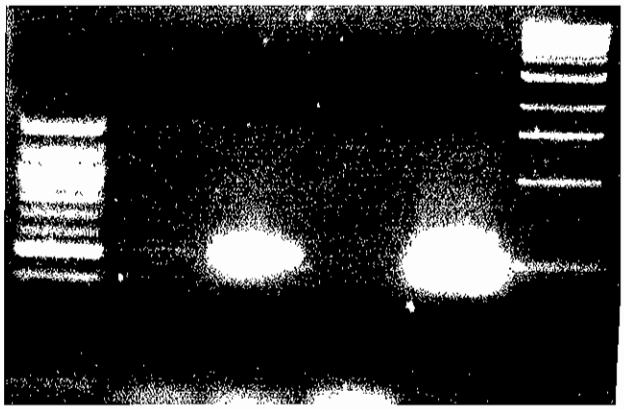

Fig 3.18. PCR analysis on *gfp* plants transformed with *ipt*. Internal *gfp*2 primers were used and a band of 510bp was expeeted. Lanes 1 and 7 eontain a 100bp and 1KB ladder respectively. Lane 2 is line I1, lane 3 is I2, lane 4 is the negative control (H2O) and lane 5 is the positive control (the pMSK18 vector).

3.2.2 Investigating GFP leakage in the generated transgenic plants

3.2.2.1 Protoplast isolation

Protoplasts were isolated from the transgenic plants in the hope of observing GFP fluorescing in the chloroplast. The protocol was optimized on the cv. Wisconsin $pSAG_{12}$: *ipt* / *gfp* plant tissue by varying the amount of tissue and volume of K3 protoplast islotaion medium used. It was also investigated if a K3 0.4 M sucrose solution containing macerozyme alone could be used to isolate protoplasts. The optimum conditions observed were 1 g leaf tissue in 20 ml K3 0.4 M sucrose solution containing macerozyme and cellulase.

No visible fluorescence was observed from protoplasts when placed under a UV microscope. When the filters on the microscope were adjusted, fluorescence was observed but this could also be seen in the wild type protoplasts. It was decided that the cv. Petit Havana *gfp* plants (provided by John Gray, Cambridge) were not a strong expresser of the *gfp* gene rendering it impossible to visually observe GFP leakage during senescence. Quantitative analysis of the GFP protein in leaf discs and their media was undertaken to investigate whether GFP leakage would be delayed in gfp plants containing $pSAG_{12}$: *ipt*

3.2.2.2 GFP leakage from detached leaf discs into medium

Nine Leaf discs (per plate) were floated on 30 ml H_2O and left in darkness in this experiment. GFP was quantified in the media (Fig. 3.19) and the leaf discs (Fig. 3.20)

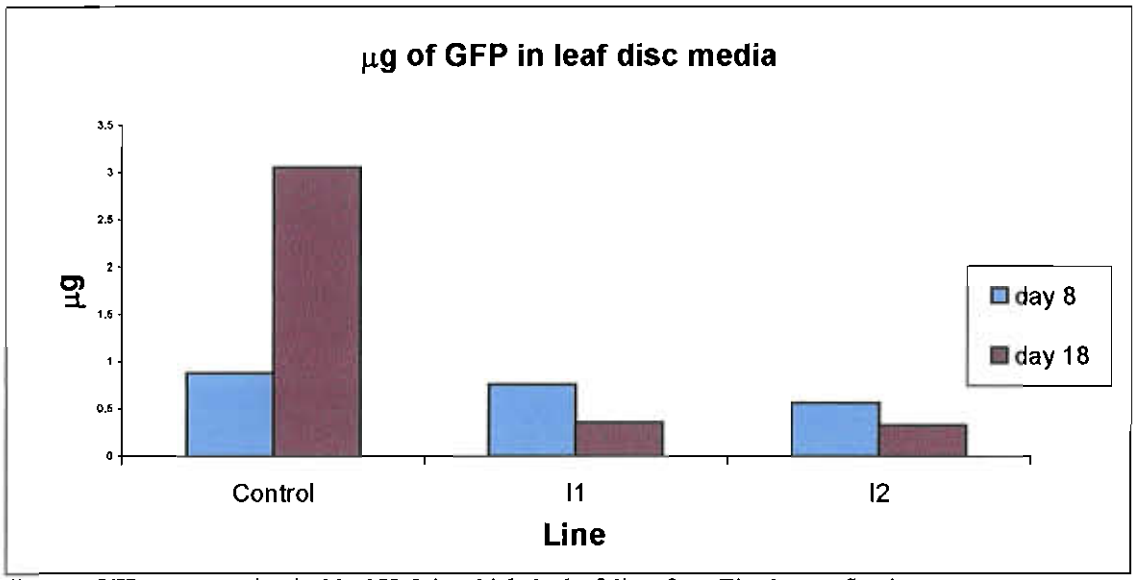

Fig. 3. .GFP concentration in 30ml H_2O in which the leaf discs from Fig. 3 were floating on.

Measurements were taken at day 0 and day 18. The control plant contains only the *gfp* gene.

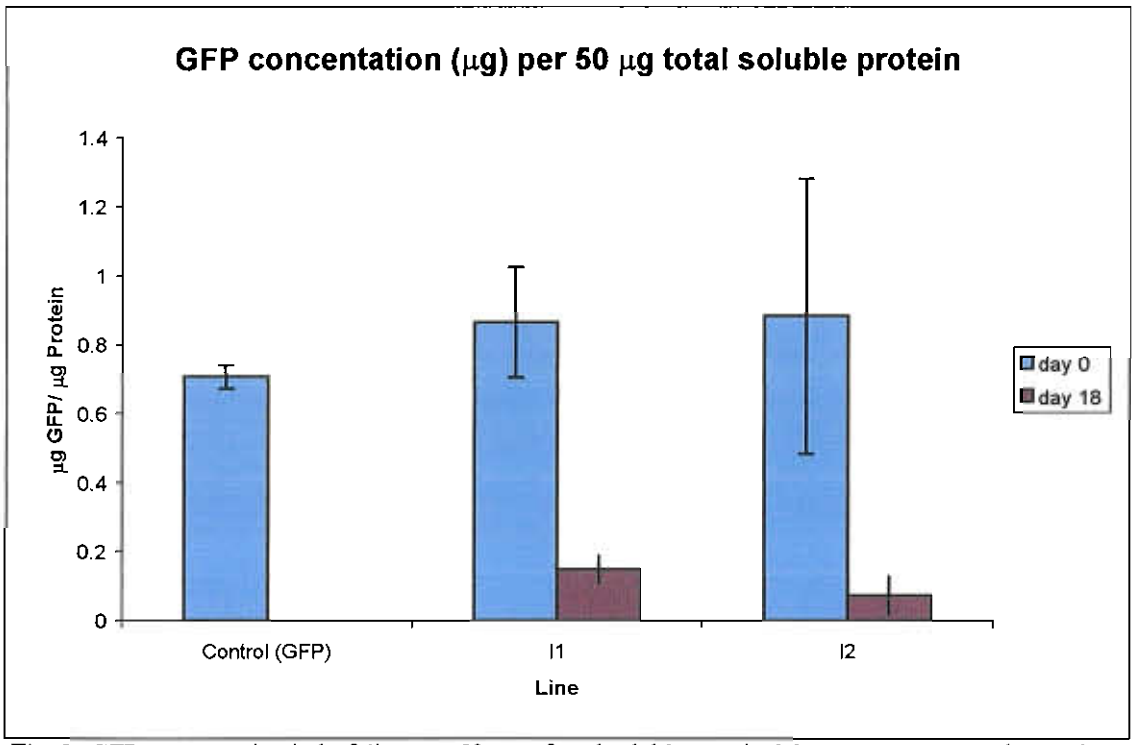

Fig. 3. .GFP concentration in leaf discs per 50 mg of total soluble protein. Measurements were taken at day 0 and day 18. The control plant contains only the *gfp* gene.

The concentration of GFP in the media increased from 0.8 to 3 μg in the control leaf discs. Less GFP was detected in the media of plants containing the $pSAG_{12}$: *ipt* cassette along with the *gfp* gene (Fig. 3.19). Simultaneously GFP was being measured in the leaf discs and at day 18 no GFP could be detected in the discs of the control plant, while low concentrations were still detectable in the wild type leaf discs (Fig. 3.20).

Chlorophyll analysis was carried out alongside this experiment and the results are shown in Fig.3.21. I1 behaved similarly to the control plant, in terms of chlorophyll retention. Line I2 contained a third more chlorophyll at the beginning of the experiment and appears to be more capable of chlorophyll retention.

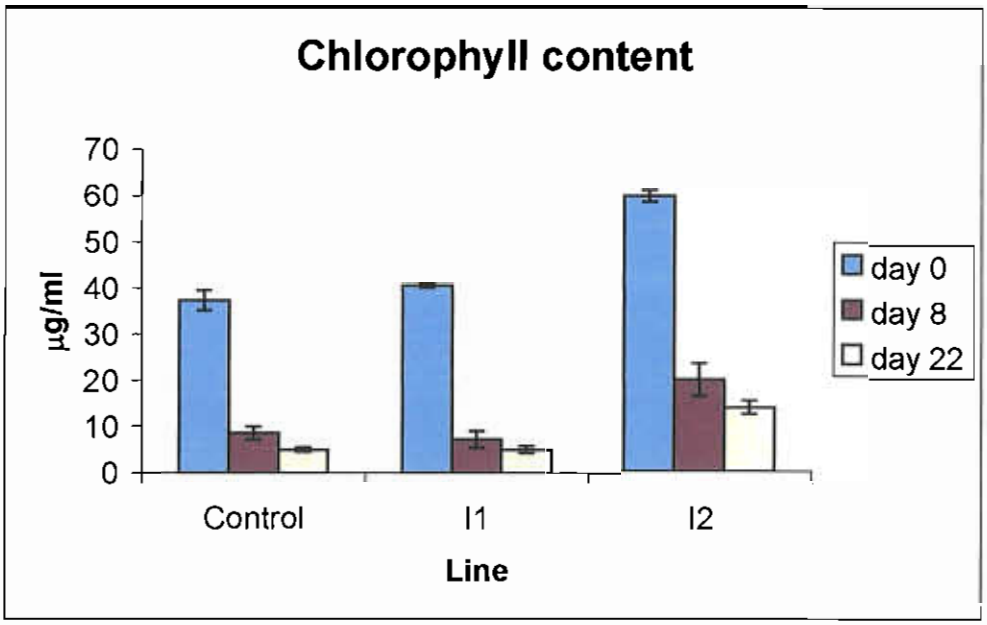

Fig. 3.21. Chlorophyll content (μgl^{-1}) in leaf discs from control plant, line I1 and I2. Chlorophyll content was measured 8 and 22 days after detachment.

Discussion

4.1 Characterisation of transgenic apple plants

The role of cytokinins in delaying the process of senescence in plants is well established and has received additional support in recent years through investigations with transgenic plants with modified cytokinin metabolism (Gan and Amasino, 1995; Chen *et al.*, 2001). These studies have mostly utilized annual species, such as tobacco (Gan and Amasino, 1995) and lettuce (McCabe *et al.*, 2001), although similar plants have also been partially characterized in the biennial crop, cauliflower (Eugene Kane, personal communication). To date, little is known about whether a similar role for cytokinins can be ascribed to a deciduous tree species, which exhibits simultaneous, as opposed to sequential, senescence. The cytokinin autoregulation system, first devised by Gan and Amasino (1995), in which a cytokinin synthesis gene (*ipt*), is placed under control of a senescence associated gene promoter (SAG12), and introduced into transgenic plants, would appear to be a useful tool to investigate this process. Such plants were previously produced with the apple cultivar Greensleeves, and provided the main source of material for the current study. The main purpose of this work was to explore, over several growing seasons whether the cytokinin autoregulation system is operational in apple trees, either at the level of leaf senescence and leaf fall in intact trees, or in the senescence process in detached leaves.

4.1.1 Morphological characterization of older transgenic apple plants

In November 2002, when wild type apple plants had lost, or partially lost, their leaves, 9 of the 23 transgenic plants had green leaves (Table 3.2). 6A(3) retained its green leaves the longest of the transgenic plants (Fig. 3.1). The following year also saw a number of

lines capable of retaining their leaves longer than the wild type. 6G(3) and 4B2(3) are two examples (Fig. 3.2). It would appear, from observation, that in some of transgenic plants, the pSAG$_{12}$:*ipt* cassette is in fact having an effect on senescence. It was decided to investigate what was occurring at a cellular level and this was achieved using various physiological assays. The height of greenhouse grown apple trees was also measured (Table 3.1) but no significant difference was found between the height of wild type and the transgenic plants.

4.1.2 Molecular characterisation of transgenic apple plants

Molecular characterisation using PCR for *ipt* was first carried out on all of the available pSAG$_{12}$:*ipt* lines, to ensure the presence of the pSAG$_{12}$:*ipt* cassette. Of the 27 lines tested, 18 were found to be positive in the presence of *ipt*. The 9 greenhouse grown lines were included in this 18 and were the lines used in subsequent assays. Fig. 3.3 is a representative of these PCR reactions and shows the presence of a 355bp band for the *in vivo* lines 14B, 37C and 47A, conferring the presence of *ipt*

4.1.3 Chlorophyll analysis

The chlorophyll content of leaves was measured by punching leaf discs from detached leaves and floating them on H_2O in darkness or light. The reasoning behind this is that model systems for the study of senescence are usually carried out on leaf segments left in the darkness (Quirino, 1999). Dark incubation is known to switch anabolism to catabolism unlike the subtle transition of metabolism in natural senescence (Smart, 1994). It was important to bear this in mind when interpreting the results and for this

reason assays was carried out in light and dark conditions. There are drawbacks in the use of detached leaves as a study model. The first being that the sinks for the export of broken down macromolecules are removed and secondly, it is most likely that the senescence observed is due to the stress of detachment rather than age (Becker and Apel, 1993). However, significant results can be yielded from detached leaf experiments, especially when comparing wild type leaves to those of transgenic plants already known to display a delay in senescence. It is also more feasible than only being able to carry out experiments once yearly (in the case of a deciduous species).

Chlorophyll assays were carried out in 2002 and 2004. In the 2002 assay, 13 of the 23 transgenic plants tested retained their chlorophyll longer than the wild type, some to a more significant extent than others (Figs.3.5 and 3.6). 4B2(1), 6A(3), 6D(1), 6D(3) and 6r were 4 of the better lines at chlorophyll retention each retaining 48%, 54%, 51%, 74% and 74% of their original chlorophyll content, respectively, compared to the 20%/32% retention of the wild type discs (Table 3.3). Chlorophyll loss is generally accepted to be a visual signal of the onset of senescence (van Staden *et al.*, 1988), so it would appear that the $pSAG_{12}$: *ipt* cassette is having a similar effect in a deciduous species as has been observed in several herbaceous species (Gan and Amasino, 1995; McCabe *et al.*, 2001) Jordi *et al.*, reported in 2000, a dramatic retention in chlorophyll in detached older leaves of $pSAG_{12}$: *ipt* tobacco plants.

In the 2004 assay, transgenic leaves showed an increase in chlorophyll retention capability (Figs. 3.7 and 3.8), perhaps because they were removed in the early summer as

opposed to the autumn picked leaves of the 2002 assay. The two wild type plants retained 56% and 74% of their original chlorophyll content 34 days after detachment. 9 out of 23 transgenic plants retained more than 56% and another 9 of the remaining plants retained more than 74%. The more capable plants, in terms of chlorophyll retention, were 6B(1), 6G(2), 6G(3), 15B(1), 4B2(3) and 2A3(3) (Table 3.3). A 4B2 plant also appeared in the highest scorers of the 2002 chlorophyll assay.

In the 2002 assay, leaves were removed from nodal positions 1, 5 and 9 (with preference for younger leaves but older leaves being sufficient in their absence) and this is most likely the reason for the bigger variance in individual results than in the 2004 assay (where leaf age was the main criteria regardless of nodal positions). The younger leaves of the 2004 assay contained more chlorophyll than the older leaves of 2002 (Figs. 3.5, 3.6, 3.7 and 3.8). In 2004, wild type leaves contained more chlorophyll, in general, that the transgenic ones while wild type leaves in 2002 generally had less chlorophyll at Day 0 than the transgenic leaves (Figs. 3.7, 3.8, 3.9 and 3.10). It may be that younger $pSAG_{12}$: *ipt* leaves contain lower chlorophyll concentration than the wild type (Fig. 3.7 and 3.8) and as wild type chlorophyll concentration decreases as the leaf ages, levels are maintained in the transgenic leaves. It could also be that the transgene is losing its effect over growth seasons (Herschbach and Kopriva, 2002). Further studies, outside of the scope of the current project, could be an investigation into this, in the form of a repeated experiment in the autumn to see if the results are similar to those found for 2002.

4.1.4 Total soluble protein measurement

Protein levels are decreased during senescence, as molecules are broken down and transported out of the senescing leaf (Quirino, 1999). Jordi *et al.*, reported in 2000 that elevated *ipt* expression in the leaves of tobacco plants was less effective in delaying protein degradation than chlorophyll loss. Total soluble protein levels measurements were made, to further investigate the progress of the senescence process. Measurements were made on younger leaves in August 2003 (Fig. 3.9). The time frame of the experiment did not allow for any conclusive results and total soluble protein content was maintained at a fairly constant level during the experiment.

However, when this experiment was repeated on detached young leaves of all the greenhouse plants in May 2004, several lines exhibited a delay in the protein degradation normally associated with senescence (Table 3.4). In this experiment, wild type plants retained 56% and 35% of their original protein content 15 days after detachment, while plants 6D(2), 6D(3), 2A3(3) retained 99%, 95% and 80%, respectively, in comparison. The transgenic plant 2A3(3) also retained 81% of its original protein content 34 days after detachment in the parallel chlorophyll assay carried out (Table 3.3). The leaves used in the assay in August 2003 were younger than those of the 2004 assay, this may be a reason for the decline in protein levels after 15 days in the 2004 assay.

4.1.5 Ascorbate peroxidase activity

Ascorbate peroxidase activity is known to increase in plant cells during stress (He and Gan, 2002). As senescence in detached leaves will be stress related, it was decided an appropriate experiment to measure ascorbate peroxidase activity in detached leaves since senescence of the leaves will be stress related. In 2003, ascorbate peroxidase activity was measured in 0.3 g segments of younger leaves and was fairly constant in the time frame of the experiment (Fig. 3.10). After 5 readings and at day 24 post detachment, ascorbate peroxidase levels were similar to the original values. This experiment was repeated on all detached young leaves of all transgenic and wild type greenhouse grown apple plants (75 plants in total) in May 2004. Ascorbate peroxidase levels were lower since 0.3g segments of tissue were removed immediately before each reading. In the 2003 assay, 0.3g segments were floated on H_2O, the wounding assumingly contributing to the higher ascorbate peroxidase activity (ascorbate peroxidase activity ranging from 177 to 401 μMm^{-1} in the 2004 assay (Table 3.5) compared to 1.32 to 2.06 mMm^{-1} in 2004 (Fig. 3.10).

In 2004, values for Ascorbate peroxidase activity were taken at day 0 and day 15 (Table 3.5). After 15 days, the values with smaller standard error bars tend to be lower that the values at day 0. It is difficult to conclude anything from this data since there appears to be no consistency throughout the values and a significant proportion of these values have very large error bars. This is possibly due to the large sample number of this particular experiment. The experiment might well yield interesting results there was a smaller

sample number containing only those transgenic lines showing an ability for high chlorophyll and total soluble protein retention.

4.2 Investigating GFP leakage in *gfp* transformed plant cells exhibiting delayed senescence

A parallel investigation was included, a novel experimental system, using the model species, tobacco, to further probe the cellular events taking place during the senescence process. Transgenic plants containing autoregulated *ipt* in the nucleus and *gfp* in the chloroplast were generated in order to study GFP leakage from the chloroplast during senescence. Since the cytoplasm and chloroplast contain different proteases, certain proteins may be more suited to the chloroplast environment (Bogorad, 2000). A recombinant protein may find the chloroplast a more favourable environment, making chloroplast transformation more preferable than nuclear transformation. Since the chloroplast is known to be one of the first organelles to be dismantled during senescence, a chloroplast transformant containing the $pSAG_{12}$: *ipt* cassette will have obvious advantages over those without it. Other advantages of chloroplast transformation include the fact that DNA can be directed to a specific chromosomal site unlike nuclear transformation where random integration into the genome is observed. Random integration is problematic since the location at which a foreign gene is placed on a chromosome can affect how strongly it is expressed. Uniform results in expression studies of transgenic plants are obviously more preferable (Bogorad, 2000; Pena and Seguin, 2001). Another merit is that the transferal of the transgene to nearby growing

relatives can be avoided, since chloroplasts of angiosperms are generally only maternally transmitted (Bogorad, 2000).

4.2.1 Generation of tobacco plants containing autoregulated *ipt* in the nucleus and *gfp* in the chloroplast

Since chloroplast transformants occur at a much lower frequency than nuclear transformants (Svab *et al.*, 1990), nuclear (*ipt*) transformation carried out alongside chloroplast (*gfp*) transfomantion. *N. tabacum* Petit Havana *gfp* (chloroplast transformed) plants (John Gray, Cambridge) were subjected to *Agrobacterium*-mediated transformation using the vector pVDH396 (Fig. 2.1), resulting in 10 nuclear *ipt* transformants (named I1- I10). *N. tabacum* Wisconsin $pSAG_{12}$:*ipt* (nuclear transformed) plants (Gan and Amasino, University of Wisconsin) plants were subjected to biolistic transformation using the vector pZS:*gfp* (Fig. 2.3.), resulting in 2 *gfp* chloroplast transformants. Nuclear transformants were obtained first and lines I1, I2 and a control plant (containing only *gfp*) were potted up.

4.2.2 Quantitative GFP analysis

Leaf discs from lines I1, I2 and a control plant were floated on H_2O and left in darkness (3.2.2.2). GFP measurements were made on leaf discs and the media to determine the level of containment of the recombinant protein. In leaf disc measurements at Day 0, GFP concentration was equivalent to 1.6 % of the total soluble protein concentration. Newell *et al.* reported, in 2003, a 0.06 % of total soluble protein concentration in plants transformed with the same vector, pMSK18. GFP could not be detected in the control

discs 18 days after detachment while small quantities were still detectable (Fig. 3.), suggesting that recombinant protein leakage is more likely in the control plant than those exhibiting delayed senescence. Strengthening this is the fact that there were high levels of GFP detected in the media of the control plant at Day 18, compared to the low levels detected in the media of the $pSAG_{12}$:*ipt* plants, I1 and I2 (Fig 3.).

Chlorophyll analysis was also carried out on detached leaf discs of I1, I2 and a control plant left floating on H_2O (Fig. 3.). Line I1 ($pSAG_{12}$: *ipt*) has similar levels of chlorophyll to the control plant (*gfp*). There is also no significant difference in chlorophyll retention. Chlorophyll levels were significantly higher (78.2 μgml^{-1} compared to 28.7 μgml^{-1} in the control) in Line I2 and levels are being maintained for longer (13.7 μgml^{-1} at Day 18 compared to 4.9 μgml^{-1} in the control), indicating that the $pSAG_{12}$: *ipt* cassette appears to be functioning properly in the line I2, since studies have shown that elevated endogenous expression of *ipt* has shown an increased biosynthesis of chlorophyll in transgenic tobacco plants (Sa *et al.*, 2000).

References

Conclusions

pSAG$_{12}$:*ipt* has been shown to delay senescence in several herbaceous species. This study investigated if the cassette had a similar effect on a deciduous species, apple. Several physiological assays were carried out and various pSAG$_{12}$:*ipt* lines demonstrated ability for chlorophyll and protein retention longer than that of the wild type plants. During the autumn of two years, transgenic apple plants also retained their leaves longer than the wild type. A parallel study was the investigation of recombinant protein leakage from the chloroplast during senescence. Plants transformed with pSAG$_{12}$:*ipt* demonstrated an increased ability to contain the recombinant protein in the chloroplast longer than the wild type plant, offering an obvious advantage for the production of chloroplast transformants.

Armitage P, Walden R and Draper J (1988). Vectors for the transformation of plant cells using *Agrobacterium*. In: Plant Genetic Transformation and Gene Expression. A Laboratory Manual. (Draper J, Scott R, Armitage P and Walden R. Eds), Blackwell Scientific Publication, NY.

Asada K (1997). The role of ascorbate peroxidase and monodehydroascorbate reductase in H_2O_2 scavenging in plants. In: Oxidative Stress and the Molecular Biology of Antioxidant Defenses, Cold Spring Harbor Laboratory Press, NY, pp. 715- 735

Blumenthal A, Kuznetzova L, Edelbaum O, Raskin V, Levy M and Sela I (1999). Measurement of green fluorescence protein in plants: quantification, correlation to expression, rapid screening and differential gene expression. *Plant Science* 142: 93- 99

Bogorad L (2000). Engineering chloroplasts: an alternative site for foreign genes, proteins, reactions and products. *Tibtech* 18: 257- 263

Borlaug NE (1997). Feeding a world of 10 billion people: the miracle ahead. *Plant Tissue Culture and Biotechnology* 3: 119- 127

Botella JR (2000). Biotechnological approaches to control postharvest problems. In Quality Assurance in Agricultural Produce. (Johnson GI, To LV, Duc ND and Webb MC, Eds), ACIAR Proceedings 100

Buchanan-Wollaston V, Earl S, Harrison E, Mathas E, Navabpour S, Page S and Pink D (2003). The molecular analysis of leaf senescence- a genomics approach. *Plant Biotechnology Journal* 1: 3-22

Casper SJ and Holt CA (1996). Expression of the green fluorescent protein- encoding gene from a tobacco mosaic virus- based vector. *Gene* 173: 69- 73

Chen LFO, Hwang JY, Charng YY, Sun CW and Yang SF (2001). Transformation of broccoli (*Brassica oleracea* var. *italica*) with isopentenyltransferase gene via *Agrobacteriom tumefaciens* for post- harvest yellowing retardation. *Molecular Breeding* 7: 243- 257

De Mesa MC, Jimenez- Bermudez S, Pliego- Alfaro F, Quesada MA and Mercado JA (2000). *Agrobacterium* cells as microprojectile coating: a novel approach to enhance stable transformation rates in strawberry. *Australian Journal of Plant Physiology* 27: 1093- 1100

Dellaporta SL, Wood J and Hicks (1983). A plant DNA minipreparation. *Plant Molecular Biology Reporter* 1: 19-21

Field TS, Lee DW and Holbrook NM (2001). Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of Red- Osier dogwood. *Plant Physiology* 127: 566-574

Finer JJ, KR Finer, T Ponappa (1999). Particle bombardment-mediated transformation. In: Current Topics in Microbiology and Immunology, "Plant Biotechnology: New Products and Applications" (J Hammond, PB McGarvey, V Yusibov Eds.), Springer-Verlag, Heidelberg 240: 59-80

Frey JE (1999). Genetic flexibility of plant chloroplasts. *Nature* 398: 115-116

Fridovich I (1997). Oxygen toxicity: a radical explanation. *Journal of Experimental Biology* 201: 1203- 1209

Gan S and Amasino RM (1995). Inhibition of leaf senescence by autoregulated production of cytokinin. *Science* 270: 1986-1988.

Gan S and Amasino RM (1997). Making sense of senescence. *Plant Physiology* 113: 313-319.

Gasser CS and Fraley RT (1992). Transgenic crops. *Scientific American* 266: 62-69

Ghosh S, Mahoney SR, Penterman JN, Peirson D and Dumbdroff EB (2001). Ultrastructural and biochemical changes in chloroplasts during *Brassica napus* senescemce. *Plant Phyiology and Biochemistry* 39: 777- 784

Harker FR, Gunson FA and Jaeger SR (2003). The case for fruit quality: an interpretive review of customer attitudes, and preferences for apples. *Postharvest Biology and Technology* 28: 333- 347

Hedden P and Phillips AL (2000). Manipulation of hormone biosynthetic genes in transgenic plants. *Current Opinion in Biotechnology* 11: 130-137

Hershbach C and Kopriva S (2002). Transgenic trees as tools in tree and plant physiology. *Trees* 16: 250- 261

Hibberd JM, Linley PJ, Khan MS and Gray JC (1998). Transient expression of green fluorescent protein in various plastid types following microprojectile bombardment. *The Plant Journal* 16: 627- 632

Hurng WP and Kao CH (1994). Lipid peroxidation and antioxidative enzymes in senescing tobacco leaves during post flooding. *Plant Science* 96: 41- 44

Jordi W, Schapendonk A, Davelaar E, Stoopen GM, Pot CS, de Visser R, van Rhijn JA, Gan S and Amasino RM (2000). Increased cytokinin levels in transgenic $pSAG_{12}$:*ipt* tobacco plants have large direct and indirect effects on leaf senescence, photosynthesis and N partitioning. *Plant Cell and Environment* 23: 279- 289

Matile, P (1982). Chloroplast senescence. In: Crop Photosynthesis: Spatial and Temporal Determinants (Baker N and Thomas H, Eds), Amsterdam: Elsevier Science, pp. 413- 440

McCabe MS, Garratt LC, Schepers F, Jordi WJRM, Stoopen GM, Davelaar E, van Rhijn JHA, Power JB and Davey MR (2001). Effect of PSAG12-IPT gene expression on development and senescence in transgenic lettuce. *Plant Physiology* 127: 505-516

McCabe MS, Power JB, de Laat AMM and Davey MR (1997). Detection of single-copy genes in DNA from transgenic plants by nonradioactive southern blot analysis. *Molecular Biotechnology* 7: 79-84.

Miller CO, Skoog F, von Saltza MH and Strong M (1955). Kinetin, a cell division factor from deoxyribonucleic acid. *Journal of Ameriacan Chemical Society* 77: 1329- 1324

Mok DWS and Mok MC (2001). Cytokinin metabolism and action. *Annual Review of Plant Physiology. Plant Molecular Biology* 52: 89- 118

Noh YS and Amasino RM (1999). Identification of a promoter region responsible for the senescence- specific expression of SAG12. *Plant Molecular Biology* 41: 181-194

Nooden LD (1988). The phenomenon of senescence and aging. In: Senescence and Aging in Plants, (Nooden LN and Leopold AC, Eds.) San Diego, CA: Academic Press, pp. 1- 50

Newell CA, Birch-Machin I, Hibberd JM and Gray JC (2003). Expression of green fluorescent protein from bacterial and plastid promoters in tobacco chloroplasts. *Transgenic Research* 1205: 1-4

Pandi- Perumal SR, Seils LK, Kayumov, Ralph MR, Lowe A, Moller H and Swaab DF (2002). Senescence, sleep, and circadian rhythms. *Ageing Research Reviews* 1: 559-604

Pena L and Seguin A (2001). Recent advances in the genetic transformation of trees. *Trends in Biotechnology* 19: 500 – 506

Pennell RI and Lamb C (1997). Programmed cell death in plants. *The Plant Cell* 9: 1157-1168

Puite KJ, Schaart JG (1996). Genetic modification of the commercial apple cultivars Gala, Golden Delicious and Elstar via an *Agrobacterium tumefaciens*- mediated transformation method. *Plant Science* 119: 125- 133

Quirino BF, Noh YS, Himelblau E and Amasino RM (2000). Molecular aspects of leaf senescence. *Trends in Plant Science* 5: 278-282

Quirino BF, Normanly J and Amasino RM (1999). Diverse range of gene activity during *Arabidopsis thaliana* leaf senescence includes pathogen- independent induction of defense- related genes. *Plant Molecular Biology* 40: 267- 278

Sa G, Mi M, He- Chun Y, Ben- ye L, Guo- feng L and Kang C (2001). Expression of *ipt* gene expression on the physiological and chemical characteristics of *Artemisia annua* L. *Plant Science* 691- 698

Shi WM, Muramoto Y, Ueda A and Takabe T (2001). Cloning of peroxisomal ascorbate peroxidase gene from barley and enhanced thermotolerance by overexpressing in *Arabidopsis thaliana*. *Gene* 273: 23- 27

Smart CM, Scofield SR, Bevan MW and Dyer TA (1991). Delayed leaf senescence in tobacco plants transformed with tmr, a gene for cytokinin production in *Agrobacterium*. *The Plant Cell* 3: 647-656.

Svab Z and Maliga P (1993). High- frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proceedings of National Academic Science USA* 90: 913- 917

Svab Z, Hajdukiewicz P and Malaga P (1990). Stable transformation of plastids in higher plants. *Proceedings of National Academic Science USA* 87: 8526- 8530

Thomas H (2002). Ageing in plants. *Mechanisms of ageing and development* 123: 747- 753