INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

DISSERTATION

Cytokinin Regulation During Flower Senescence in *Petunia x hybrida* 'V26' Transformed With SAG12-*ipt*

Submitted by

Hsiang Chang

Department of Horticulture and Landscape Architecture

In partial fulfillment of the requirements
For the Degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
Fall, 2002

COLORADO STATE UNIVERSITY

November 4, 2002

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY HSIANG CHANG ENTITLED CYTOKININ REGULATION DURING FLOWER SENESCENCE IN PETUNIA X HYBRIDA 'V26' TRANSFORMED WITH SAG12-IPT BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate work

Advisor

Co-Advisor

Department Head

ABSTRACT OF DISSERTATION

CYTOKININ REGULATION DURING FLOWER SENESCENCE IN *PETUNIA X HYBRIDA* 'V26' TRANSFORMED WITH SAG12-*IPT*

Previous studies have shown that either exogenous application or increased endogenous production of cytokinins delays senescence in plants. Treatment of petunia flowers with trans-zeatin and trans-zeatin riboside significantly increased flower longevity. Elevated endogenous cytokinin levels have been accomplished by the introduction of the *ipt* gene from Agrobacterium into plants, and leaves of plants transformed with SAG12-*ipt* exhibit delayed senescence. In this study, *Petunia x hybrida* 'V26' transformed with SAG12-*ipt* were used to investigate the role of cytokinins in flower senescence. Two independently transformed lines of petunia have been identified based on increased flower longevity (IPT22 and IPT34). IPT22 and IPT34 flowers exhibited delayed senescence following pollination and during natural senescence, lasting 6 to 9 days longer than non-transgenic *Petunia x hybrida* 'V26' wild type (WT). The presence of the *ipt* construct in transgenic lines was confirmed by PCR. *Ipt* transcripts were detected in corollas by RT-PCR in transgenic lines but not wild type. The quantification of *ipt* expression was accessed by real time RT-PCR. Higher endogenous cytokinin levels were detected in IPT22 and IPT34

corollas compared to corollas from WT. The elevated cytokinin levels resulted in altered ethylene biosynthesis in SAG12-*ipt* petunia corollas compared to WT. SAG12-*ipt* flowers also showed less sensitivity to exogenous ethylene application and required higher concentrations of ethylene to induce wilting and autocatalytic ethylene production. The expression of the senescence-related gene, *Phcp1*, in SAG12-*ipt* verses wild type was also investigated, the result was not cytokinin down-regulated as previous study.

Hsiang Chang
Department of Horticulture
and Landscape Architecture
Colorado State University
Fort Collins, CO 80523
Fall, 2002

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my academic supervisor, Dr. Michelle L. Jones, who offered me the opportunity to work in her laboratory with her advice, encouragement, and great help throughout my graduate study program. I also thank the members of my doctoral committee, Dr. Carol A. Ishimaru, Dr. Leigh E. Towill, and Dr. Stephen J. Wallner, for their discussion and carefully review of research proposal and this manuscript. In addition, special thanks to the people in the Ohio Agricultural Research Center / Ohio State University for their assistance during my study in Ohio. Brennic J. Langston, Joyce C. P., and Sarah J. Negley were patient, helpful, and fun to work with.

I would also like to express my appreciation to Dr. David G. Clark, University of Florida, for providing plant material, Dr. Gary M. Banowetz, University of Oregon-ARS for assisting with cytokinin and ABA measurements, and Dr. Troy Chen for statistical analysis.

I thank my parents for their support. I also owe Aki Tawa and Kumiko Yokoyama a debt of gratitude with regard to my project. I wish to express my gratitude to

Lawrence Wei-Chun Lo and Travis S. Walker whose care and inspiration have always been with me through this study. Finally, deeply appreciate to my lovely puppies, Sumi Chang and Hime Chang, for their being with me through every tough and happy moment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Literature Review

1.1 Introduction

Senescence is defined as those processes that follow physiological maturity and lead to the death of a whole plant, organ, tissue, or cell (Watada *et al.*, 1984). The process in polycarpic plants is very different than that of monocarpic plants. The senescence of polycarpic plants can only occurs in reproductive tissues, while the rest of the plant continues to develop. Although senescence is the very last phase of the development, it is still very valuable to study it for academic and economic purposes.

For the study of senescence, flowers are excellent models. Flower petals are often the plant organ with the shortest life span, and as such provide a useful tissue for studying the mechanisms underlying control of senescence (Lawton *et al.*, 1989). Floral senescence is generally rapid, and the symptoms are easy to observe. Petals are relatively short lived, and this affects the commercial value of the flowers. Therefore, research in flower senescence will not only provide information which will increase post-harvest flower longevity, but will also provide knowledge of the physiological and biochemical events underlying the control of petal senescence (Borochov and Woodson, 1989).

Flower senescence is a complex process and has been intensively studied based on morphological changes, physiological events, biochemical reactions, and further at the molecular level. Studies have confirmed that senescence is hormone regulated and dependent on mRNA and protein synthesis (Reid and Wu, 1992). Physical changes in the cell membranes leading to the loss of electrolytes and water from the flower petals (Borochov and Woodson, 1989) are involved in senescence. Pollination accelerates senescence in some flowers (Stead, 1992). Pollination-induced senescence may be manifested differently in different flowers, causing wilting and fading in carnation and petunia, promotion of anthocyanin synthesis in *Cymbidium* (Arditti and Flick, 1976), or abscission in sweet peas, snapdragon, geranium (Wallner *et al.*, 1979), *Digitalis* (Stead and Moore, 1979, 1983), and *Linum* (Addicott, 1982; Halevy and Mayak, 1981). Flower senescence does not happen randomly, the onset and the process are genetically regulated.

Senescence is a highly controlled sequence of events comprising the final stage of development. Cells remain viable and show tight metabolic regulation until the end of senescence. This regulation requires new gene expression (Smart, 1994). Accidental death occurs through necrosis and its symptoms are markedly different from those of senescence, which is considered a form of programmed cell death. Senescence is also different from aging. Medawar (1957) provided a convenient distinction by defining aging as those changes that occur with time, without reference to death as a consequence, while senescence always ends in death. In Xu and Hanson (2000), plant senescence is also addressed as a type of programmed cell death (PCD). One important function of plant PCD is to remobilize metabolites from dying organs to developing organs, this

benefits the plant on the whole level (Smart, 1994). From this concept, the corolla and style become valueless after compatible pollination, while the ovary still has its fate to complete reproduction. During flower senescence, PCD is occurring differently among flower organs with nutrients relocated from the styles and corolla to the developing ovary.

Senescence in plants exhibits some similarity to programmed cell death in animals, which has been more intensively studied. Examples and results from animal research have directed recent plant studies in this area. One important characteristic of PCD is DNA fragmentation that has been documented both in apoptotic animal cells (Mittler and Lam, 1995; O'Brien *et al.*, 1998) and senescing flowers (Xu and Hanson, 2000). Despite this observation there is little evidence that the molecular and biochemical events responsible for the altered morphology and/or cell death are identical in plant and animal systems, because the plant cells are surrounded by complex cell walls and containing large central vacuoles that contribute unique mechanisms for PCD in plants (Rubinstein, 2000).

In the termination of flowers, there are two mechanisms. One is petals abscise even before the majority of their cells initiate a cell death program (Clark *et al.*, 1997), so the abscission may occur before or during the mobilization of nutrients to other parts of the plant. In the other case, petals are more persistent, their cells deterioration and nutrient remobilization occurs while petals are still part of the flower (Leshem *et al.*, 1986; Stead and van Doorn, 1994). The flower senescence is accomplished by increased activity of many hydrolytic enzymes that degrade macromolecules and enable the

remobilization of nutrients from petals to the developing ovary (Baumgartner *et al.*, 1975). Other biochemical and molecular changes occur well before the remobilization of nutrients is evident (Lesham *et al.*, 1986; Thompson *et al.*, 1997). Decreased membrane fluidity from carnation petals was observed, and occured before visual symptoms of senescence were obvious (Thompson *et al.*, 1997). Differential permeability induced by lipoxygenase activity and reactive oxygen species (ROS) was also observed during the programmed cell death of daylily petals (Panavas and Rubinstein, 1998).

Among PCD events, protein degradation is a common feature during organ senescence (Smart, 1994, Callis, 1995). To achieve this goal, proteolysis plays a role in nitrogen (amino acid) remobilization during senescence. The breakdown amino acids of these proteins are then transported from the senescing organ to other parts of the plant. Upon the anthesis of daylily flowers, the corolla changes dramatically from a sink to a source for transported materials, and carbohydrates and amino acids continue to be exported many hours after the flower has wilted (Bieleski, 1995). In plant cells, almost all of the proteins are degraded by the proteasome or proteases in the vacuole. Four major classes of proteases: cysteine, serine, aspartic acid and metalloproteases exist in plant cells. Among them, serine and cysteine proteases are localized in the vacuole. Cysteine proteases (cysteine endopeptidases) play an important role in PCD (Solomon *et al.*, 1999) and have been widely studied with about 60 cysteine protease genes isolated from plants to date (Ueda *et al.*, 2000). Cysteine protease mRNAs accumulated during various developmental stages such as seed germination in chickpea (Cervantes *et al.*, 1994), anther dehiscence (Koltunow *et al.*, 1990), and senescence of various organs (Drake *et al.*,

1996; Cercos *et al.*, 1999). In addition to increased degradation of existing proteins, protein synthesis may also begin to decline after anthesis (Woodson and Handa, 1987). However, the results from separation of petal proteins reveal a decrease in higher-molecular-weight proteins while lower-molecular-weight proteins increase (Woodson and Handa, 1987; Courtney *et al.*, 1994). The decreased levels in daylily petals appear to be in both membrane-bound and soluble fraction proteins (Stephenson and Rubinstein, 1998), and in carnation petals are primarily in the chloroplast (Mayak *et al.*, 1998).

Since cellular proteins are degraded in PCD, this suggests that a range of various proteinases may be activated simultaneously at multiple sites in the cell during PCD. An increase in activity of several classes of proteinases is detected using different inhibitors and substrates as criteria, and there are numerous proteinase isoforms within each class (Stephenson and Rubinstein, 1998). Stephenson and Rubinstein (1998) also suggested that the proteinases are newly translated during senescence and/or they are activated by proteins that are newly translated.

Many proteinase genes have been identified as senescence associated genes. The evidence of the involvement of proteinase genes in PCD is that several senescence-induced cDNAs have been sequenced and identified with homology to proteinase genes. For example, Jones *et al.* (1995) cloned a cysteine proteinase from carnation by amplifying a specific cDNA through PCR. RNA gel blot analyses indicated that increases in transcript abundance corresponded to pollination-induced ethylene production that leads to senescence. The transcription level was inducible and elevated by exogenous

ethylene treatments and was inhibited by NBD (2,5-norbornadiene), which is an ethylene inhibitor. In daylily petals, two cDNAs also showed a strong homology to cysteine proteinases (Guerrero *et al.*, 1998).

1.2 Corolla senescence and ethylene

The plant hormone ethylene is thought to play a primarily role in the regulation of senescence (Abeles *et al.*, 1992). The increased level of ethylene serves to coordinate the biochemical processes of petal senescence, including the transcriptional activation of several senescence-related genes (Lawton *et al.*, 1990; Woodson *et al.*, 1993). Its gaseous nature makes it unique as a plant hormone. Gases can be ideal messengers for physiological control mechanisms because of their ability to diffuse freely through cell membranes and the cytoplasm to reach receptor(s) (Abeles *et al.*, 1992). This is especially important in plant systems because plants do not have a centralized circulation system to transport hormones like that of animals. The other advantage of a gaseous hormone is after reaching the receptors and initiating a response, it is not necessary to metabolitically inactivate the molecule to cancel the signal and reset the system. The signal can be inactivated merely by stopping the production of the hormone. The hormone will then diffuse out of the tissue (Abeles *et al.*, 1992) and eventually reach non-active concentrations. Ethylene can also be biologically degraded in plant tissue (Beyer and Blomstrom, 1980).

Ethylene action requires ethylene binding to a receptor and the subsequent transduction of the signal to result in different physiological responses (Van Der Straeten

and Van Montagu, 1990). The ethylene response is either a concentration response and/or a sensitivity response. In the first case, the plant reacts to a change in endogenous ethylene production (concentration) while the sensitivity of tissues can be altered in the course of time (of development stage) in the second case. Both of these cases will be discussed further in later sections.

Increased ethylene production has been detected from senescing flowers and this elevated ethylene is required to mediate the senescence process (Nichols, 1966; Wang and Woodson, 1989). Halevy (1986b) suggested that flowers are classified into two groups-climacteric or non-climacteric, based on the presence of absence of an increased ethylene production associated with petal senescence. In carnations, petal senescence is associated with a climacteric-like increase in ethylene (Woodson, 1994). Before senescence, ethylene production is limited by low activities of both 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase, which convert AdoMet to ACC and ACC to ethylene respectively (Woodson *et al.*, 1992).

To further characterize ethylene patterns production can be described as system I and system II ethylene. System I ethylene represents the basal level of ethylene production that precedes the induction of senescence, whereas the increased ethylene produced during senescence is system II ethylene (Kende, 1993; Yang and Hoffman, 1984). In the system II, ethylene is autocatalytic in nature. The transition from system I to system II is characterized by the increases in the abundance of mRNAs encoding ACC synthase and ACC oxidase and concomitant increases in the activity of both enzymes (Park *et al.*, 1992;

Woodson *et al.*, 1992). Moreover, application of 2,5-norbornadiene inhibited ethylene production through inhibiting the activities of both ACC synthase and ACC oxidase (Wang and Woodson, 1989), and corresponding mRNA's synthesis (Woodson *et al*, 1992). Thus, expression of system II ethylene is regulated by ethylene.

In many species, pollination hastens the normal pattern of petal senescence that leads to premature petal senescence (Nichols, 1977). Thus, an intact corolla is no longer needed to attract pollinators after successful pollination. Nichols *et al.* (1983) demonstrated that the initiation of the change occurs because of the elevated ethylene production by pollination, and these increases in ethylene were detected from styles, ovaries, receptacles, as well as petals. Pollination induced corolla senescence was observed in petunia (Gilissen *et al.*, 1984; Botha and Whitehead, 1992; Singh *et al.*, 1992a) and in carnation (Larsen *et al.*, 1995). Ethylene production from styles was observed at 2, 12, 24 HACP with petals starting to senescence after 48 HACP (Larsen *et al.*, 1995). The application of NBD (2,5-norbornadiene), an ethylene inhibitor, to pollinated carnation flowers delayed pollination accelerated flower senescence (Jones and Woodson, 1997), further confirming the role of ethylene in regulating flower senescence

In addition to pollination, exogenous application of ethylene also accelerates flower senescence (Uota, 1969; Barden and Hanan, 1972; Mayak and Kofranek, 1976; Whitehead and Vasiljevic, 1993), and the application of ethylene inhibitors delays the onset of petal senescence in several plants (Mor and Reid, 1981; Woodson *et al.*, 1985; Knee, 1995; Knee, 1996). The application of 1-MCP (1-methylcyclopropene, formerly

designated as SIS-X, a cyclic ethylene analog) to *Petunia hybrida* L. 'Pink Cascade' proved ethylene is involved in the regulation of the early stages of senescence (Serek *et al.*, 1995b). 1-MCP applications prolonged vase life of ethylene-sensitive cut flowers of carnation and Alstroemeria (Serek *et al.*, 1995a). Silver ions also inhibit ethylene action, delaying corolla senescence and retarding chlorophyll loss in leaves (Aharoni *et al.*, 1979). Treatments with 1-MCP were as effective in inhibiting ethylene effects as treatment with the anionic silver thiosulfate complex (STS), the standard commercial treatment in cut flowers.

A molecular approach to confirm ethylene role is to investigate *Arabidopsis* mutant that are insensitive to ethylene (Bleecker *et al.*, 1988). A mutant gene *etr1-1* was identified which acted in a dominant fashion to confer ethylene insensitivity (Chang *et al.*, 1993). Wilkinson *et al.* (1997) transformed tomato, tobacco, and petunia with *etr1-1* gene and successfully conferred ethylene insensitivity. Petunia transformed with the *Arabidopsis etr1-1* gene linked to the cauliflower mosaic virus 35S promoter (CaMV35S) exhibited delayed senescence (Gubrium *et al.*, 2000).

In some species flower senescence is accompanied by a climacteric increase in ethylene production. For example, pollination-induced petal senescence is accompanied by increases in ethylene production in petunia (Halevy *et al.*, 1984). A burst of ethylene synthesis from the flower is detected at 3 hours after compatible pollination (HACP), and a second increase in ethylene synthesis begins at 18 hours, with the first sign of senescence apparent at 36 HACP. While in the case of an incompatible pollination, the

second production peak does not occur until 3 days (Singh *et al.*, 1992a). The second peak has also been reported at 20 HACP (Whitehead *et al.*, 1984). The second longer peak appears to induce corolla senescence, because incompatible pollinations give the first peak but these flowers do not wilt sooner than non-pollinated controls (Singh *et al.*, 1992a). After pollination, an increase in ethylene synthesis is detectable from the stigma within 5 minutes (Pech *et al.*, 1987), and from styles within 1-2 hours (Nichols *et al.*, 1983). When flowers are not pollinated, there is also an increase in ethylene production prior to flower senescence. Several days later, color fading is visible and wilting begins (Whitehead *et al.*, 1984).

In addition to ethylene production rates, sensitivity to ethylene also plays an important role (Trewavas, 1982; Whitehead and Bossè, 1991; Whitehead and Vasiljevic, 1993). The increase in ethylene sensitivity of flowers during the pre-climacteric phase was found to be associated with an increase in the ability of the petal tissue to bind ethylene to membrane-bound receptor sites (Brown *et al.*, 1986; Whitehead and Vasiljevic, 1993). For example in carnation, mature flowers but not flower buds respond to exogenous ethylene treatment with accelerated corolla senescence (Lawton *et al.*, 1990). Trewavas (1982) emphasized that the differences in sensitivity to hormones, rather than the level of the hormones themselves, are the main factor controlling plant responses. In carnation, variations in senescence have been related to differences in sensitivity by comparing the sensitivity to exogenous ethylene application (Wu *et al.*, 1991); and by genetic modification of ethylene biosynthesis (Altvorst *et al.*, 1997). The role of sensitivity has also been observed in many other plants, such as orchids (Goh *et al.*, 1985; Porat *et al.*,

1995), roses (Müller *et al.*, 2000), kalanchoe (Serek and Reid, 2000), Dendrobium (Ketsa and Rugkong, 2000), English Holly (Fjeld *et al.*, 1995) and petunia (Porat *et al.*, 1993). While *Cyclamen persicum* does not respond to ethylene treatment (Halevy *et al.*, 1984), its ethylene sensitivity can be enhanced by pollination (Halevy, 1995). Paton *et al.* (1996) further suggested that changes in ethylene sensitivity during flower senescence are mediated by modulation of receptor levels during development. Pollination also enhances the sensitivity of the corolla to ethylene. A yet unidentified "sensitivity factor" is apparently transmitted from the pollinated gynoecium to the corolla rendering it sensitive to the senescence-promoting effects of ethylene (Halevy, 1986a). In conclusion, sensitivity also plays a role in regulating senescence and is not only accomplished by the ethylene burst.

The sensitivity of ethylene is especially important because the ethylene is often produced in one part of the plant and may lead to physiological responses in other parts of the plant. In order to accomplish this goal, the plant system must be able to differentially regulate ethylene perception and signal transduction. The difference of sensitivity either among various plant tissues or during a plants life cycle can also be due to the abundance and/or activation of hormone receptors. A hormone receptor is a specific cellular recognition protein that can bind hormone molecules and then initiates a sequence of events that leads to a physiological response. Studies have used *Arabidopsis* mutants defective in components of the ethylene signal transduction pathway as a model system to investigate this regulation (Guzman and Ecker, 1990; Roman *et al.*, 1995). Several mutations in the *Arabidopsis etr1* gene result in dominant ethylene insensitivity

(Bleecker *et al.*, 1988). This *Arabidopsis ETR1* protein can be divided into three domains by analogy to bacterial two-component systems (Chang *et al.*, 1993; Schaller and Bleecker, 1995). The amino-terminal sensor domain contains three putative transmembrane segments and has been shown to bind ethylene when expressed in yeast. A mutation in this domain abolishes ethylene binding (Schaller and Bleecker, 1995). This protein functions as a dimer in the membrane and the transition metal cofactor copper is required for ethylene binding (Schaller and Bleecker, 1995). The second domain shows homology to histidine kinases in bacterial two component sensing systems. This section of the ETR1 protein has been shown to have enzyme activity *in vitro* (Gamble *et al.*, 1998). The other section, the response regulator, may receive the phosphate from the histidine of the histidine kinase domain at an aspartate residue (Chang *et al*, 1993). Research from tomato also demonstrated a family of putative ethylene receptor genes, designated as *LeETR1, LeETR2, NR* (Never ripe), *LeETR4, and LeETR5* (Wilkinson *et al.*, 1995; Lashbrook *et al.*, 1998; Tieman and Klee, 1999). The *LeETR1, LeETR2, LeETR4, and LeETR5* were cloned by homology to the *Arabidopsis ETR1*, *ETR2*, and tomato *NR* genes. Among these ethylene receptor genes, *NR* and *LeETR4* tend to have a negative regulatory effect to ethylene response (Tieman *et al.*, 2000). Introduction of the *Arabidopsis ETR1-1* gene into tomato (*Lycopersicon esculentum Mill.* 'Floradade') (Chang *et al.*, 1993), tobacco (*Nicotiana tabacum* 'Samsun'), and petunia (diploid *Petunia x hybrida* 'Mitchell') (Wilkinson *et al.*,1997), as well as in *Petunia x hybrida* 'V26' (Gubrium *et al.*, 2000) confers ethylene insensitivity.

For those flowers that are insensitive to ethylene, such as daylily, ABA plays an important role in regulating senescence. In leaves of detached, senescing tobacco (Even-Chen and Itai, 1975) and rice (Philosoph-Hadas *et al.*, 1993), increased ABA levels were detected. In the senescence of flowers, elevated ABA levels were detected in advanced senescence stages of roses (Mayak and Halevy, 1972; Borochov *et al.*, 1976; LePage-Degivry *et al.*, 1991), 12 hours before anthesis in daylily (Panavas *et al.*, 1998), and in senescing carnations (Eze *et al.*, 1986). The exogenous application of ABA accelerated flower senescence of rose (Müller *et al.*, 1999) and prematurely up-regulated events that occur during natural senescence, such as loss of differential membrane permeability, increases in lipid peroxidation and the induction of proteinase and RNase activities in daylily (Panavas *et al.*, 1998). Exogenous ethylene treatment was also found to increase the ABA content in rose, while pre-treatment with 1-MCP (1-methylcyclopropene) delayed ABA promoted flower senescence, suggesting that the effect of ABA is at least partly mediated by ethylene (Müller *et al.*, 1999). Grossmann and Hansen (2001) also proposed ethylene-triggered ABA would play a role in plant growth regulation.

1.3 Cytokinins and senescence

Cytokinins delay leaf senescence in many species (Dyer and Osborne, 1971; Venkatarayappa *et al.*, 1984). Richmond and Lang (1957) applied cytokinins to a detached cutting, single leaf or a localized area that returned green color, and resulted in mobilization of a labeled amino acid to the treated area (Mothes and Engelbrecht, 1961). A classical demonstration for cytokinins effects on senescence is the appearance of 'green islands'. The formation of 'green islands' can be obtained by the application of

cytokinins to half of a bean leaf infected with the rust *Uromyces phaseoli* while the other untreated half results in promoted senescence (Király *et al.*, 1967). Green islands on detached barley leaves infected with powdery mildew have a lower photosynthetic rate than uninfected tissue while the surrounding senescing tissues is unable to photosynthesize (Coghlan and Walters, 1992).

The ability of cytokinins to delay senescence is partially due to its influence on many other metabolic pathways. Exogenous application of kinetin delayed the loss of both chlorophyll and protein from leaves (Richmond and Lang, 1957) and slowed the decline of nucleic acids (Dyer and Osborne, 1971). Cytokinin also stimulates chloroplast formation (Fletcher *et al.*, 1973), and the synthesis of chloroplast proteins such as the light-harvesting chlorophyll *a/b* binding protein (Axelos *et al.*, 1984). The transient rise in respiratory rate associated with senescence is also prevented by cytokinins (Tetley and Thimann, 1974). Recent studies suggested that cytokinins are involved in the control of senescence at the level of gene expression. Teyssendier *et al.* (1985) demonstrated a 10-fold increase in the level of LHCP II (light-harvesting chlorophyll *a/b* binding protein) mRNA in kinetin-treated tobacco suspension cultured cells compared with control cells.

Most of the early cytokinin studies of senescence were focused on application in leaves. In experiments with carnations, flower senescence was delayed by exogenous cytokinin application. This result was associated with reduced ethylene production and decreased sensitivity to ethylene (Eisinger, 1977; Cook *et al*, 1985; Mor *et al.*, 1983). Endogenous cytokinins in the petals are also a factor to in flower. Mayak and Halevy

(1970) demonstrated that the cytokinin content in young rose petals was higher than in old petals, and that short-lived varieties contained lower endogenous cytokinin levels than long-lived varieties. In addition, the endogenous cytokinin contents can be further modulated by ethylene during petal senescence by degrading or inactivating cytokinins (Taverner *et al.*, 1999).

Many studies have shown that both the exogenous application (Richmond and Land, 1957) and increased endogenous production of cytokinins delay leaf senescence (Gan and Amasino, 1995; Jordi *et al.*, 2000; Ooms *et al.*, 1991). The ability to transform plants with foreign genes has made it possible to alter endogenous cytokinin levels. For example, introduction of the cytokinin producing *ipt* gene from *Agrobacterium* has been utilized to elevate endogenous cytokinin levels. This *ipt* gene codes for the enzyme, isopentenyl transferase, which catalyzes the rate-limiting step in cytokinin biosynthesis. (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984). This step is where AMP is incorporated into Δ^2-isopentenyl pyrophosphate to form isopentenyl AMP. The introduction of the *ipt* gene into plants is associated with increased cytokinin content (Smart *et al.*, 1991; Li *et al.*, 1992; Hewelt *et al.*, 1994; McKenzie *et al.*, 1994; Gan and Amasino, 1995; Faiss *et al.*, 1997; Clark *et al.*, 2002). These approaches have advantages over studies utilizing external applications of cytokinins, as it avoids the need to consider the efficiency of uptake and transport. It also provides more direct evidence for the role of cytokinin in senescence than just correlative data. Molecular engineering of endogenous cytokinin production makes it possible to investigate the physiological role of cytokinins in the senescence process.

Although cytokinins can be obtained by breaking down tRNA, it is generally accepted that the majority of the cytokinin synthesis begins with the reaction catalyzed by isopentenyl transferase (Fig. 1.1) (Gan and Amasino, 1996), or iptase (EC 2.5.1.8) (Chen and Ertl, 1994), also called cytokinin synthetase (Kaminek, 1992), or dimethylallylpyrophosphate:AMP transferase (DMA transferase) (Akiyoshi *et al.*, 1984). The activity of this enzyme can be detected in plant extracts but several attempts to purify it have failed due to instability (Chen and Ertl, 1994). Thus the *ipt* gene from the plant pathogenic bacterium *Agrobacterium tumefaciences* has been used to in investigate the physiological effects of cytokinins. Not until recently has the gene coding for this rate-limiting enzyme been identified in plants (Kakimoto, 2001; Takei *et al.*, 2001; Zubko *et al.*, 2002).

Transforming the experimental plant material with the *Agrobacterium ipt* (isopentenyl transferase) gene with its constitutive promoter, and/or without selecting the proper prompter to regulate the *ipt* gene will result in over expression in plant cells (Wang *et al.*, 1997). If ipt expression is initiated without any regulation, it will cause overproduction of cytokinins in all plant organs throughout development, and result in the inability to regenerate plants from tissue culture. The result is abnormal plant development, thereby precluding the study of cytokinin over-expression within the whole plant (McKenzie *et al.*, 1998). Symptoms for overproduction in these transgenic plants are failure to form roots, production of callus at the base of tissue culture plants, limited shoot elongation, and enhanced lateral branching. Various degrees of shortening of internodes, decreased leaf size, reduced apical dominance and poor rooting were observed from *in vitro* grown

potato plants transformed with *ipt* (Ivana *et al.*, 1997). In order to obtain elevated endogenous cytokinins in plants with normal development, a proper promoter is required to manipulate endogenous cytokinin levels. Many studies of the ipt gene constructed with various promoters based on specific research goals are summarized in a review (Gan and Amasino, 1996). Promoters used range from the relatively week native ipt promoter (Higgins, 1995; Groot *et al.*, 1995) to the strong cauliflower mosaic virus (CaMV) 35S promoter (Smigochi and Owens, 1988).

In order to overcome the problems caused by constitutive cytokinin over-production, an auto-regulated construct was developed with a senescence-specific promoter that results in the suppression of leaf senescence when introduced into plants (Gan and Amasino, 1995). Using this construct cytokinin production is specially targeted to senescencing organs and is negatively auto-regulated to prevent cytokinin over production. Once cytokinins are produced and accumulate to a level that delay senescence, the promoter will be shut off to prevent over production of cytokinins. Among the identified senescence-associated genes (SAGs) from *Arabidopsis thaliana* (Lohman *et al.*, 1994), SAG_{12} is highly senescence specific, therefore the SAG_{12} promoter ($PSAG_{12}$) was joined to the coding region of *ipt* to form the chimeric gene $PSAG_{12}$-*ipt* (Gan and Amasino, 1995).

SAG12 belongs to one of four classes of senescence-associated genes. Genes belonging to the first class are considered 'housekeeping' genes. These genes control the primary metabolic activities for maintaining living cells functions. The properties of the

second class of genes are regulatory and are expressed at the initiation of senescence, controlling its timing and rate of progress. The third class of genes showing enhanced expression during senescence, e.g. enzymes of pigment breakdown. The last class of genes encodes proteins that remobilize storage products, e.g. enzymes of gluconeogenesis (Smart, 1994).

Among the model systems for studies of flower senescence, petunia was selected because petunia meets many requirements in our study. For example, petunia is ethylene sensitive, and pollination hastens the senescence. The senescence symptoms are obvious, and a transformation protocol for petunia has been well established (Jorgenson *et al.*, 1996). Petunia lines transformed with the SAG_{12}-*ipt* construct were obtained from Dr. David Clark at the University of Florida. All of these transgenic petunia plants appeared to have normal growth and development. However, in our preliminary experiments, flower longevity of two transgenic lines was significantly increased over wild type. Since phenotypic evidence implies the regulation and function of the SAG_{12}-*ipt* gene may play an important role in retarding flower senescence, further studies were performed to 1). Reveal the expression of this gene during the process of flower senescence, and 2). Determine how SAG_{12}-*ipt* increased cytokinin levels effects ethylene production and 3). Delays the senescence process. It is expected that the results from this study will provide new insight into the hormonal relation among cytokinin, ethylene, and ABA, and how they interplay during flower senescence.

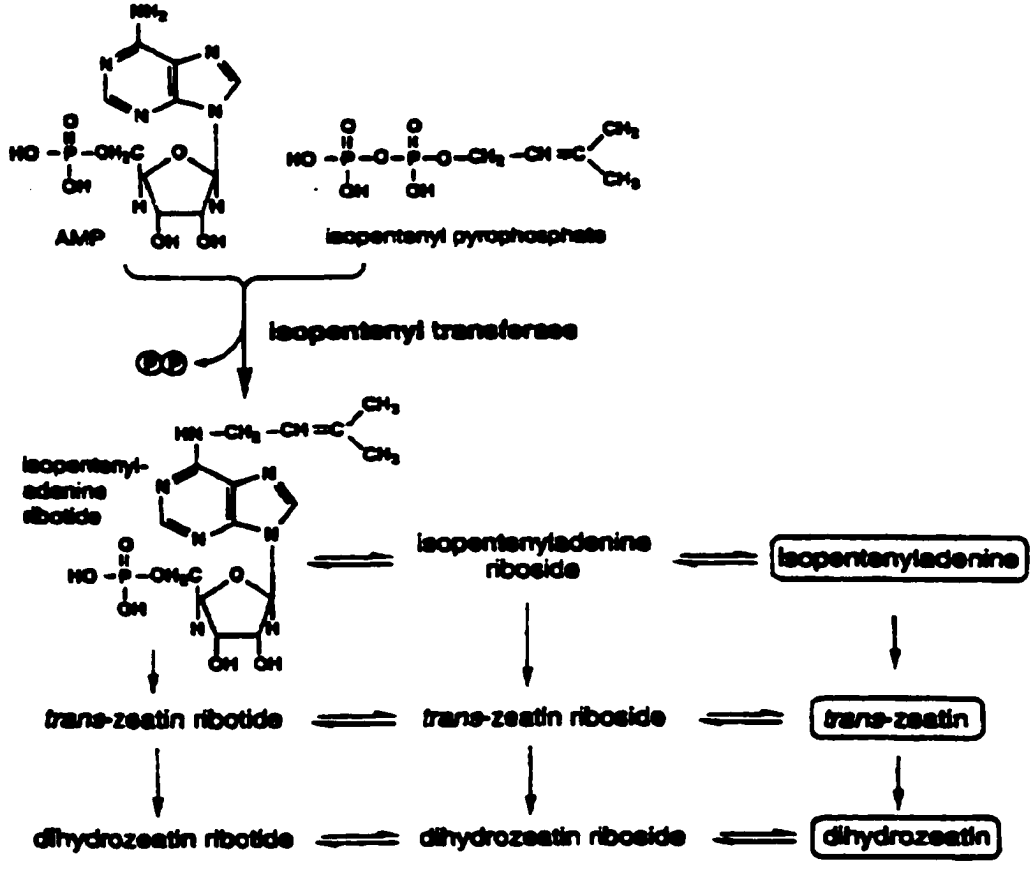

Fig. 1.1 Principle pathways of cytokinin biosynthesis.

Chapter 2

Expression of SAG12-*ipt* increases cytokinin production and delays corolla senescence in transgenic petunias

2.1 Abstract

The expression of the *ipt* gene under the control of a senescence-associated gene promoter (SAG12) has been determined in petunia (*Petunia x hybrida* 'V26') corollas. The existence of the construct was confirmed by PCR. The expression of the chimeric SAG12-*ipt* was quantified by Real Time RT-PCR. The cytokinin levels were determined by an HPLC-immunoassay method. The results from the lines IPT22 and IPT34 showed expression of the *ipt* gene markedly elevated the cytokinin levels in senescing corollas. Flower senescence was retarded up to 6 days relative to wild type flowers after pollination. During natural senescence, IPT22 lasted 10 days longer, and IPT34 lasted 7 days longer than wild type flowers.

2.2 Introduction

The role of cytokinins in plant development has been investigated by either exogenous application or associating endogenous cytokinin levels with different phases during plant

development. Exogenous cytokinin applications have proven effective in delaying senescence in carnations (Maclean and Dedolph, 1962; Heide and Qydvin, 1969; Mayak and Dilley, 1976a; Mayak and Kofranek, 1976; Upfold and Van Staden, 1990), roses (Mayak and Halevy, 1970; 1974), Gerbera (Van Meeteren, 1979), and *Petunia* (Taverner *et al.*, 1999).

Studies based on external applications are subject to criticism because the application is complicated by the problems of uptake, transport and metabolism of the supplied cytokinins, and is influenced by developmental stage of experimental flowers (Kelly *et al.*, 1985; Bosse and Van Staden, 1989; Upfold and Van Staden, 1990). It can therefore be difficult to draw any decisive conclusion. To avoid these issues, plant materials transformed with the cytokinin biosynthesis gene *ipt* from *Agrobacterium tumerfaciens* have provided a new approach to the study of the regulatory role of cytokinins. However, transformed plants with the *ipt* gene and its native promoter resulted in extreme production of cytokinins and consequently root development was suppressed. Other promoters have been constructed with the *ipt* such as the 35S cauliflower mosaic virus promoter (Smigocki and Owens, 1988) and promoter of the gene encoding the small subunit of Rubisco (Beinsberger *et al.*, 1992). These fusions overproduced cytokinins and the transformed tobacco plants couldn't develop roots. In order to regain root development, Hewelt *et al.* (1994) and Eklof *et al.* (1996) transformed tobacco with a promoterless *ipt* gene. Beside these approaches, heat shock promoters have been widely used (Medford *et al.*, 1989; Smart *et al.*, 1991; Smigocki, 1991; Ainley *et al.*, 1993; van Loven *et al.*, 1993). Plants transformed with this construct produces higher cytokinin

levels for a short period of time (duration) after heat shock treatment. It is probably not possible to maintain constant elevated cytokinin levels in this way and the heat shock treatments can also affect study results (van Loven *et al.*, 1993).

It is important to design a chimeric *ipt* gene construct that will be developmentally regulated in plants and confine expression to fit in particular research purpose. In order to target the role of cytokinins in flower senescence in our study, the senescence-specific promoter SAG12 cloned from *Arabidopsis thaliana* joined with the *Agrobacterium ipt* (Gan and Amasino, 1995) gene was used to investigate cytokinins effects on senescence. The SAG12-*ipt* will express in senescence specific manner and expression level will be decrease once cytokinins are accumulated up to certain levels to affect the senescence. The negative feedback control will prevent cytokinins over-produced that results in abnormal plant development.

Plants transformed with SAG12-*ipt* exhibited delayed leaf senescence (Gan and Amasino, 1995; Jordi *et al.*, 2000; Zhang *et al.*, 2000; McCabe *et al*, 2001), and elevated cytokinin levels also enhanced flooding resistance (Zhang *et al.*, 2000) and drought tolerance (Clark et al., 2002). Although Zhang *et al.* (2000) and Schroeder *et al.* (2001) reported a delay in flower senescence further data on flowers was not included in their investigations Our objective is to utilize SAG12-*ipt* petunias to study flower senescence and provide information on how cytokinins regulate flower senescence. The major objectives of this chapter are: 1). Determine if SAG12-*ipt* plants have delayed flower senescence. This will be accomplished by comparing the flower longevity during natural

aging (no pollination) and following pollination in WT and transgenic lines. 2). Determine expression patterns of the ipt gene during senescence. This will confirm whether the SAG12 promoter is functioning and determine if expression is senescence specific. 3). Determine if cytokinin levels are higher in transgenic corollas and identify what forms of cytokinins accumulated. If the ipt gene is expressed it is hypothesized that flowers will have higher cytokinin levels and exhibit delayed corolla senescence.

2.3 Materials and Methods

2.3.1 Plant material and growing conditions

Petunia x hybrida 'V26' previously transformed with P_{SAG12}-*ipt* (Gan and Amasino, 1995) were obtained from Dr. David Clark at the University of Florida. Two independently transformed lines with a delayed flower senescence phenotype were selected for the studies. Progeny from these two transformed lines with increased flower longevity were selected for subsequent experiments because they also showed delayed leaf senescence and contained a single copy of the *ipt* transgene (Clark *et al.*, 2002). These two transgenic lines were designated I-1-7-22 (IPT22) and I-3-18-34 (IPT34). One hundred percent of T4 seedlings were previously confirmed by ELISA contained the NptII protein, indicating that both lines were homozygous for the transgene construct.

Seeds of IPT22, IPT34, and WT (Wild type-'V26') were germinated in commercial 6-pack germination trays with a transparent cover to retain the moisture. Plantlets were then moved to 4 inches pots after 4 weeks, and fertilized twice a week with N at 300 ppm from 15N-5P-15K CalMag (The Scotts Co., Marysville, Ohio). The greenhouse condition

of day/night period was set to16/8 hours, temperature set at 25 °C. Foliage fungicide, pot and soil fungicide (Banrot) were applied every other Thursday.

2.3.2 PCR analysis

Polymerase chain reaction (PCR) was conducted to confirm the existence of the SAG12-*ipt* gene construct by amplifying the extracted genomic DNA from petunia. Genomic DNA was extracted from young leaves using a modified CTAB extraction protocol (Fulton *et al.*, 1995). Ten young leaves (≈ 1.3 g) were ground in liquid nitrogen, quickly moved to centrifuge tubes with 20 ml pre-warmed CTAB extraction buffer, and incubated for 30 minutes at 65°C. Fifteen ml chloroform was added and mixed gently for 15 minutes. After spinning for 10 minutes at 8000 rpm, the supernatant (≈ 15ml) was decanted to a new tube. Fifteen ml of ice-cold isopropanol (2-propanol) was added, tubes were incubated on ice for 5 to 10 minutes followed by centrifuging at 8000 rpm for 10 minutes at 4°C. The DNA pellet was collected with a bent Pasteur pipette and moved to a micro-centrifuge tube. The DNA pellet was dissolved in 100ul TE buffer and 1ul of RNAse A was added. After 20 minute incubation, 10ul 3M Na-acetate and 200ul 95% ethanol were added and samples were precipitated at –20°C. After at least 30 minutes, the samples were centrifuge at 10,000 rpm at 4°C for 10 minutes. The supernatant was pipetted out and the DNA pellet was washed with 70% ethanol then dried. The DNA pellet was dissolved in 100ul TE buffer, and stored at 4°C. DNA was quantified on a Spectrophotometer (Beckman DU-600) using A_{260}. For PCR analysis, one microgram of DNA was amplified for 35 cycles of denaturation at 94° C for 1 min, annealing at 60° C for 1 min, and elongation at 72° C for 1 min using a Mastercycler gradient thermocycler

(Eppendorf, Hamburg, Germany). Forward (5'- GCG TCT AAT TTT CGG TCC AA-3') and reverse (5'- TCC ATA TCT GCG TCA AGC TG- 3') primers specific to the *ipt* sequence were constructed to amplify a 596 bp segment of the *ipt* gene. The amplified DNA samples were electrophoresed on a 1.0% agarose gel that contained ethidium bromide at 90 volts for two hours. The results were documented by exposing the gel under UV light using Bio Imaging System (Synoptics LTD, Cambridge, UK).

2.3.3 Visual flower senescence scoring

Visual flower senescence was scored for both aging of un-pollinated flowers (in future, this refers to natural senescence) and after pollination. Emasculation was performed one day before anthesis. Flower corollas were slit with a sharp razor blade and anthers were carefully removed to prevent self-pollination. On the day of flower opening (anthesis), 6 flowers from different plants were either self-pollinated or remained unpollinated for the observations of pollination-induced senescence and natural senescence respectively. Flowers were visually scored everyday at the same time of day for the degree of senescence using a rating scale. Ratings were as follows: 3, intact flowers without any visual symptoms of senescence; 2, flowers showing initial symptoms of flower senescence including wilting of petal margins; and 1, flowers severely wilted. Senescence scoring was presented as a 50% senescence (S_{50}) rating (Bichara and Van Staden, 1993) indicating the time after pollination or after anthesis at which 50% of the flowers were assigned a particular senescence score. Senescence pictures were taken and documented. All visual senescence scorings were repeated 3 times. Data presented here is

from one set of the experiments. Another senescence data were also collected. The times to show first visual symptom and total wilt. The statistical method as described below: For each data set, an omnibus one-way ANOVA was conducted at $\alpha = 0.05$. If the omnibus F-test is significant, a post hoc pairwise comparison based on Scheffe's procedure will be conducted.

2.3.4 Exogenous cytokinin application

To confirm that cytokinin has an effect on senescence in petunia flowers, exogenous application experiments were performed twice with two types of cytokinins, trans-zeatin and trans-zeatin riboside. These two cytokinins were chosen because they have previously been found to be preferentially accumulated in *ipt* transformed plants (Redig *et al.*, 1996; Faiss *et al.*, 1997; Schmulling *et al.*, 1999; Jordi *et al.*, 2000).

Flowers were emasculated one day before anthesis as previous described. Wild type flowers were harvested on the day of anthesis and corollas were carefully removed in distilled water and moved to solution containing various concentrations of either trans-zeatin and trans-zeatin riboside. The concentrations of the first set of experiments included 0.00, 0.01, 0.05, 0.10, 0.20, 0.30, 0.40, 0.50 mM of *trans*-zeatin, plus a transgenic control group incubated in distilled water. The solution was changed every 24 hours. The basal part of corolla was cut daily with a sharp blade to aid the absorption of solution. The second experiment was performed twice with various concentrations of *trans*-zeatin (tZ) (Acros Organics, New Jersey) and *trans*-zeatin riboside (tZR) (Sigma, St. Louis, MO). The concentrations were 0.00, 0.05, 0.1, 0.2, 0.4 mM (in water), plus a

transgenic control group concentration of 0.00 mM. Results were accessed every 24 hours until corollas were totally wilted by senescence scoring as previous described. Individual pictures were taken accordingly. Statistical analysis for the corolla to show the first time visual symptom and total wilt were also performed according to previous described.

2.3.5 RNA extraction

Tissues were frozen in liquid nitrogen and stored in -80°C until needed. The total RNA from corollas, ovaries, and styles was extracted using the Guanidine method (Chirgwin *et al.*, 1979) and TRIZOL reagent (Gibco BRL, Rockville, Maryland). Tissue was ground in a pre-cooled mortar and pestle with liquid nitrogen with 10X 5M Guanidine extraction buffer. The ground mixture was then filtered through miracloth into a 50 ml centrifuge tube with the support of a funnel. A half volume of ethanol was added and samples were mixed carefully and stored in a -20°C freezer over night to precipitate RNA. After centrifuging 15 minutes at 14k rpm, the supernatant was discarded and the pellet was re-suspended with 5 ml 4M Guanidine solution, and half volume (2.5 ml) of ethanol. Samples were stored in a -20°C freezer over night to precipitate RNA. After centrifuging 15 minutes at 14k rpm, the supernatant was discarded carefully and 10 ml of ethanol was added to the pellet to remove guanidine by centrifuging another 15 minutes at 14k rpm, the supernatant was removed and a second ethanol wash and centrifuge step were done. RNA was obtained by adding 3 ml of depc (Diethyl Pyrocarbonate)-treated (0.1%) water with centrifuging. The depc treated water containing the RNA was collected into Corex Tubes, coverd with parafilm, and stored in a -20°C freezer over night to precipitate RNA.

After centrifuging 15 minutes at 10k rpm, supernatant was removed and 3 ml 70% ethanol was added. Samples were centrifuged for 15 minutes at 10k rpm, the supernatant was discarded and 100 μL of depc-treated was added. Extracted total RNA was quantified by a Spectrophotometer using A_{260} (Beckman DU®640, Fullerton, CA). RNA samples were then diluted to $1\mu g.\mu l^{-1}$, and all the RNA samples were stored at –80°C. Quality control was monitored by running RNA samples on a denaturing agarose gel.

2.3.6 Real Time RT-PCR analysis

Based on the results from preliminary experiments, the designed primer sets, the primer concentration, and Tm were investigated by performeing regular PCR. Two hundred fifty nanograms of total RNA samples were reverse transcribed and amplified using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, California) with the iCycler iQ real-time PCR Detection System (BioRad, Hercules, California). The RNA sample was first reverse transcribed for 30 min at 50° C. Then the reaction was incubated at 95° C for 15 min to activate the HotStar Taq DNA polymerase. A total of 45 cycles of PCR was conducted with denaturation at 94° C for 15 sec, annealing at 60° C for 30 sec, and elongation at 72° C for 30 sec. The result obtained from the iCycler was the calculated value of the Threshold Cycle (Ct). This is the cycle at which the amplification plot crosses the threshold, i.e., at which there is the first clearly detectable increase in fluoresence (from SYBR Green) by adjusting the threshold to a value above the noise baseline level, and located in the log-linear range of the PCR. By calculating these Ct values against an external standard RNA curve (Lightcycler Control RNA Kit; Roche Diagnostics, Mannheim, Germany), relative amounts of transcript were obtained.

Primers were constructed to amplify a 152 bp amplicon of *ipt*. These *ipt* primers were forward 5'- GCC TCT GGT GAA GGG TAT CA –3'; *ipt* reverse 5'- CCG CAC TCC AAT AAC TGC TT –3'. Samples were run in triplicate with similar results. Data presented is for one experiment.

2.3.7 Cytokinin measurements

Corollas from 6 flowers were collected at 0, 12, 18, 24, 36, 48, and 72 hap and after 12 h ethylene treatment and instantly frozen by dipping in liquid nitrogen. Corollas were lyophilized for 12 hours. Tissue (approximately 100 mg per sample) was ground in liquid N_2 and cytokinins were extracted in 100% ethanol for 30 min. After nine volumes of 40 mM ammonium acetate (pH 6.5) were added to the extract, cytokinins were isolated on C18 SepPaks (Waters Assoc., Bedford, MA), purified and quantified in triplicate samples using a previously described combined HPLC-immunoassay method (Banowetz, 1992). The immunoassay utilized monoclonal antibodies tZR3 (Trione *et al.*, 1985) and iPA3 (Trione *et al.*, 1987), prepared against the ribosides of zeatin and isopentenyl adenosine, respectively. These antibodies react with free bases, 9-glucoside and 9-ribosides of zeatin, dihydrozeatin (tZR3) and isopentenyl adenine (iPA3). All cytokinin quantities are expressed as zeatin riboside (ZR) or isopentenyl adenosine (iPA) equivalents because ZR and iPA were used to generate standard curves from which the cytokinin content of samples was extrapolated. This analysis was conducted in the lab of Dr. Gary M. Banowetz at the Oregon State University-ARS.

2.4 Results

2.4.1 Confirmation of transgenic petunia plants

To confirm that the transgenic lines did contain the *ipt* gene insertion, PCR using *ipt*-specific primers was performed (Fig. 2.1). The 100 bp molecular weight markers were used to verify the size of bands amplified from genomic DNA samples and control groups. The negative control (-) contained distilled water rather than genomic DNA, and the positive control (+) contained the *ipt* construct that was previously isolated from plasmid DNA. The size of amplified bands from both transgenic lines and positive control was 596 bp, which was expected according to the designed primer set. This confirmed the presence of the *ipt* gene in IPT22 and IPT34 plants and its absence in WT plants.

2.4.2 Comparison of flower longevity

During natural senescence (aging without pollination treatment) 50% of WT flowers exhibited the first visual symptom of senescence at 120 hours after anthesis (Fig. 2.2). The distal portion of corolla lost turgidity and was floppy. This stage defined as stage 2. Compared with WT flowers, both IPT22 and IPT34 did not reach stage 2 until 264 hours after anthesis. All the flowers from transgenic lines and WT reached stage 1 within 24 hours after stage 2. Table 2.2 showed the days to first visual wilting symptom and total wilted without pollination. Both IPT22 and IPT34 flowers lasted twice longer than the WT.

Pollination hastened senescence and WT flowers showed the first visual senescence symptom (stage 2) by 36 hours after pollination (hap), and were completely wilted (stage 1) within the next 12 hours (Fig. 2.3). In contrast, pollination did not accelerate the senescence of IPT 22 as observed in WT. Fifty percent of IPT22 flowers were not at stage 2 until 264 hap. After 24 hours at stage2, the IPT22 flowers reached stage 1. IPT34 flowers also lasted longer than WT, yet reached stage 2 72 hours earlier compared with IPT22 flowers. IPT34 corollas remained in stage 2 for 72 hours, and stage 1 for 24 hours. Table 2.3 showed the days to first visual wilting symptom and total wilted after pollination. Both IPT22 and IPT34 lasted longer than WT for the first visual symptom and total wilted.

Although in either natural or pollination induced senescence, IPT22 and IPT34 lasted longer in stage 3 with their corolla shape intact and unwilted, their color faded before wilting (stage 2) (Fig. 2.4) while the WT wilted to stage 1 without any loss of color, and remained a deep purple. Following pollination, some of the corollas remained rigid (without senescence symptom, yet with a little fade of color) toward the end of stage 3 even the basal part of the corolla was partially pushed away by the development of the ovary (Fig. 2.5).

2.4.3 Response of exogenous cytokinin applications

The excised WT corollas treated with *trans*-zeatin (tZ) exhibited delayed senescence (Fig. 2.6). The group without cytokinin treatment and the lowest concentration (0.01 mM) wilted dramatically from stage 3 to totally wilt at day 4. The other concentration

treatments, 0.05, 0.10, 0.20, 0.30, 0.40, 0.50 mM retarded and exhibited similar senescence, but were not statistically different except for 0.30 mM treatment for the days to total wilted (Table 2.4). In the other experiment, treatments with tZ and tZR of WT flowers, both effectively delayed senescence (Fig. 2.7, Fig. 2.8, Table 2.5, Table 2.6). The cytokinin treatments increased the flower longevity by various degrees, and the transgenic IPT22 incubated in distilled water also showed extended vase life. Although exogenous application enhanced the flower longevity, no conclusion could be drawn about the best concentration to delay senescence. This result suggested there were problems in absorption and translocation of the exogenous cytokinins.

2.4.4 Quantification of *ipt* expression

Real time RT-PCR showed the *ipt* gene was successfully transcribed in the corolla of IPT22 and IPT34, while it was not in WT corollas. The expression of *ipt* gene existed as early as anthesis, even in flower buds (data not shown). In the corolla of IPT22, the mRNA levels reached the first major peak at 18 hap, followed by an expression drop at 24 hap. A higher peak was observed at 36 hap that was almost 5 fold compared with 24 hap, then the expression went down at 48 hap. Another peak was present at 72 hap. In IPT34 corollas, the expression levels were sustained until it decreased at 36 hap and increased at 48 hap. The highest mRNA levels were detected at 72 hap (Fig. 2.9).

2.4.5 Cytokinin analysis of corolla

The total cytokinin levels were elevated in transgenic lines (IPT22, IPT34) compared with WT. This result demonstrated that the introduced *ipt* construct in petunia corollas not only successfully produced the isopentenyltransferase, but also this enzyme was functioning during senescence. In IPT22, the total cytokinin content through the time course following pollination was higher than WT. The total cytokinin contents increased from 18hap and decreased at 36hap (still higher than WT) peaking at 48hap. In IPT34, the total cytokinin content were all higher than WT during senescence (at all hap). The production pattern in IPT34 was different from IPT22 at 18 and 24 hap where no increased production peaks were detected, yet the level was still significantly higher than in WT (Fig. 2.10). Among all the cytokinins measured (Table 2.1), Z (Fig. 2.11) and ZR (Fig. 2.12) were the major contributors to the total cytokinin calculated. The Z and ZR production patterns were similar to total cytokinin content, while the other measured cytokinins exhibited different patterns across time. Although iP (Fig. 2.13), iPA (Fig 2.14), and diHZR (Fig. 2.15) were produced in a relatively lower amounts, higher levels at 0 hap accounted for the elevated total cytokinin contents in both IPT22 and IPT34. iP and iPA levels were also significantly higher at 36hap which corresponded to the first wilting of WT flowers, followed by the higher accumulated levels of Z, Zr, and diHZR at 48hap. This suggested the higher levels of ip type cytokinins at 36hap were converted to Z types. The accumulations of ip-9G (Fig. 2.16) from both transgenic lines were not always higher following pollination. This suggested other elevated active cytokinins did not convert to ip-9G, and WT flowers had a higher tendency to accumulate ip-9G.

2.5 Discussion

Cytokinins have clearly been shown to have a critical role in plant development. One of the prominent effects is retarding senescence. With the transformation of Agrobacterium *ipt* gene into plants, numerous experiments have demonstrated that elevated endogenous cytokinins have significantly delayed natural leaf senescence (Smart *et al.*, 1991; Li *et al.*, 1992; Hewelt *et al.*, 1994; Gan and Amasino, 1995; Jordi *et al.*, 2000; McCabe *et al.*, 2001) and the senescence caused by water stress (Zhang *et al.*, 2000; Clark *et al.*, 2002). There were also observations of cytokinins effects on prolonged flower longevity (Bossè and van Staden, 1989; Cook *et al.*, 1985; Mor *et al.*, 1983), however, no further investigation on cytokinin effects on flower senescence (Zhang *et al.*, 2000; Schroeder *et al.*, 2001). In our research, petunia transformed with the autoregulated SAG12-*ipt* construct significantly delayed flower senescence. The period from anthesis to senescence was lengthened by 5 to 11 days. Arabidopsis transformed with SAG12-*ipt* also exhibited delayed flower senescence from 7 to 12 days (Zhang *et al.*, 2000). Thus transformation with the SAG12-*ipt* construct delayed flower senescence.

Exogenous cytokinin application delayed flower senescence in carnations (MacLean and Dedolph, 1962; Mayak and Dilley, 1976a; Mayak and Kofranek, 1976; Cook *et al.*, 1985; Upfold and Van Staden, 1990). The senescence of petunia corollas was also delayed by exogenous cytokinin application with 6-benzylaminopurine (BAP) at 2 and 20 μM and zeatin riboside at 0.2 mM (Taverner *et al.*, 1999). In our exogenous application study, the concentrations of zeatin riboside from 0.05 to 0.4 mM were also effective in

delaying corolla senescence. Delayed corolla senescence occurred in zeatin treatments, while the corollas in the highest concentration senesced a little earlier. This might be due to an inhibitory effect of the highest level. Zeatin and zeatin riboside, which are the two predominantly accumulated cytokinins in SAG12-*ipt* corollas, delayed the senescence of WT flowers (Table 2.1). Other cytokinin applications, such as 5-10 μg ml^{-1} kinetin (Eisinger, 1977), 5 mg L^{-1} BA (benzyladenine) (Cook *et al.*, 1985), and 0.1 mM BA, kinetin, and zeatin (Mor *et al.*, 1983) also significantly retarded flower senescence.

To monitor the activity of *ipt* expression through time after pollination and determine the specificity of the SAG12 promoter, real time RT-PCR was performed. Firstly, the data confirmed that the *ipt* gene was transcribed in both transgenic lines. Secondly, differences of expression following pollination were detected between two transgenic lines IPT22 and IPT34. Although both IPT22 and IPT34 appeared healthy and normal, the preliminary data showed the *ipt* transcript was detected in early flower buds and also young developing leaves (data not shown). This suggested the SAG12 promoter was functioning but not completely regulated in the senescence specific manner. It can be up-regulated during senescence, but expression was not confined to senescencing tissues. This was also reported for young plants of tobacco transformed with SAG12-*ipt* based on the little difference in morphology, distribution of cytokinins, sugars, proteins, and chlorophyll (McCabe, *et al.*, 2001). The SAG12 promoter was also found to be repressed by sugar (Suc, Glc, and Fru), auxin (indole acetic acid), and cytokinins such as kinetin and benzyl adenine, but not by adenine (Noh and Amasino, 1999). In our experiment, the *ipt* expression was different in IPT22 and IPT34 following pollination. There are several

possible reasons for the differences between these two transgenic lines. The SAG12 promoter may require certain types of cytokinins or concentrations above a certain threshold to repress the SAG12 promoter.

Endogenous cytokinins accumulate in plants because of the expression of the *ipt* gene from *Agrobacteruim tumerfaciens* (Brzobohartý *et al.*, 1994; Gaudin *et al.*, 1994; Motyka *et al.*, 1996). In order to have a better understanding of the *ipt* genes effects on cytokinin production in petunia, various cytokinins were analyzed. No increased iP-type cytokinins detected in either *ipt* transgenic *Arabidopsis* or tobacco plants (Redig *et al.*, 1996; Faiss *et al.*, 1997). This suggested that the likely enzymatic product was rapidly metabolized in higher plants to form the zeatin-type cytokinins. In contrast to this, transgenic *ipt* moss *Physcomitrella patens* preferentially accumulated iP-type cytokinins (Reutter *et al.*, 1998). Recently an isopentenyltrasferase homologue (sho) has been cloned from petunia (Zubko *et al.*, 2002). Plants transformed with the sho gene accumulated iP type over Z type cytokinins. This result suggested iP types are also important cytokinin forms in petunia. While the SAG12-*ipt* transformed petunia preferentially accumulated zeatin and zeatin riboside, elevated levels of iP-type were also found in both transgenic lines IPT22 and IPT34 (Fig 2-13, 2-14). In the corolla of transgenic petunia IPT22 after pollination treatment, the patterns of accumulated levels of iP forms (Fig 2-13, 2-14) were closely associated with the *ipt* expression pattern (Fig 2-9). These results reflected the fact that the iP type cytokinin was the immediate product catalyzed by the *ipt* construct. This might suggest that the plant system had not had time to convert iP to Z type cytokinins. From 0 hap to 12 hap, the higher levels of the iP form were then metabolized to Z form.

It is possible to reach a certain point that the cytokinins would be over produced up to an abnormal level. Even in transgenic plants, the endogenous hormone levels are monitored and controlled by various homeostatic mechanisms such as conjugation or oxidation, in addition to control by promoters. Elevated endogenous cytokinin produced by the heat shock promoter was higher at 4 hours after heat shock treatment compared with 24 hours after treatment. This suggested that there is an efficient pathway for their degradation in the leaf and that *iso*-pentenyl transferase has a high rate of turnover (Smart *et al.*, 1991). Chatfield and Armstrong (1986) have provided some evidence that external application of cytokinins to callus tissue results in increased cytokinin oxidase activity. It will be of interest to investigate the behavior of cytokinin oxidase, the cytokinin-degrading enzyme during conditional over-production of endogenous cytokinins in petunia flowers.

As elevated cytokinins delays flower senescence, there is a question about the sites of cytokinin production. The construct SAG12-*ipt* is, however, also expressed in other tissues, especially leaves (data not shown) which have been shown to have a potential for cytokinin synthesis (Singh *et al.*, 1992b). Roots are the main location for cytokinin production (Letham, 1994), and roots of petunia transformed with the *sho* (Shooting) gene contained the highest concentration of most cytokinins (Zubko *et al.*, 2002). While delayed senescence might be due to the relocation of cytokinins produced from tissues other than the corolla itself, Faiss *et al.* (1997) suggested that cytokinin activity in *ipt* transformants may be more restricted to the site of synthesis, even in the case that

cytokinins produced by *sho* transformed plants might be able to migrate into adjacent tissue (Zubko *et al.*, 2002).

In summary, many reports have indicated that exogenously applied cytokinins can delay the senescence of flowers (MacLean and Dedolph, 1962, Heide and Oydvin, 1969; Mayak and Kofranek, 1976). Eisinger (1977) proposed that cytokinins act as natural anti-senescence compounds in flowers, and that the declining endogenous cytokinin levels could serve as a trigger for senescence initiated by increased ethylene production. It has been shown previously that cytokinins may retard flower senescence by blocking ethylene biosynthesis (Eisinger, 1977; Mor *et al.*, 1983; Rasche and Eisinger, 1984; Cook *et al.*, 1985). In our study, cytokinin effects in delaying senescence of flowers were also confirmed by extending flower life significantly in transgenic lines in both natural and pollination induced senescence. It is clear that we employed an experimental system where elevated endogenous cytokinins accumulated during flower senescence and this challenged the constitutive hormone metabolic system in plants. However, the results presented here could reflect the processes that occur during flower senescence and demonstrate the positive correlation of cytokinin content and the delayed flower senescence. The conclusions from these experiments are as follows 1). Petunias transformed with SAG12-*ipt* exhibited delayed flower senescence during natural and pollination induced senescence. 2). Expression of the ipt gene was detected in flower buds and during the senescence of corollas, suggesting that the SAG12 promoter was functioning, however, was not senescence specific in petunia flowers. 3). Higher endogenous cytokinins levels accumulated in transgenic petunias. The predominant

cytokinin forms were Zeatin and Zeatin riboside, confirming findings with previous transgenic plants transformed with ipt increasing the endogenous cytokinins in petunias therefore delayed flower senescence.

Treat-ment	Plant Line	Z-9-G	Z	DiHZ + diHZ-9G	ZR	diHZR	iP-9G	iP	IPA
12 het	WT	15.18±3.26[a]	<l	<l	8.12±0.28	<l	3.61±0.22	12.6±1.93	9.72±1.14
	IPT22	7.44±1.47	758.74±225.6	5.86±1.13	2014.85±711.65	11.5±3.8	3.45±0.34	12.65±1.49	15.1±0.25
	IPT34	<l	789.34±95.73	6.13±0.65	1597.76±331.4	9.02±1.7	2.03±0.16	14.64±0.38	20.76±0.3
0 hap	WT	<l	<l	<l	6.83±0.3	2.32±0.43	2.07±0.28	6.66±0.28	6.68±0.42
	IPT22	<l	<l	<l	7.25±0.4	3.32±0.44	2.59±0.12	10.48±0.62	14.71±1.46
	IPT34	<l	<l	<l	5.41±0.32	6.6±10.2	2.06±0.16	11.35±0.88	17.32±1.12
12 hap	WT	6.23±0.75	<l	<l	4.03±0.37	<l	3.94±0.37	12.85±0.57	4.03±0.34
	IPT22	16.84±1.22	<l	<l	12.32±0.72	1.5±0.42	2.72±0.1	9.98±0.03	6.82±0.43
	IPT34	9.33±1.22	<l	<l	3.66±0.78	1.98±0.41	1.97±0.12	13.13±1.08	10.56±1.06
18 hap	WT	<l	<l	<l	3.7±0.72	<l	2.41±0.17	9.34±0.15	5.1±0.44
	IPT22	<l	31.12±4.46	<l	118.02±6.19	<l	2.38±0.5	11.17±0.82	11.15±0.86
	IPT34	<l	2.63±0.64	<l	8.35±0.39	1.28±0.16	2.13±0.07	10.15±0.21	9.11±0.24
24 hap	WT	11.41±0.87	<l	<l	<l	<l	4.47±0.32	11±0.18	2.95±0.13
	IPT22	11.97±1.16	162.22±29.51	<l	126.85±15.71	1.5±0.16	2.96±0.28	9.84±0.74	8.58±0.79
	IPT34	<l	24.2±0.51	<l	35.33±2.7	<l	2.27±0.35	10.65±1.72	7.11±0.81
36 hap	WT	<l	<l	<l	<l	<l	3.24±0.35	10.76±0.99	5.35±0.57
	IPT22	<l	4.43±0.26	<l	20.17±1.27	2.03±0.12	4.29±0.38	15.83±1.04	14.94±1.49
	IPT34	<l	6.59±0.67	<l	31.81±1.5	1.16±0.07	3.17±0.53	14.29±0.81	17.51±0.35
48 hap	WT	9.38±1.46	<l	<l	1.18±0.13	2.27±0.26	1.56±0.07	11.11±0.82	1.59±0.17
	IPT22	21.34±0.85	180.65±28.86	<l	502.64±77.01	5.08±1.05	2.05±0.53	6.6±0.29	5±0.08
	IPT34	16.29±0.99	260.8±39.89	<l	390.4±52	5.44±0.82	1.37±0.08	7.32±1.23	7.04±0.87
72 hap	WT	<l	<l	<l	<l	<l	<l	4.75±0.89	4.41±0.37
	IPT22	<l	211.63±17.35	<l	228.98±14.64	6.1±0.89	2.57±0.4	13.99±2.31	12.52±1.3
	IPT34	<l	112.46±6.75	<l	127.05±17.87	2.6±0.4	1.86±0.39	5.26±0.74	5.54±2.06

Table 2.1 Cytokinin content of petunia corollas following pollination (hap) or 12 hours treatment with 2 μL L^{-1} ethylene (het). Six corollas were collected from six plants of IPT22, IPT34 and wild type. Cytokinin content is expressed in ng g^{-1} DW. Cytokinins included Z-9-G, zeatin-9-glucoside; Z, zeatin; diHZ + diHZ-9G, dihydrozeatin and dihydrozeatin-9-glucoside; ZR, zeatin riboside; diHZR, dihydrozeatin riboside; iP-9G, isopentenyl-9-glucoside; iP, isopentenyl adenine; iPA, isopentenyl adenosine.

[a]Values represent average of 3 replicates ± SEM.

Table 2.2 Mean number of days (± SEM) to first visual wilting symptom and total wilted without pollination. WT, IPT22 and IPT34 flowers were examined on plants.

Plant line	Days to first visual wilting symptom	Days to total wilted
WT	5.2 ± 0.37 a[z]	6.2 ± 0.37 a
IPT22	11.2 ± 0.20 b	14.4 ± 0.75 b
IPT34	11.0 ± 0.00 b	12.8 ± 0.37 b

[z]Means followed by the same letter within each column are not significantly different at $p < 0.05$ by Scheffe's Procedure.

Table 2.3 Mean number of hours (± SEM) to first visual wilting symptom and total wilted after pollination. WT, IPT22 and IPT34 flowers were examined on plants.

Plant line	Days to first visual wilting symptom	Days to total wilted
WT	36.0 ± 0.00 a[z]	96.0 ± 0.00 a
IPT22	225.6 ± 24.71 b	302.4 ± 5.88 b
IPT34	196.8 ± 4.80 b	288.0 ± 0.00 c

[z]Means followed by the same letter within each column are not significantly different at $p < 0.05$ by Scheffe's Procedure.

Table 2.4 Mean number of days (± SEM) to first visual wilting symptom and total wilted. WT flowers were excised and incubated in tZ solutions and IPT22 in distilled water.

tZ (mM)	Days to first visual wilting symptom	Days to total wilted
0.00	4.0 ± 0.00 a[z]	4.0 ± 0.00 a
0.01	3.7 ± 0.33 a	4.0 ± 0.00 a
0.05	4.0 ± 0.00 a	6.7 ± 0.33 ab
0.10	4.0 ± 0.00 a	6.3 ± 0.33 ab
0.20	3.7 ± 0.33 a	6.0 ± 0.00 ac
0.30	4.0 ± 0.00 a	7.7 ± 0.88 bcd
0.40	3.0 ± 0.00 a	5.7 ± 0.67 ad
0.50	3.7 ± 0.33 a	6.7 ± 0.67 ad
IPT22	10.0 ± 0.00 b	11.0 ± 0.00 e

[z]Means followed by the same letter within each column are not significantly different at $p < 0.05$ by Scheffe's Procedure.

Table 2.5 Mean number of days (± SEM) to first visual wilting symptom and total wilted. WT flowers were excised and incubated in tZ solutions and IPT22 in distilled water.

tZ (mM)	Days to first visual wilting symptom	Days to total wilted
0.00	3.0 ± 0.00 a[z]	6.0 ± 0.00 a
0.05	5.7 ± 0.33 b	10.0 ± 1.00 b
0.10	6.0 ± 0.00 b	8.3 ± 0.67 ab
0.20	6.0 ± 0.00 b	8.7 ± 0.88 ab
0.40	5.7 ± 0.33 b	6.0 ± 0.00 a
IPT22	6.7 ± 0.33 b	9.0 ± 0.00 ab

[z]Means followed by the same letter within each column are not significantly different at $p < 0.05$ by Scheffe's Procedure.

Table 2.6 Mean number of days (± SEM) to first visual wilting symptom and total wilted. WT flowers were excised and incubated in tZR solutions and IPT22 in distilled water.

tZR (mM)	Days to first visual wilting symptom	Days to total wilted
0.00	3.0 ± 0.00 a[z]	6.0 ± 0.00 a
0.05	5.3 ± 0.88 ab	8.0 ± 0.58 ab
0.10	5.7 ± 0.33 b	8.3 ± 0.88 ab
0.20	6.0 ± 0.00 b	6.7 ± 0.33 ab
0.40	5.3 ± 0.33 ab	6.0 ± 0.00 a
IPT22	6.7 ± 0.33 b	9.0 ± 0.00 b

[z]Means followed by the same letter within each column are not significantly different at $p < 0.05$ by Scheffe's Procedure.

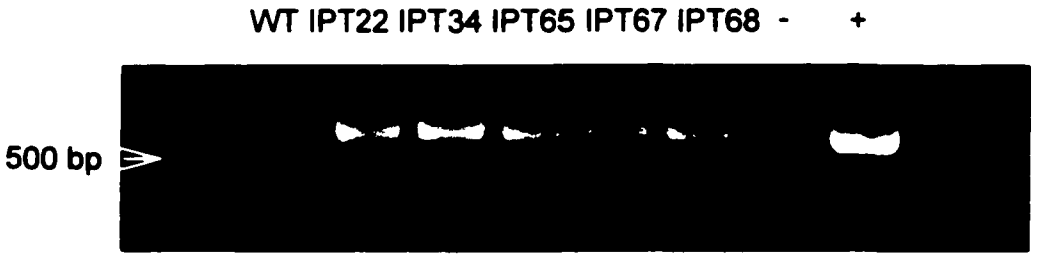

Fig. 2.1 PCR result of the *ipt* gene from genomic DNA extracted from young leaves of WT, transgenic IPT22, IPT34, IPT65. IPT67, and IPT68.

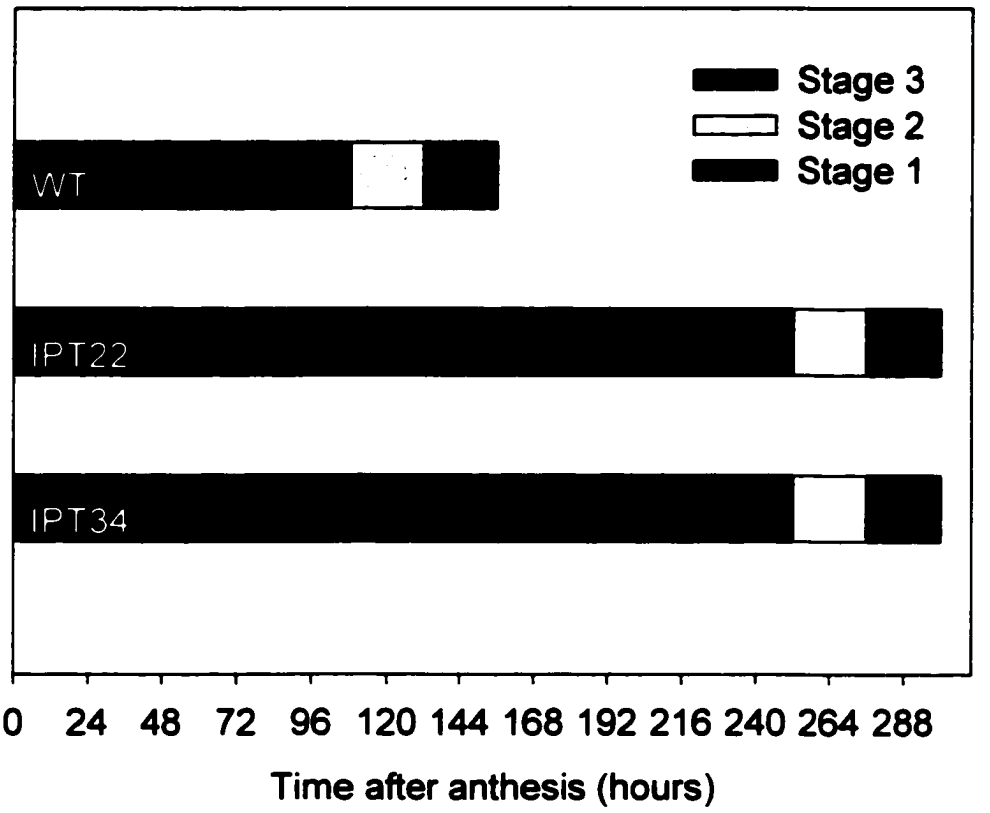

Fig. 2.2 Visual senescence ratings of WT, IPT22 and IPT34

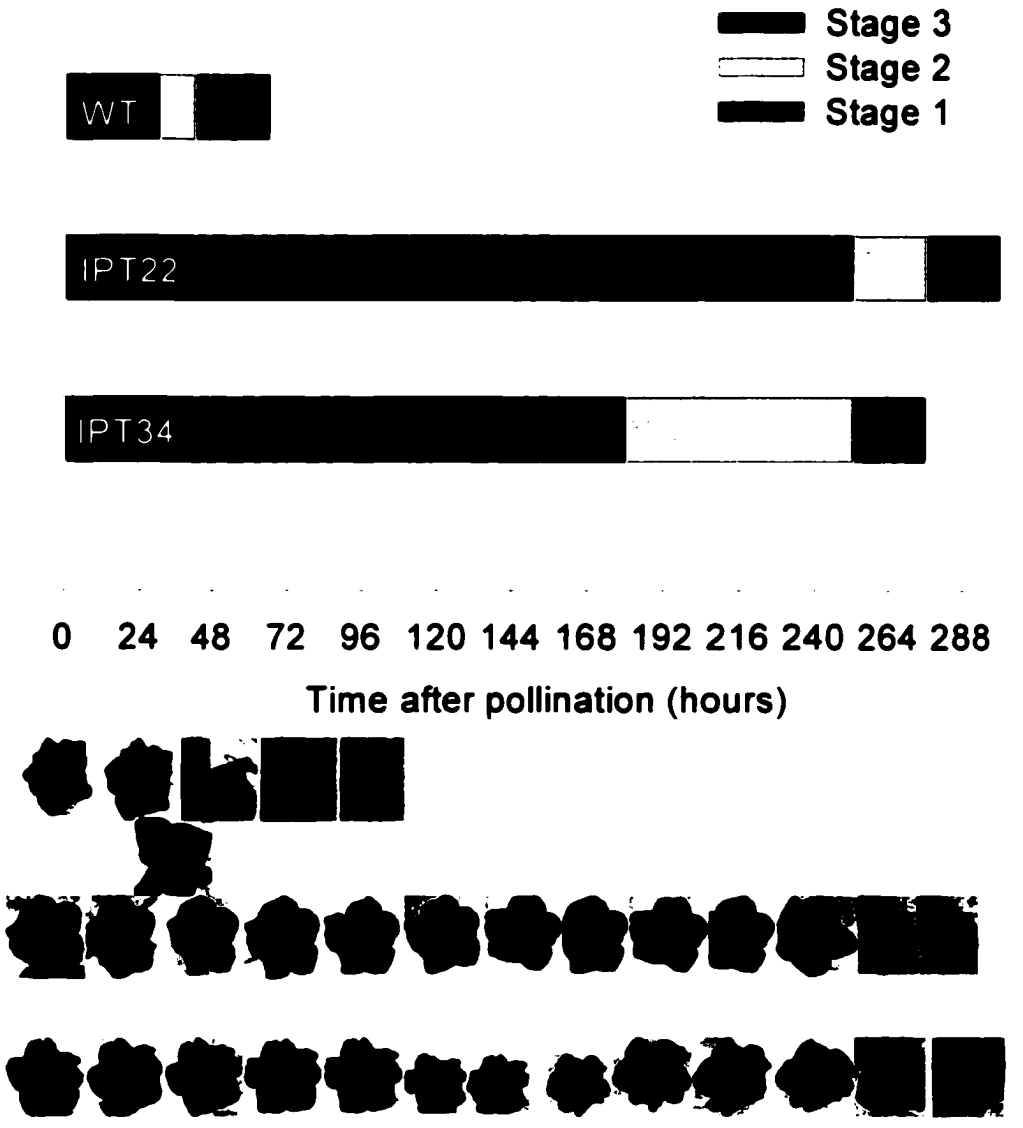

Fig. 2.3 Visual senescence ratings of WT, IPT22 and IPT34 during pollination induced senescence.

A. B.

Fig. 2.4 Visual symptom of color fading before wilting in transgenic lines. A. Picture taken at the day of anthesis. B. Ten days after pollination

Fig. 2.5 Corolla was partially pushed away by the development of ovary.

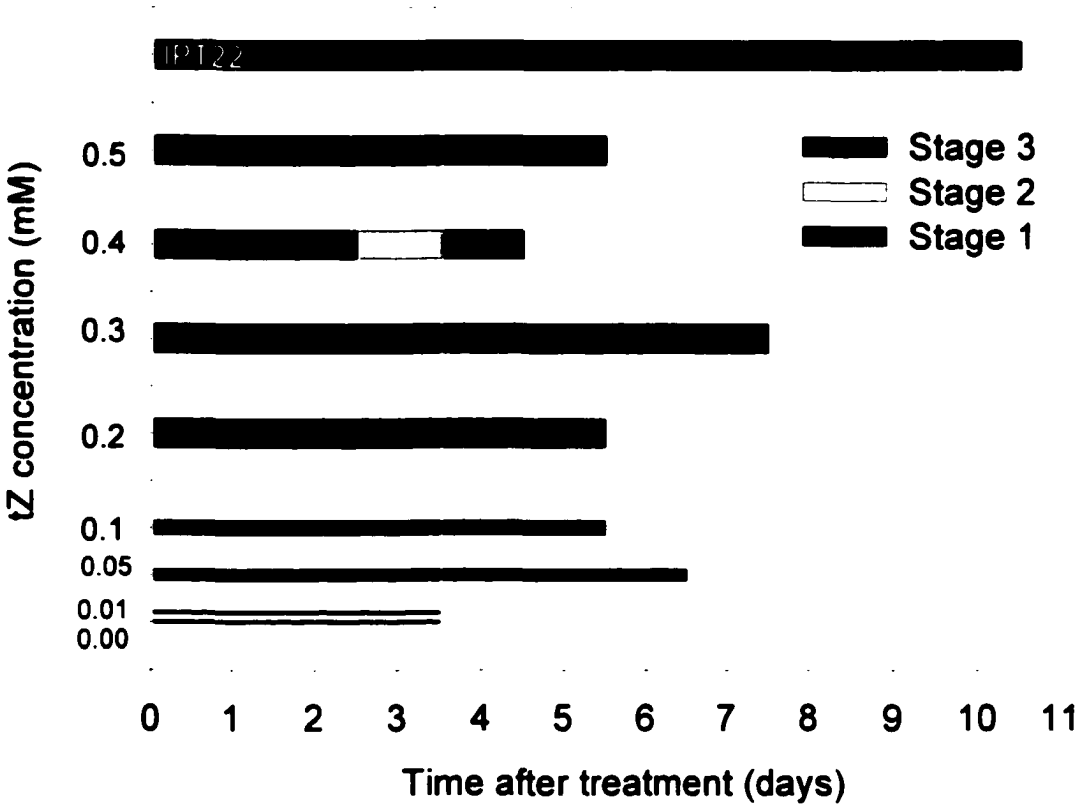

Fig. 2.6 Senescence ratings of WT corollas following exogenous applications of tZ compared to IPT22 corollas in distilled water.

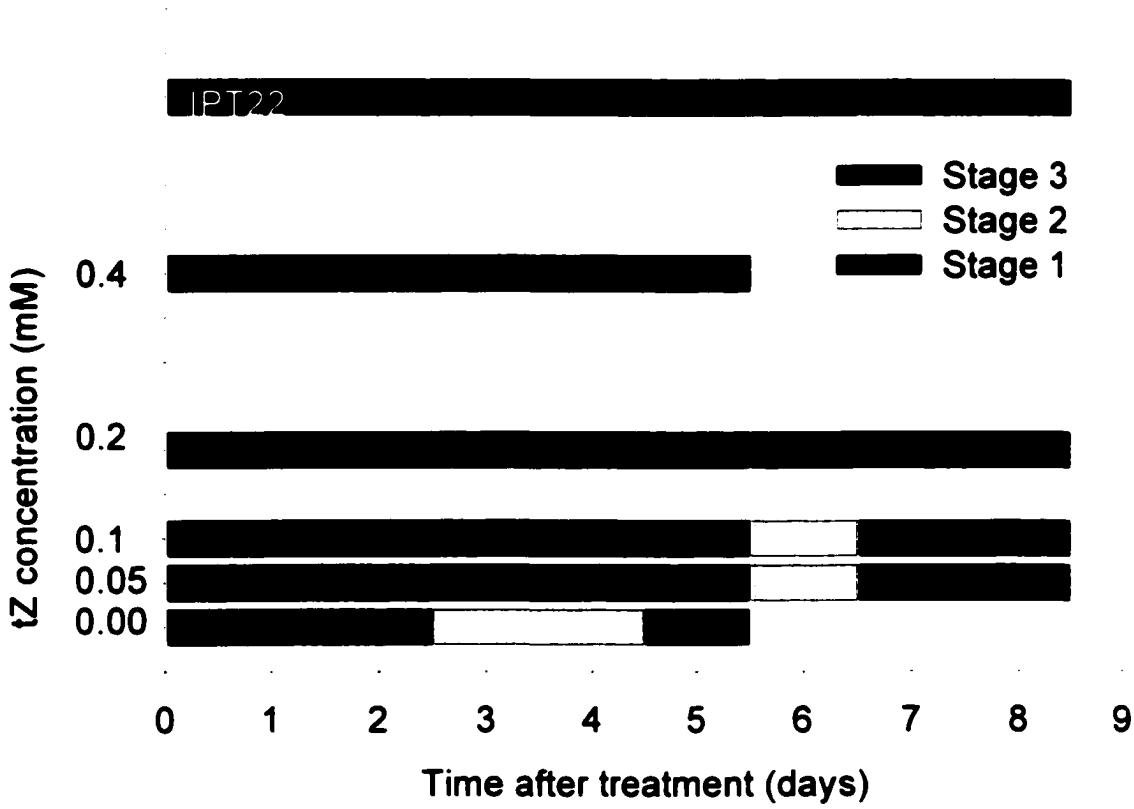

Fig. 2.7 Senescence ratings of WT corollas following exogenous applications of tZ compared to IPT22 corollas in distilled water.

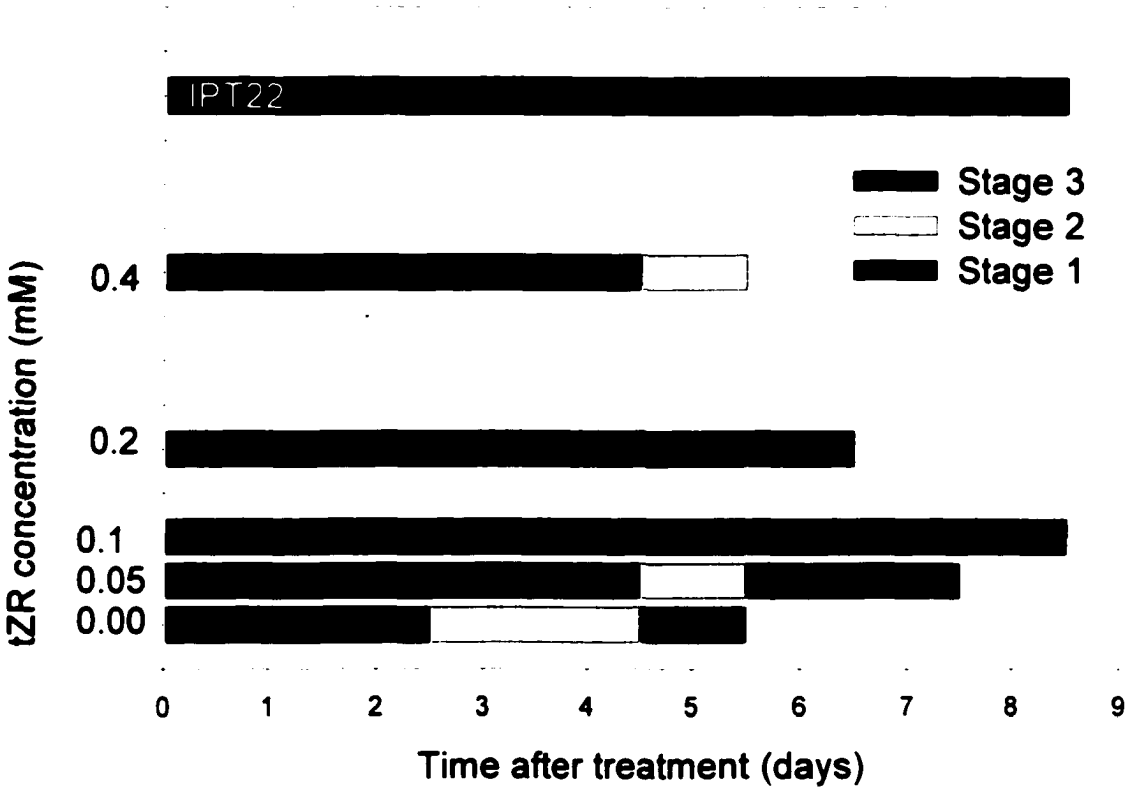

Fig. 2.8 Senescence ratings of WT corollas following exogenous applications of tZR compared to IPT22 corollas in distilled water.

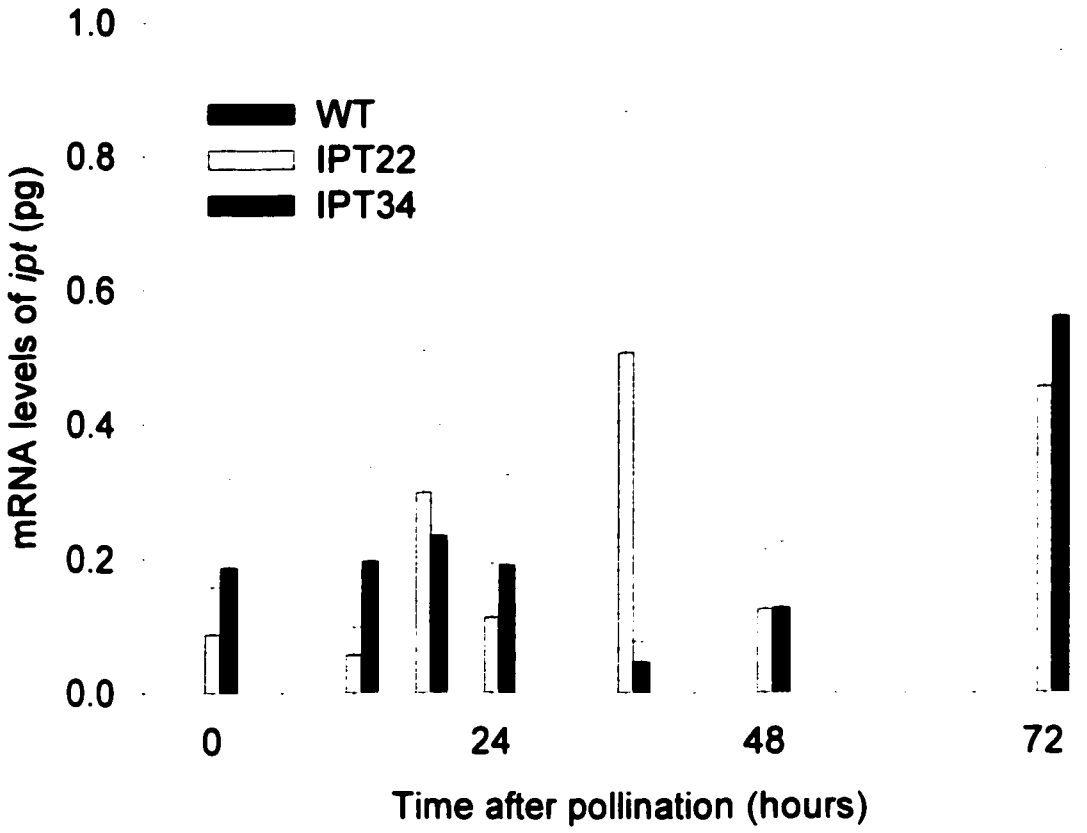

Fig. 2.9 Real time RT-PCR quantification of relative mRNA levels of *ipt* in WT, IPT22, and IPT34 corollas at 0, 12, 18, 24, 36, 48, and 72 hours after pollination (hap).

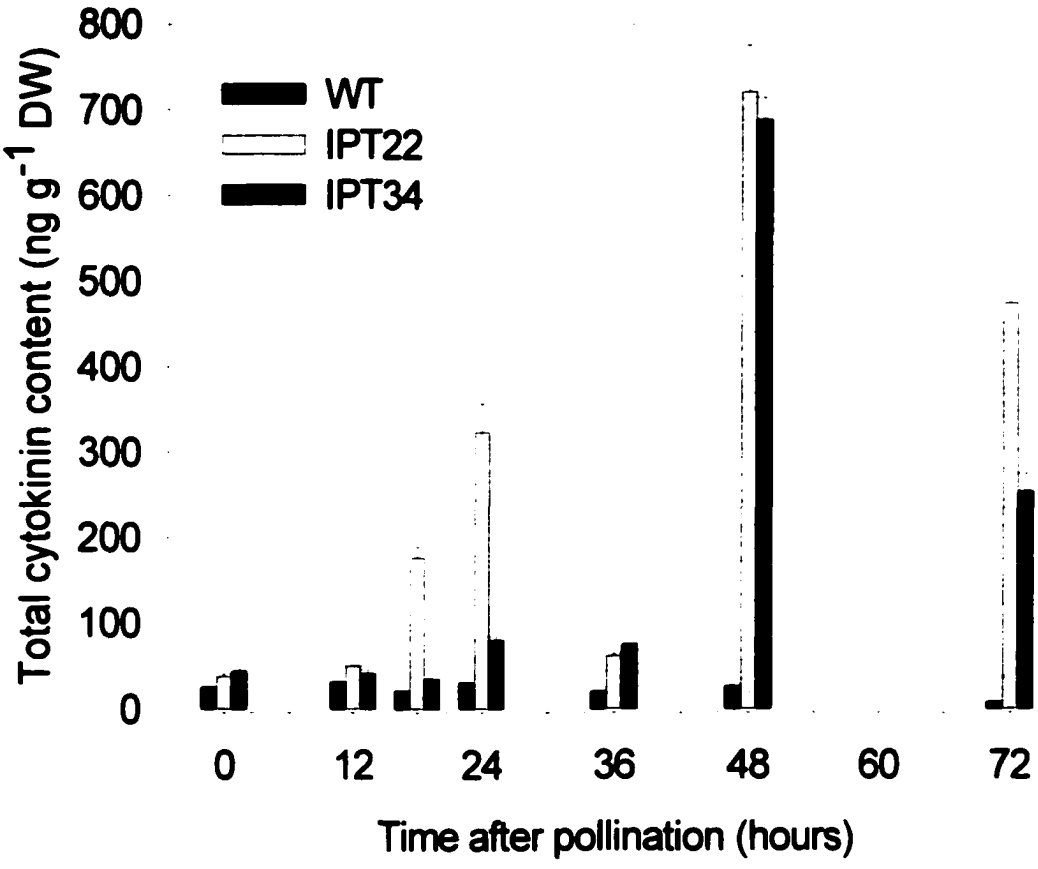

Fig. 2.10 Quantification of endogenous total cytokinin in corolla of WT, IPT22, and IPT34 at 0, 12, 18, 24, 36, 48, and 72 hap. The values presented here are the average of 3 replicates ± SEM.

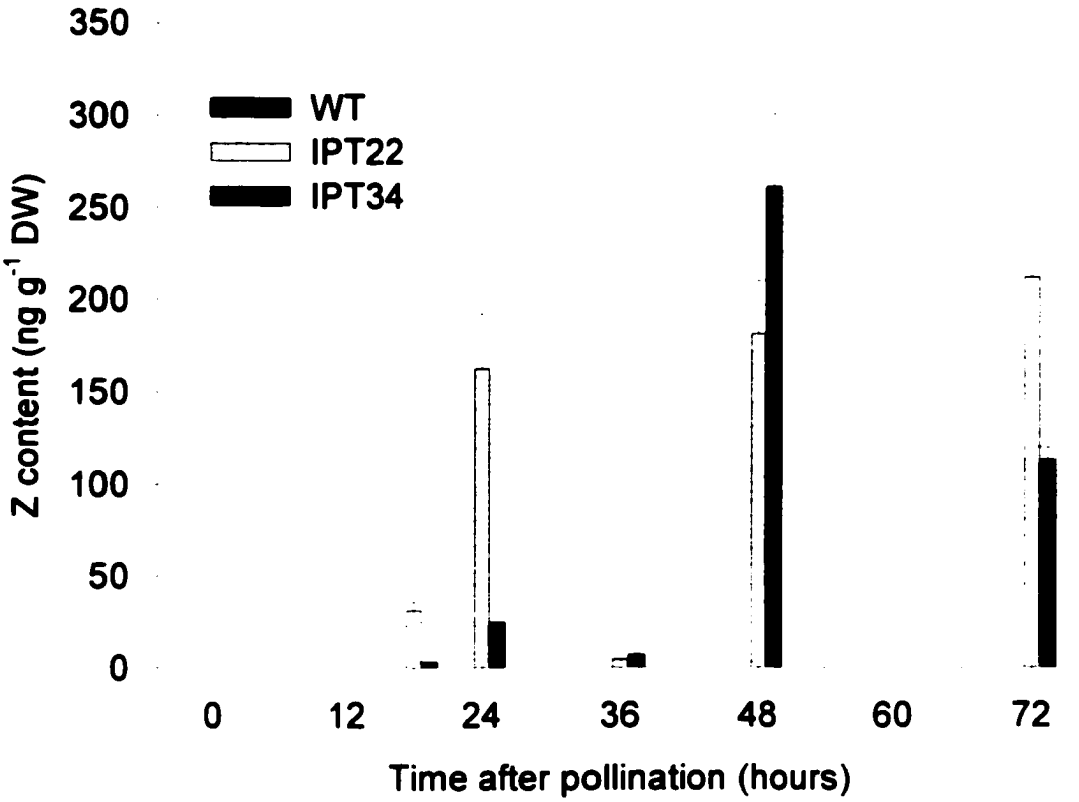

Fig. 2.11 Quantification of endogenous total Z in corolla of WT, IPT22, and IPT34 at 0, 12, 18, 24, 36, 48, and 72 hap. The values presented here are the average of 3 replicates ± SEM.

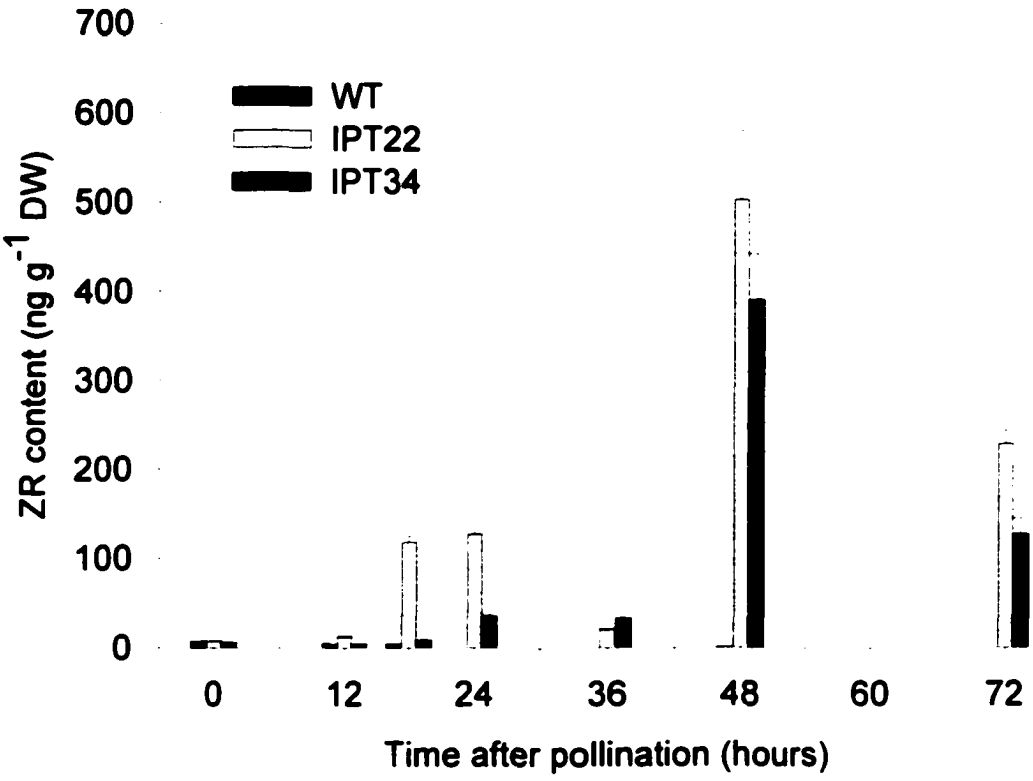

Fig. 2.12 Quantification of endogenous total ZR in corolla of WT, IPT22, and IPT34 at 0, 12, 18, 24, 36, 48, and 72 hap. The values presented here are the average of 3 replicates ± SEM.

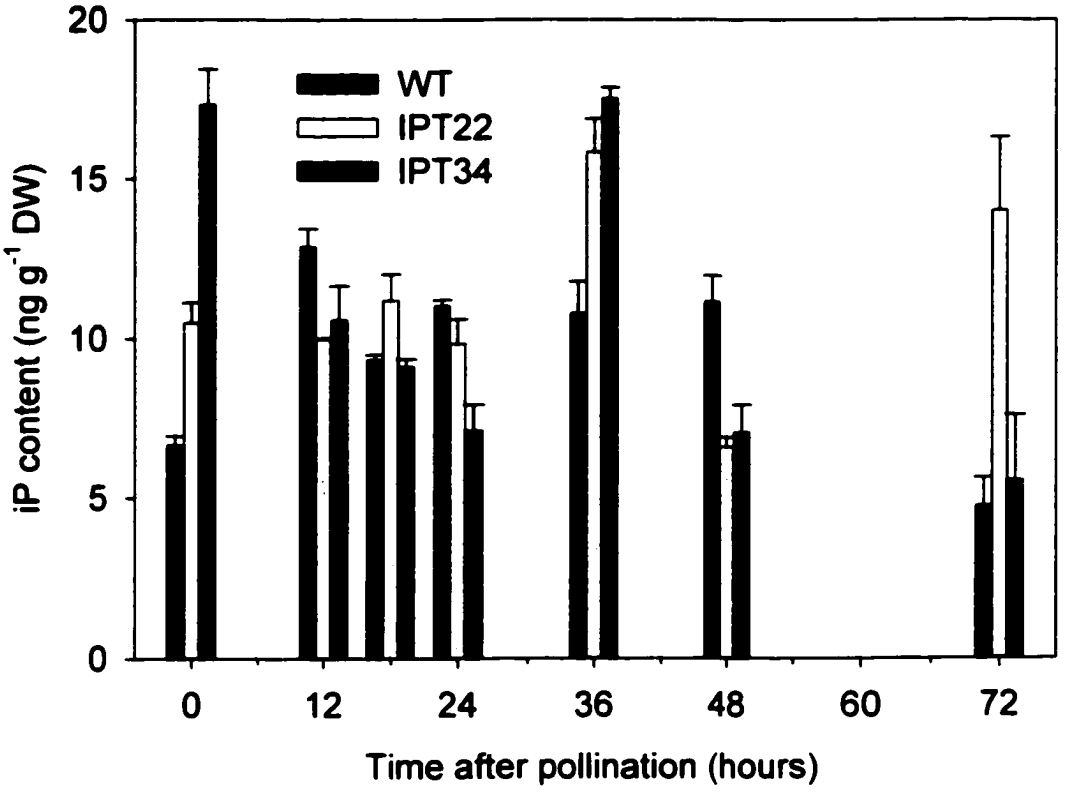

Fig. 2.13 Quantification of endogenous total iP in corolla of WT, IPT22, and IPT34 at 0, 12, 18, 24, 36, 48, and 72 hap. The values presented here are the average of 3 replicates ± SEM.

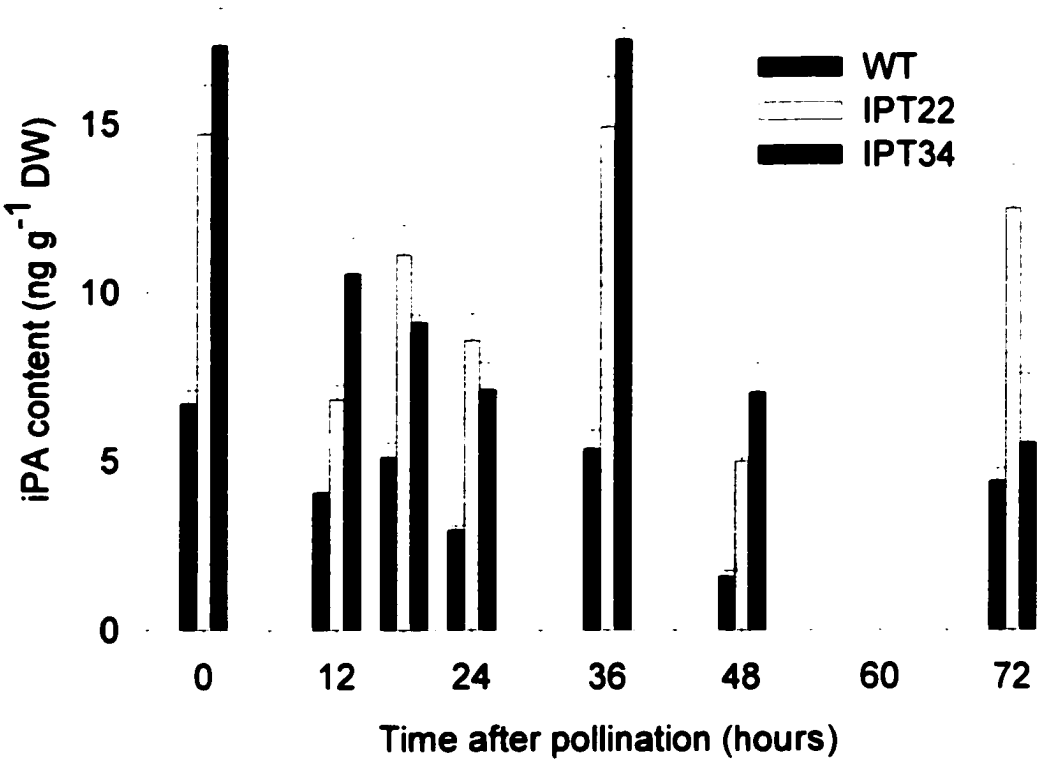

Fig. 2.14 Quantification of endogenous total iPA in corolla of WT, IPT22, and IPT34 at 0, 12, 18, 24, 36, 48, and 72 hap. The values presented here are the average of 3 replicates ± SEM.

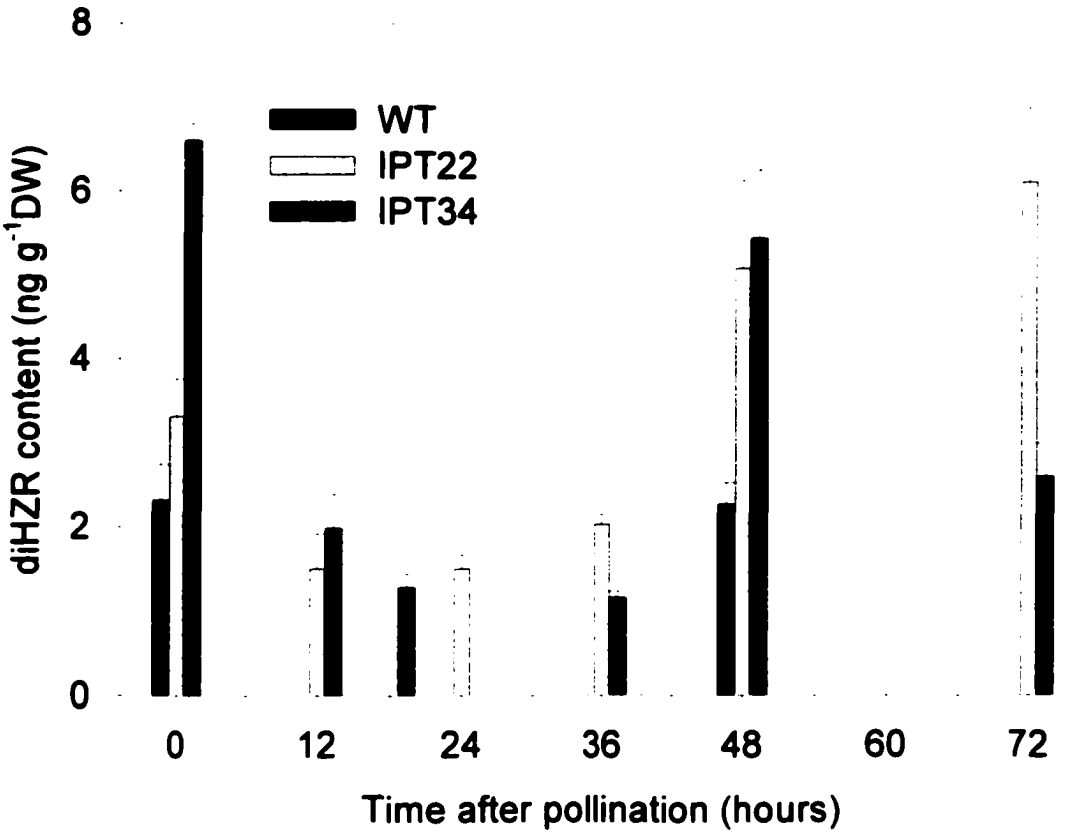

Fig. 2.15 Quantification of endogenous total diHZR in corolla of WT, IPT22, and IPT34 at 0, 12, 18, 24, 36, 48, and 72 hap. The values presented here are the average of 3 replicates ± SEM.

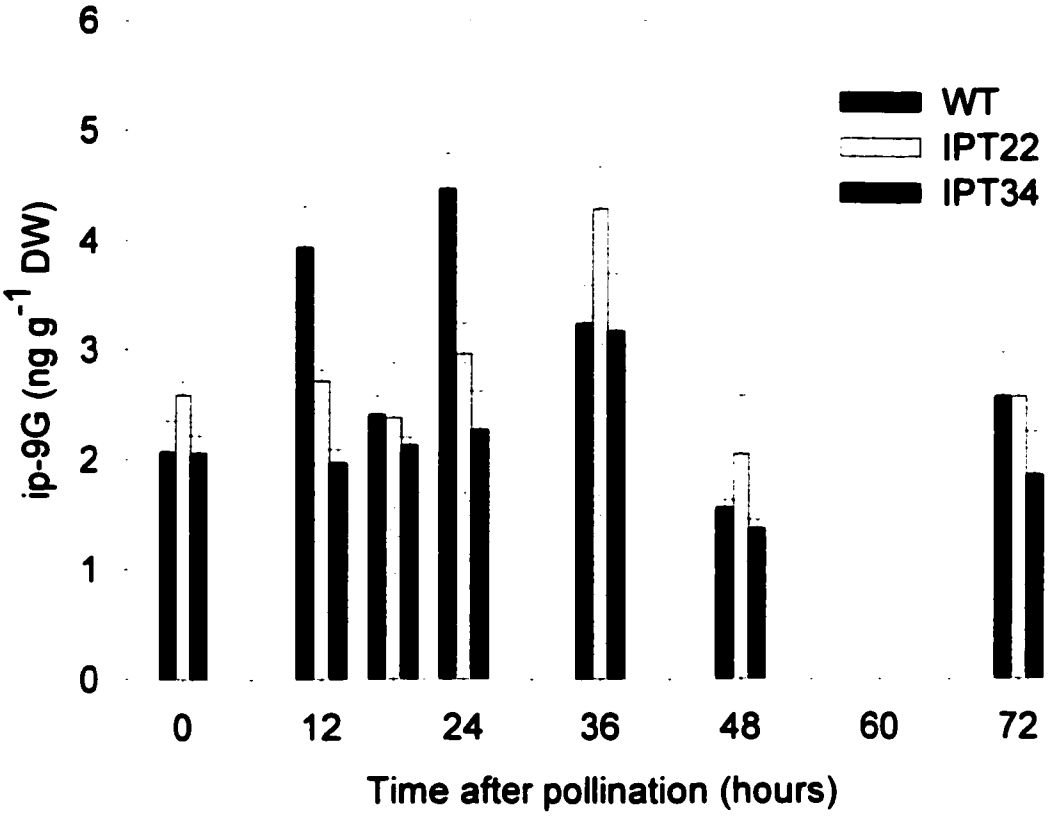

Fig. 2.16 Quantification of endogenous total ip-9G in corolla of WT, IPT22, and IPT34 at 0, 12, 18, 24, 36, 48, and 72 hap. The values presented here are the average of 3 replicates ± SEM.

Chapter 3

The interaction of elevated cytokinins with ethylene and ABA in SAG12-*ipt* petunias

3.1 Abstract

The interaction of cytokinins and ethylene and their regulation of senescence in SAG12-*ipt* petunias was investigated. The elevated endogenous cytokinins detected in transgenic corollas delayed senescence by altering ethylene production during both natural senescence and pollination-induced senescence. Moreover, cytokinins also altered the ethylene sensitivity of petunia corollas. These transgenic petunias required higher concentrations and extended ethylene treatments to initiate senescence. Ethylene treatment also resulted in altered up-regulation of the senescence-related gene *Phcp1* in IPT22 and IPT34 versus WT corollas. Increases in ABA detected during the late stages of corolla senescence in WT flowers were not detected in the transgenic corollas, suggesting that ABA level was suppressed by the high endogenous cytokinin levels in the early stage of flower senescence. Ethylene production and the corolla's sensitivity to ethylene play an important role in regulating petunia corolla senescence.

3.2 Introduction

Plant growth and development are regulated by the interaction of multiple plant hormones. These interactions involve both changing the balance among hormones and the plants sensitivity to hormones.

Exogenous application of ethylene to many types of flowers promotes petal senescence (Crocker and Knight, 1908; Zimmerman *et al.*, 1931). The visual symptoms of flower petals exposed to ethylene, such as wilting, are indicative of a loss in turgor (Nichols, 1968; Hanson and Kende, 1975; Mayak *et al.*, 1977). In the case of carnation, Lieberman *et al.* (1964) demonstrated that exogenous application of ethylene accelerated the loss of water from petals normally associated with senescence, and that water absorption rates were decreased within 2 hours of exposure to 2 μl/liter ethylene even though wilting symptoms were not observed until 6 hours after ethylene treatment (Mayak *et al.*, 1977). Treating carnation flowers with ethylene also led to an earlier increase in membrane permeability (Borochov and Faragher, 1983; Faragher and Mayak, 1984; Sylvestre and Paulin, 1987), and an earlier decline in polar lipid-bound fatty acids (Sylvestre and Paulin, 1987). Treatment with ACC (1-aminocyclopropane-1-carboxylic acid), the immediate precursor of ethylene, also increased ethylene production and accelerated visual symptoms that were associated with a decline in membrane fluidity, ATPase activity and sucrose uptake (Adam *et al.*, 1983).

The response of flowers to ethylene is dosage dependent, and acceleration of senescence is influenced by the concentration of exogenous ethylene, the duration of treatment, and the age of the flower. In carnation it has been determined that exogenous application of ethylene to induce corolla senescence is dependent on the concentration and duration of ethylene treatment (Uota, 1969; Barden and Hanan, 1972; Mayak and Kofranek, 1976). The response to ethylene treatment is affected by the age and the developmental stage of the tissue at the time of ethylene treatment (Halevy *et al.*, 1984). The sensitivity of corollas to ethylene treatments increased with age and thus more mature corollas responsed to lower concentration of ethylene (Barden and Hanan, 1972; Kende and Hanson, 1976; Mayak and Kofranek, 1976; Woodson *et al.*, 1985). Trewavas (1982) has suggested that plant tissues responses to hormones, such as ethylene, is due to sensitivity rather than the concentration of the hormone, and that sensitivity is the limiting factor in determining the responses of the plant. Other factors affecting the sensitivity of corollas to ethylene, include sucrose (Mayak and Kofranek, 1976), inorganic solutes (Hanson and Kende, 1975), and cytokinins (Mayak and Kofranek, 1976; Mor *et al.*, 1983; Cook *et al.*, 1985). These have all been found to decrease the sensitivity of the corolla to ethylene.

While ethylene appears to play a prominent role in petal senescence, other plant hormones have been implicated in petal senescence although their roles in this process are less clear (Borochov and Woodson, 1989). Cytokinins are considered to be anti-senescent hormones, as their application delays leaf senescence (Richmond and Lang,

1957; Kende, 1964; Van Staden *et al.*, 1968). Not only has the exogenous applications of cytokinins delayed corolla senescence (Mayak and Dilley, 1976a; Eisinger, 1977; Mor *et al.*, 1983; Kelly *et al.*, 1985; Cook *et al.*, 1985; Bossè and Van Staden, 1989), but endogenous levels of cytokinins declined with the aging of petals (Mayak and Halevy, 1970; Van Staden and Dimalla, 1980; Van Staden *et al.*, 1987). The interactions between cytokinins and ethylene were demonstrated when the application of cytokinins and cytokinin-like compounds altered ethylene production by delaying the ethylene-climacteric (Apelbaum and Katchansky, 1978; Mor *et al.*, 1983; Cook *et al.*, 1985).

Cytokinins also have an effect on the pathway of ethylene biosynthesis. Mor *et al.* (1983) demonstrated that carnation petals pretreated with BA (N^6-benzyl-adenine) were not able to convert ACC to ethylene, and did not produce increased levels of ethylene even when treated with exogenous ethylene. Other cytokinins, including zeatin, and kinetin also inhibited ethylene biosynthesis in this experiment. Similar results were found in intact flowers pretreated with BA (Cook *et al.*, 1985). In addition to cytokinins retarding and/or disabling ethylene production during the senescence of flowers, cytokinins also decrease the sensitivity of petals to ethylene. In the experiment of Eisinger (1977), carnations treated with kinetin were significantly less sensitive to exogenous ethylene. Thus cytokinins have been proposed to be an anti-senescence factor in flower petals (Eisinger, 1977; Mor *et al.*, 1983; Cook *et al.*, 1985).

In contrast to the cytokinins, ABA (abscisic acid) generally accelerates petal senescence and the climacteric production of ethylene (Mayak and Dilley, 1976b; Ronen

and Mayak, 1981; Borochov and Woodson, 1989; Vardi and Mayak, 1989; Müller *et al.*, 1999). Carnation flower petals pretreated with ABA were more sensitive to external ethylene treatments that resulted in an advanced ethylene climacteric and subsequent petal wilting (Ronen and Mayak, 1981). During flower senescence higher endogenous ABA levels have also been detected in roses (Mayak and Halevy, 1972; Borochov *et al.*, 1976) and carnations (Nowak and Veen, 1982; Eze *et al.*, 1986). Nowak and Veen (1982) demonstrated that increased ABA levels were detected in carnation petals before the loss of fresh weight, and the elevated ABA levels could be prevented by pretreating with silver thiosulfate. It was confirmed that higher ABA levels paralleled the increase of ethylene and the onset of irreversible wilting in carnation petals. Additionally, a small temporary increase in ABA was noted in the preclimacteric petals (Hanley and Bramlage, 1989). Unlike carnation where the endogenous ABA and senescence are dependent on ethylene production and sensitivity, the senescence of some other flowers, such as Amaryllidaceae, Liliaceae, Iridaceae and Asteraceae, do not appear to be regulated by ethylene (Woltering and Van Doorn, 1988; Van Doorn and Stead, 1994). In daylily, which is a model system for ethylene insensitive senescence, application of ABA hastened senescence-associated events, such as ion leakage, lipid peroxidation, and the activities and patterns from both proteinases and nucleases on activity gels (Panavas *et al.*, 1998). Moreover, five of six cDNAs cloned by differential display are prematurely up-regulated 3- to 45-fold by ABA (Panavas *et al.*, 1999). This evidence suggests that endogenous ABA levels are important for the progression of senescence in daylily flowers, which are not sensitive to ethylene, and also equally important for the regulation of senescence in ethylene sensitive flowers.

Cytokinins are generally considered to be antagonists of ABA with these two hormones having opposing effects in several developmental processes including stomatal opening (Blackman and Davies, 1984), cotyledon expansion and seed germination (Thomas, 1992). Cytokinin (N^6-benzylaminopurine)-treated cotyledons also had faster greening and plastid biosynthesis, whereas abscisic acid had an inhibitory effect on these processes (Kusnetsov *et al.*, 1998). There is a biochemical basis for cytokinin-abscisic acid (CK-ABA) antagonism. Cytokinins appear to antagonize many physiological processes thought to be mediated, all or in part, by ABA (Cowan *et al.*, 1999). For example, ABA induces leaf senescence and eventually abscission. The ABA induced senescence is reversed by exogenous application of cytokinins. Cytokinin-mediated release of seed dormancy contrasts with ABA inhibition of germination (Salisbury, 1994). However, little has been explored regarding their effects on flower senescence.

The purpose of this chapter is to determine how cytokinins delayed senescence in the SAG12-*ipt* transformed petunia corollas. The objectives are: 1). Determine the effect of increased cytokinins on ethylene production, by comparing the ethylene production patterns obtained from WT and IPT petunias flowers following pollination and during natural senescence. 2). Determine if SAG 12-ipt plants have altered sensitivity to ethylene. If the delayed flower senescence observed in IPT petunia is not due to decreased ethylene production and/or delayed production peaks, the IPT petunia might have altered sensitivity to ethylene. Petunia flowers will be treated with exogenous ethylene. To evaluate their response or sensitivity to this treatment ethylene production,

rates of flower senescence and expression of the senescence-related gene Phcp1 (*Petunia hybrida* cysteine protease 1) will be determined. 3). Determine if ABA is involved in flower senescence. These experiments will be conducted because plant development is not usually regulated by a single hormone but by the interaction among, or between multiple hormones.

3.3 Material and methods

3.3.1 Plant Material and growing conditions

Petunia plants (*Petunia hybrida* 'V26') wild type (WT) and previously transformed with P_{SAG12}-*ipt* (Gan and Amasino, 1995) (IPT22, and IPT34) were grown in a greenhouse. The cultural practice was as described in chapter 2.

3.3.2 Ethylene production of petunia flowers

The biosynthesis of ethylene by whole flower on the plant was measured during natural senescence and following pollination. Ethylene production from corollas removed from the plant at various times after pollination was also measured. Flowers were emasculated before anther dehiscence (one- to two days before anthesis). To measure ethylene production from the same whole flowers on the plant, one flower was enclosed in a 450 mL chamber. To prevent any leakage, siliconized acrylic latex (Elmer's Squeez'N Caulk, Elmer's productions, INC., Columbus, OH) was generously applied all over any possible openings. Four flower replicates were performed. The chamber was sealed with a rubber cap for one hour at every sampling time point before an air sample was withdrawn (Fig. 3.1). A 1ml air sample was obtained by syringe at 0, 3, 6, 12, 18, 24,

36, 48, 72, 96, and 120 hap for the pollination-induced senescence, or collected every 24 hours at the same time of the day for natural senescence. After the air sample was collected the sealing cap was removed. Air samples were analyzed using a gas chromatograph equipped with an Haysep R packed column and flame ionization detector (Varian, Walnut Creek, California).

To pollinate flowers, the stigmatic surface of a flower was dusted with pollen from a freshly dehisced anther from the same plant. To determine ethylene production from corollas after pollination, 12 flowers were sampled at 0, 12, 18, 24, 36, 48, and 72 hap. Three corollas as a group were weighted and carefully twisted into a 25 mL vial then sealed with a screwed cap with a rubber septum for 20 minutes. One mL of air sample was removed from the vial and the ethylene level was determined with a gas chromatograph as described. Experiments were conducted three times with similar results. Data presented here are from one experiment and represents an average of 4 ethylene measurements ± SEM (ppb for whole flower or nl g^{-1} hr^{-1} for corolla).

3.3.3 Ethylene sensitivity

To determine ethylene sensitivity, petunia flowers were treated with exogenous ethylene. Experiment 1: Flower samples were treated with 2 ppm ethylene, and flower samples were collected according to the time points up to 48 hours, and ethylene production were also measured. Experiment 2: After 2 ppm ethylene treatment for 12 hours, flower samples were removed and ethylene production was measured. Experiment 3: Study of visual symptom by exogenous ethylene treatment. For the exogenous

application of ethylene, these flowers were sealed in a 24L air-tight chamber (Fig. 3.2) until sampling time points. For the ethylene sensitivity experiments, emasculated petunia flowers were harvested the day of anthesis, and 12 flowers of each line were grouped into 4 culture tubes with distilled water (Fig. 3.3).

Ethylene was introduced by syringe injection into the chamber to obtain a final concentration of 2 ppm, and air was injected for the air control group. Flowers were removed at 0, 3, 6, 9, 12, 18, 24, 36, and 48 het (hours after ethylene treatment), visual symptoms were observed, and flowers sat in air in the hood for 20 minutes to allow treated ethylene to diffuse away. After removal of styles, stigma, and ovary, only corollas were sampled for ethylene production. Immediately after the gas sample was taken from the vial, the corolla samples were quickly frozen in liquid nitrogen and stored at -80°C for RNA extraction, cytokinin, and ABA measurements. Data represent the mean ethylene production ± SEM. All experiments were performed a minimum of three times with similar results. Data shown here are from one replicate.

For another experiment to evaluate ethylene sensitivity, flowers were treated with 2 ppm ethylene (and an air control group as previous described) in the chambers for 12 hours and removed. The visual symptoms were photo-documented every 24 hours until 96 hours after treatment. These corolla samples were also collected accordingly and frozen for RNA extraction. The RNA samples were used to determine the *Phcp1* expression for the ethylene sensitivity study.

3.3.4 Cytokinin and ABA measurements

Corolla samples were collected at 0, 12, 18, 24, 36, 48, 72 hap (hours after pollination) and 12 hours after 2 ppm exogenous ethylene treatment (see above). Six corollas were pooled together at each time point. Tissue samples were first lyophilized overnight. Tissue (approximately 100 mg per sample) was ground in liquid Nitrogen and ABA was extracted in 100% ethanol for 30 min. After nine volumes of 40 mM ammonium acetate (pH 6.5) were added to the extract, ABA was isolated on C18 SepPaks (Waters Assoc., Bedford, MA), purified and quantified in triplicate samples using a previously described combined HPLC-immunoassay method (Banowetz, 1992). The immunoassay utilized a monoclonal antibody to ABA (Banowetz *et al.*, 1994). The extraction, purification, and quantification were kindly completed in the lab of Dr. Banowetz in the University of Oregon.

3.3.5 RNA extraction

RNA was extracted from corolla samples using TRIZOL reagent (Gibco BRL, Rockville, Maryland) as described in chapter 2.

3.3.6 Quantification of *Phcp1* expression

By running preliminary experiments to optimize the parameters for PCR, conditions were transferred successfully to real time PCR settings. To determine the expression of the *Phcp1* gene, the conditions of the real-time RT-PCR settings using the iCycler iQ real-time PCR detection System were previously described in 2.3.6. Primers were constructed to amply a 112 bp amplicon of *Phcp1*. These primers: forward 5'-GAA CTT

CGC CGA TGA GAA TC-3'; reverse 5'-AGC GAA GGA GAG AGC ATG AC–3'. Samples were run in triplicate with similar results. Data presented are from one replication.

3.4 Results

3.4.1 Characterization of ethylene synthesis from the flowers of SAG12-*ipt* and Wild type petunias

The ethylene biosynthesis pattern from the whole petunia flower was determined from both natural senescence (un-pollinated) (Fig. 3.4) and pollination-induced senescence (Fig. 3.5). During natural senescence, the ethylene production for WT flowers peaked at 5 days after anthesis and then decreased at 7 days after anthesis. Another peak was observed at 10 days after anthesis, then production declined. IPT22 shared the same pattern until day 6 and then exhibited higher amounts of ethylene production. The production pattern increased until 12 days with a similar decline in production detected. .

Following pollination (Fig. 3.5), all flowers exhibited the first ethylene peak at 3 hap, followed by a second peak at 24 hap for both IPT34 and WT. WT and IPT34 exhibited a third peaked at 48 hap. Ethylene production from IPT34 flowers during this last peak was extended through 96 hap while ethylene production from WT flowers declined again at 72 hap. IPT22 did not exhibit the second production peak as IPT34 and WT, however, a latter ethylene peak similar to the third peak of IPT34 was observed.

Ethylene production from corollas after pollination is presented in Fig 3.6. The patterns were similar to the results from whole flower except there was no peak detected at 3 hap. WT corollas exhibited the first ethylene production peak at 24 hap followed by a peak at higher levels at 48 hap that coincided with the dramatic visual symptom of corolla wilting. IPT34 also exhibited the same production pattern, however, the production levels were in contrast to WT, with a second peak that was lower than the first. IPT22 did not show the first peak as observed in IPT 34 and WT, while a production peak was detected at 48 hap that was not different from WT statistically, yet was higher than IPT34. Flowers from both IPT22 and IPT34 did not show any visual symptoms of senescence within the time points (72 hap) in this experiment.

3.4.2 Ethylene production and sensitivity of petunia corolla

After exogenous application of ethylene, WT corolla ethylene production peaked at 9 and 12 hours, while transgenic IPT22 and IPT34 showed production peaks at 24 and 36 hours respectively (Fig. 3.7). However, the elevated ethylene production from IPT22 corolla was not as great as that observed from WT and IPT34, and might not display a different production peak. Visual wilting symptoms were observed in WT corolla after 48 hours of treatment with ethylene, while IPT22 and IPT34 did not exhibit any visual symptoms during the treatments (48 hours). In the air control group (Fig. 3.8), only WT exhibited a significant peak at 36 hours after treatment, while transgenic IPT22 and IPT34 did not show any significant elevated ethylene synthesis.

3.4.3 Expression of *Phcp1* and visual symptom of ethylene sensitivity

When flowers were exposed to 2 ppm ethylene for 12 hours, followed by incubation in air in order to observe the symptoms of corolla senescence, the WT corollas exhibited the first visual symptoms at 24 hours after treatment, while IPT22 and IPT34 did not show symptoms of wilting until 48 hours after that (72 hours after treatment) (Fig. 3.9). Low levels of *Phcp1* transcripts were detected at anthesis (shown as time point –12 hours after treatment) in WT and transgenic corollas (Fig. 3.10). Elevated transcripts were observed after 12 hours exogenous ethylene treatment from WT and transgenic lines (at 0 time point), where the highest level was detected in IPT34. High levels of transcript in IPT22 and IPT34 corollas were maintained until 72 hours, and declined at 96 hours after ethylene treatment was removed. At this final time point, both transgenic corollas were completely wilted (Fig. 3.9). The transcript levels in WT corollas peaked at 12 hours treatment (at 0 time point) and then declined. In the air control group (Fig. 3.11), WT did not show visual symptoms of senescence until 48 hours of treatment (treated with injection of air), and transgenic corollas did not wilt until 96 hours after treatment. The *Phcp1* transcript level of WT peaked at 48 hours after air treatment, and decrease at 72 after air treatment. In the transgenic lines, the pattern of *Phcp1* expression was similar to ethylene treatment group, but was delayed with the peak observed at 24 hours after treatment.

3.4.4 Endogenous cytokinin levels after ethylene treatment

The total cytokinin content was low in corollas of WT, IPT22, and IPT34 flowers before ethylene treatment. After treatment with 2 ppm ethylene for 12 hours, the total

cytokinin content was elevated 74 times in IPT22 and 56 times in IPT34 with only a slight increase (2 fold) detected in wild type corollas (Fig. 3.12). The Z form cytokinins contributed to the major increases detected in transgenic lines. The Z contents were 758.74 (± 225.6) and 789.34 (± 95.73), and ZR contents were 2014.85 (±711.65) and 1597.76 (±331.4) ng g^{-1} DW in IPT22 and IPT34 respectively, while low or below detectable levels were found before ethylene treatment (Table 2.1).

3.4.5 Effect of elevated cytokinin on endogenous ABA

Endogenous ABA levels were quantified to characterize the production pattern and to determine its association with pollination-induced senescence in petunia flowers (Fig. 3.13). ABA was detected at anthesis in all flowers, and increased beginning at 24 hours after pollination in WT corollas, reaching 900 and 1000 ng g^{-1} DW at 48 and 72 hours after pollination respectively. IPT22 and IPT34 did not exhibit increases in ABA similar to those detected in WT corollas and the highest levels detected were only about 200 ng g^{-1} DW.

3.5 Discussion

The purpose of these experiments was to examine the effects of elevated endogenous cytokinins on delaying senescence. Two approaches toward ethylene's interaction with cytokinins were investigated. The first approach was to characterize the ethylene production pattern since ethylene has been proven to play a regulatory role in corolla senescence. The second approach was to determine if higher cytokinin levels altered the corollas sensitivity to ethylene.

Flowers during natural senescence (Fig. 3.4) exhibited the same pattern of ethylene production in both WT and IPT22 before 6 days after anthesis (DAA). The ethylene production of WT corollas peaked at 5 and 6 DAA where the corolla exhibited visual symptom of wilting at 5 DAA and were completely wilted at 6 DAA. In the transgenic line IPT22, ethylene production increased gradually until 11 DAA when the first wilting symptoms were observed (Fig. 2.2) and decreased afterwards when the IPT22 corollas were completely wilted. The extended ethylene production from IPT22 corollas was a result of the delayed senescence of the corolla tissue, and a significant production drop was associated with the wilting and death of the corolla. The patterns of ethylene production by the flowers was climacteric-like, similar to that found previously for petunia (Whitehead and Halevy, 1989; Porat *et al.*, 1993), and other flowers such as carnation (Wu *et al.*, 1991) and orchid (Goh *et al.*, 1985). At advanced stages of senescence, the ethylene production dropped as there was no more living tissue to maintain ethylene production. Elevated endogenous cytokinins altered the ethylene production during natural senescence by delaying the ethylene climacteric and subsequently extended the life of the flowers.

Pollination hastened the corolla senescence of WT petunia flowers. The results of earlier visual wilting symptoms (Fig. 2.2, 2.3) and ethylene production patterns (Fig. 3.4, 3.6) confirmed previous observations in petunia that pollination–induced petal senescence is accompanied by earlier increases in ethylene production (Halevy *et al.*, 1984). In the whole flower ethylene production after pollination (Fig. 3.5), the first peak

observed in all flowers at 3 hap was from the gynoecium, because this peak was not observed in the ethylene production of corollas only (Fig. 3.6). This result confirmed the statement of Pech *et al.* (1987) that an increase in ethylene synthesis is detectable from the styles and stigmas of petunia (*Petunia hybrida L. 'Pink Cascade'*) within 2-3 hours after pollination. A climacteric peak of ethylene production was observed at 48 hap in WT flowers when the corolla wilted completely. This peak is believed to be the result of autocatalytic ethylene production and has been shown to be associated with corolla senescence following a compatible pollination (Singh *et al.* 1992a). In the evaluation of corolla ethylene production after pollination, the IPT34 corollas showed the same pattern as WT corollas while IPT22 did not exhibit the first peak at 24 hap (Fig. 3.5). The 2nd production peak was associated with the visual wilting symptoms in WT. This result confirmed the observation of Halevy *et al.* (1984) in petunia that the pollination-induced petal senescence is accompanied by increases in ethylene production. The timing of the climacteric production peak that leads to flower senescence varied in different studies. In our studies, ethylene peaked at 48 hap for *Petunia x hybrida* 'V26', compared to 18 hap in *Petunia inflata* 'Fries' (Singh *et al.*, 1992a) and 20 hap in *Petunia hybrida* L. 'Pink Cascade' (Whitehead *et al.*, 1984).

The delay of corolla senescence was associated with the elevated endogenous cytokinin content in both transgenic lines (Table 2.1). The ethylene production patterns of transgenic petunias, IPT22 and IPT34, were different as they had different levels of cytokinins at various time points after pollination. At 24 hap IPT34 and WT corollas had a peak of ethylene production but this peak was not detected in IPT22 corollas. IPT22

corollas had higher cytokinin levels at 24 hap than IPT34 corollas (Fig. 2.10) and this may have accounted for the inhibition of ethylene production. This result confirms that cytokinins affect ethylene production in petunia. This effect has been previously reported in carnation flowers where treatment with 5 ppm BA inhibited ethylene production (Cook *et al.*, 1985) and at 0.1 mM prevented the autocatalytic rise in ethylene production (Mor *et al.*, 1983). Kinetin application at 5 μg/ml also reduced peak ethylene production by 55% and delayed the peak by 1 day (Eisinger, 1977). The reduced peak production was also observed in IPT34 at 48 hap.

Since tissue sensitivity to ethylene is an important factor in the control of flower senescence, to understand the nature of the 'sensitivity factor' is central to the knowledge of the mechanisms involved in the control of ethylene action in flower senescence. Although the involvement of ethylene in flower senescence varied among different species, mature flowers of carnation and petunia are sensitive to ethylene and exposure to ethylene will accelerate their senescence and endogenous ethylene production (Maxie *et al.*, 1973; Whitehead *et al.*, 1984; Whitehead and Vasiljevic, 1993). Wild type and IPT flowers were treated with ethylene to determine if elevated endogenous cytokinins affected ethylene sensitivity. Changes in ethylene sensitivity were evaluated by determining the effects of exogenous ethylene treatment on flower senescence, ethylene production and expression of the senescence-related gene *Phcp1*.

Following treatment with ethylene, WT flowers had a peak of ethylene biosynthesis at 9 hours (Fig. 3.7). When isolated flowers were treated with air (controls) this ethylene

biosynthetic peak occurred at 36 hours (Fig. 3.8). This result demonstrated that the flowers of 'V26' were sensitive to ethylene and underwent accelerated ethylene biosynthesis in response to exogenous ethylene application, and confirmed 'V26' had a similar response to ethylene application as previously reported for petunia (Whitehead and Vasiljevic, 1993). The elevated endogenous cytokinins effects on ethylene sensitivity are revealed by altered patterns of ethylene biosynthesis observed in transgenic lines. Although both transgenic lines had different responses to ethylene application, their ethylene production was not accelerated as rapidly as observed in WT. Enhanced ethylene production by transgenic flowers was delayed 15 to 27 hours compared to control flowers. This result demonstrated elevated endogenous cytokinins in IPT22 and IPT34 corollas altered the sensitivity to exogenous ethylene.

In further ethylene sensitivity experiments designed to evaluate ethylene effects on accelerating corolla senescence, corollas were treated with 2 $\mu L\ L^{-1}$ ethylene for 12 hours and then evaluated over a period of 96 hours after treatment. There was a difference in flower longevity between the ethylene treatment group and the air control group, as well as the WT and transgenic lines (Fig. 3.9). This study confirmed that ethylene hastened flower senescence as previously observed in petunia (Whitehead *et al.*, 1984), carnation (Maxie *et al.*, 1973; Whitehead and Vasiljevic, 1993), and Chrysanthemum (Bartoli *et al.*, 1997). A significantly elevated cytokinin content following 12 hours of ethylene treatment (Fig. 3.12) was found in transgenic lines IPT22 and IPT34, but not in WT corollas suggesting that the elevated cytokinin levels resulted in delayed senescence by decreasing the ethylene sensitivity. This result confirmed the previous reports that

exogenous application of cytokinin decreased ethylene sensitivity in carnation (Eisinger, 1977) and petunia (Taverner *et al.*, 1999) flowers. By comparing the visual wilting symptoms in transgenic lines following ethylene treatment to the air control group, the ethylene treatment did hasten the senescence of IPT petunia flowers. This suggested IPT22 and IPT34 were not ethylene insensitive, yet their sensitivity was decreased by elevated endogenous cytokinins. Our studies of ethylene treatments not only demonstrated the importance of ethylene sensitivity in flower senescence, but also proved that delayed senescence observed in IPT petunias was a result of elevated cytokinins decreasing ethylene sensitivity.

Cysteine proteases are associated with programmed cell death in plants (Koltunow *et al.*, 1990; Granell *et al.*, 1992; Ye and Varner, 1996; Xu and Chye, 1999; Solomon *et al.*, 1999; Wagstaff *et al.*, 2002). In order to further characterize PCD in petunia flower senescence, transcript levels of *Phcp1* that encodes a petunia cysteine protease (Tournaire et al., 1996) were examined in the ethylene sensitivity experiments (Fig. 3.10; 3.11). Our results showed the transcript peak pattern of WT was shifted toward earlier time points after ethylene treatment compared with air control. This confirmed the strong association between *Phcp1* transcripts and senescence in WT petunia. Ethylene treatment resulted in the upregulation of *Phcp1* transcripts in both WT and IPT corollas. Transcript levels then decreased in WT flowers as they began to show visual symptoms of senescence. In contrast, IPT flowers which were not wilting until 96 hours after removal from treatment maintained higher levels of transcript. This result suggested that accumulated higher cytokinins in corollas after 12 hours ethylene treatment (Fig. 3.12) delayed the

senescence process and this was accompanied by an altered pattern of gene expression for the cysteine protease, *Phcp1*. However, the down regulation of this gene by cytokinin was not observed as previously reported (Tournaire *et al.*, 1996).

ABA has also increased flower senescence (Borochov and Woodson, 1989; Bianko *et al.*, 1991; LePage-Degivry *et al.*, 1991; Garello *et al.*, 1995; Panavas *et al.*, 1998). Moreover, in these flowers, ABA applications led to an early increase in ethylene evolution and an acceleration of flower senescence. In our study, elevated ABA levels were also detected in WT corollas and peaked at 48 and 72 hours after pollination when flowers were severely wilted. Transgenic lines had relatively low levels of ABA through all time points (Fig. 3.13). Corolla wilting caused by the loss of water might lead to elevated ABA levels due to water stress in petals. In roses and carnations ABA levels also did not increase until the last stages of senescence associated with decreases in water potential (Mayak and Halevy, 1972; Eze *et al.*, 1986).

The suppressed production of ABA in transgenic lines may suggest interactions between ABA and cytokinins, but previous reports on this issue remain limited for flower senescence. There have been observations that cytokinins act as antagonists of ABA with opposing effects in several developmental processes including the regulation of stomatal opening (Blackman and Davies, 1984), seed germination and cotyledon expansion (Thomas, 1992). Moreover, Grossmann and Hansen (2001) proposed ethylene triggered ABA might be involved in other processes that coincide with a strong stimulation of ethylene biosynthesis. Our results also suggested that elevated ABA, detected in late time

points rather than the ethylene peak observed in earlier time points that coincided the corolla senescence, was a response to senescence rather initiating the senescence process.

In our SAG12-*ipt* petunia studies, the results confirmed and demonstrated the anti-senescence effects of elevated endogenous cytokinins. Cytokinins delayed senescence by effecting ethylene biosynthesis, decreasing ethylene sensitivity, as well as suppressing ABA production. These results obtained from the petunia as a model system can be applied to improve other crops that are sensitive to ethylene, and further provide an understanding of the hormonal regulation of senescence that is necessary to improve post harvest flower quality. Applications to bedding plants to enhance blooming period and cut flowers to increase their vase life will significantly increase the value of these flowers.

The conclusions that can be drawn from these experiments include: 1). The elevated endogenous cytokinins in IPT22 and IPT34 flowers altered ethylene production patterns compared to WT, but did not significantly decrease overall rates of production. The ethylene production was not totally suppressed by the elevated endogenous cytokinins in corolla. 2). The delayed corolla senescence of IPT22 and IPT34 flowers was not only due to altered ethylene production but also decreased sensitivity to ethylene. The expression of *Phcp1* as a molecular indicator of ethylene sensitivity or responsiveness was not as informative as was expected and this may be due to its regulation by multiple plant hormones. 3). These results will greatly benefit the horticultural industry, as the information obtained from the SAG12-ipt transformed plants with delayed leaf and

flower senescence serve as a model system and this technology can be used in other flower crops that are sensitive to ethylene in order to delay senescence.

The regulation of the SAG12 promoter in petunia was not strictly senescence specific. The leaky regulation of the ipt gene by the sag12 promoter has previously been reported in petunia (Clark et al., 2002). The SAG's were original cloned from senescence leaves, not flowers. From the viewpoint of development, the flower is a determinate structure while the leaves tend to have a longer life span. When senescence takes place, it is likely that senescence is regulated differently in these tissues. If a SAG could be cloned from flowers this might provide better regulation of transgene expression in flowers,. Although it is senescence specific in *Arabidopsis*, it may not function the same way in other organisms. The regulation in our transgenic petunia was not clear, but it was functioning somehow, since the transgenic lines did not show abnormal phenotypes all the time. The *Agrobacterium ipt* was confirmed to function in petunia system as previous studies in another plants, such as tomato, lettuce, tobacco. As the result of transformation, elevated cytokinins were detected. By utilizing this chimeric SAG_{12}-*ipt* construct in our experiment, we successfully demonstrated that elevated endogenous cytokinins delayed flower senescence by altering ethylene production as well as sensitivity to ethylene, and by suppressing ABA accumulation in the corolla. These results confirmed the cytokinins have anti-senescence effects in flowers.

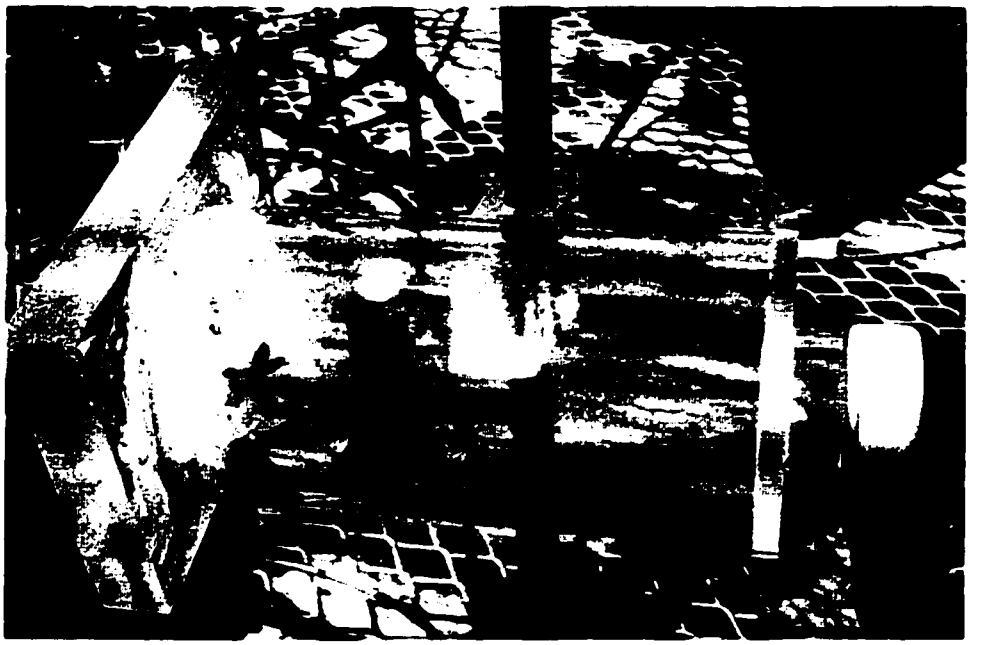

Fig. 3.1 Whole flower enclosed in a 450 ml chamber for ethylene measurement.

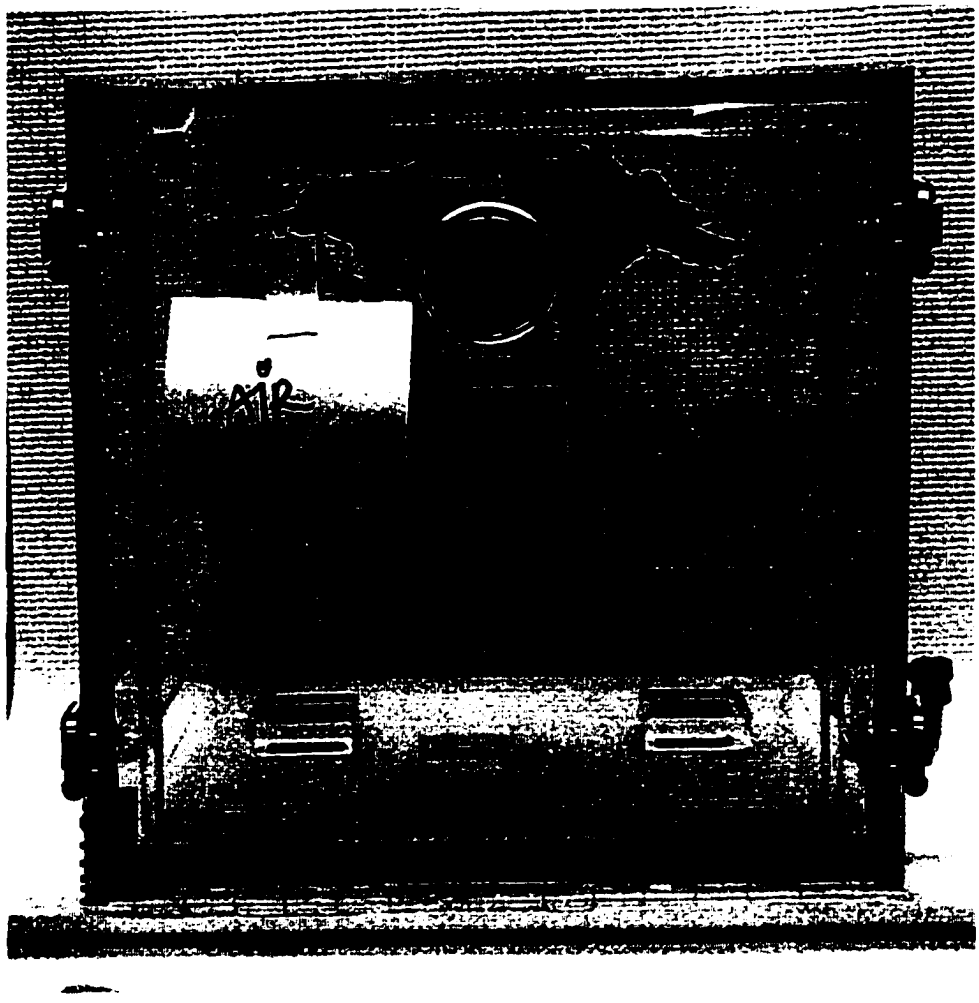

Fig. 3.2 The 24 L air-tight chamber for exogenous ethylene treatment.

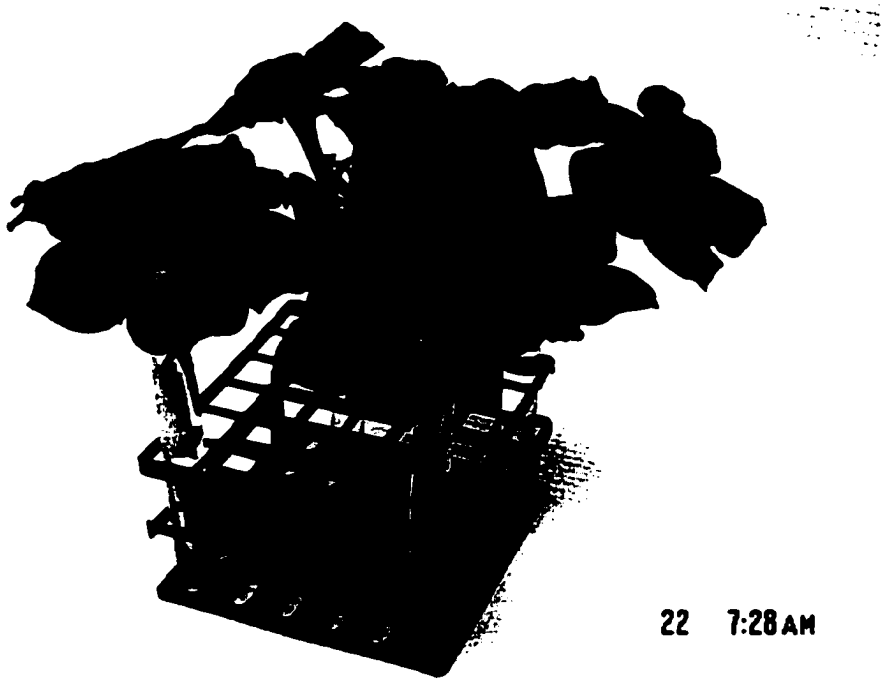

Fig. 3.3 Twelve flowers incubated in distilled water for exogenous treatment

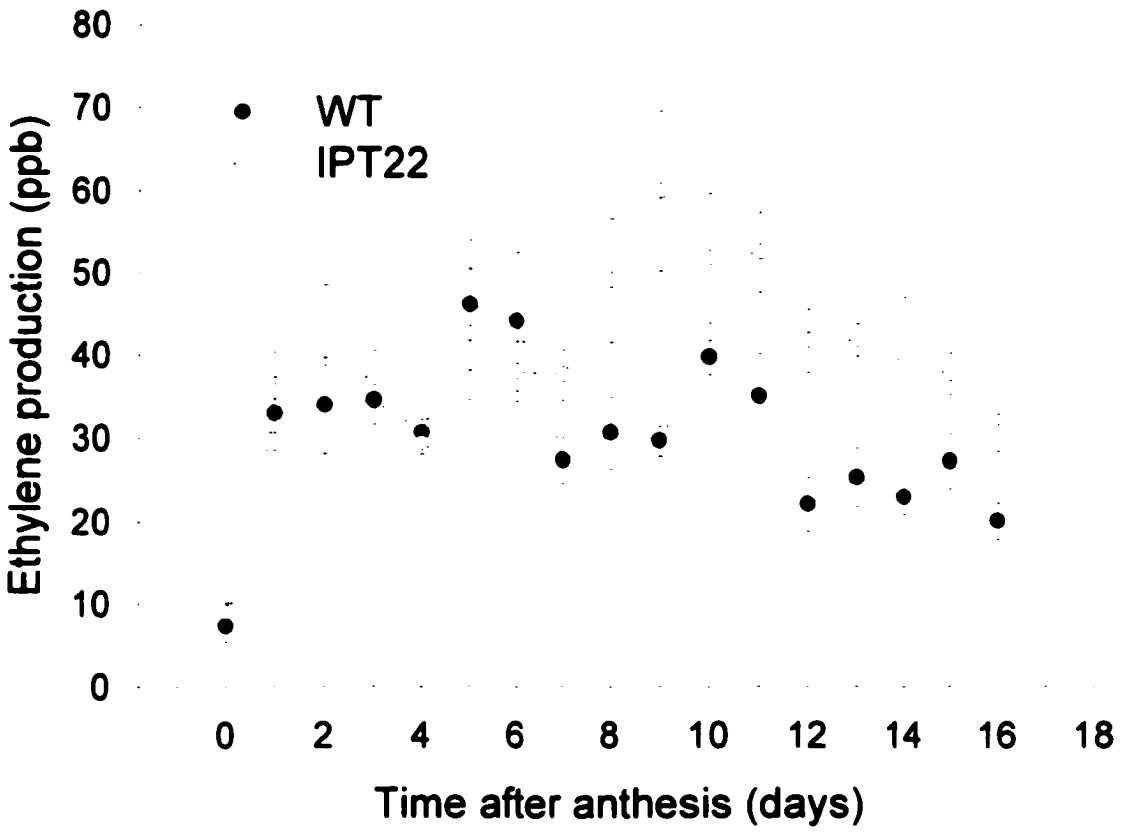

Fig 3.4 Ethylene production from whole flower of WT, IPT22 after anthesis. The values represented here are the average of four replicates ± SEM.

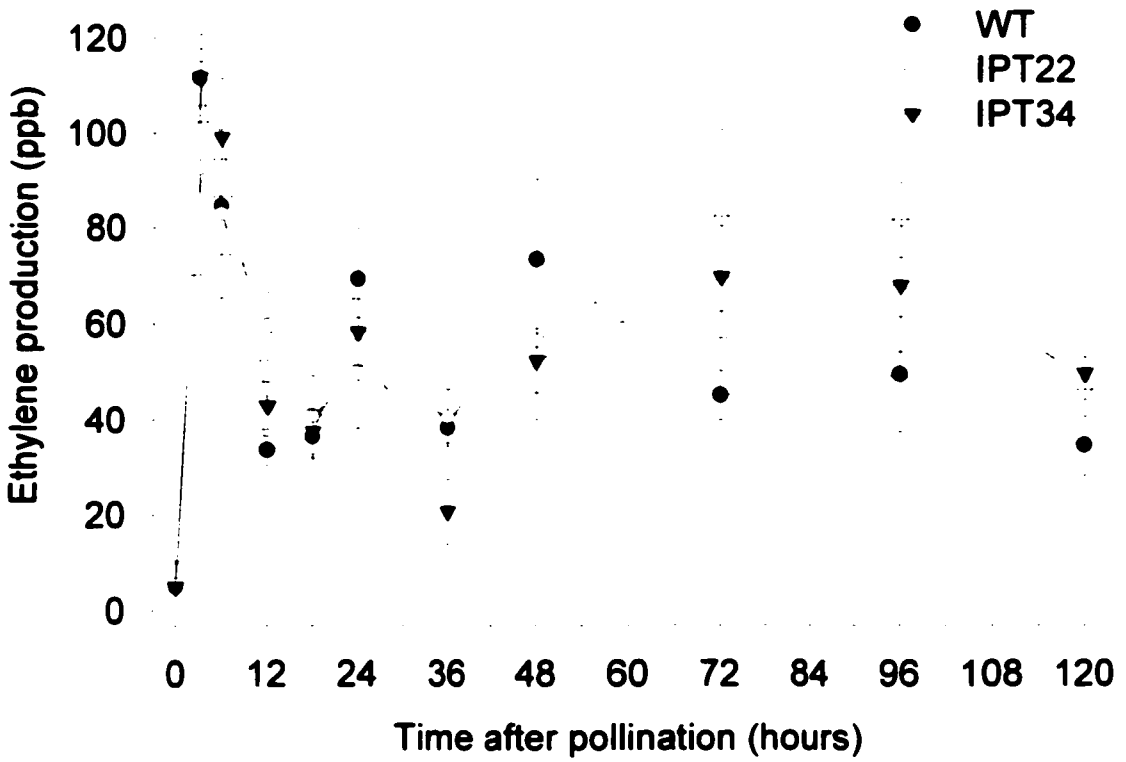

Fig 3.5 Ethylene production from whole flower of WT, IPT22, and IPT34 after pollination. The values represented here are the average of four replicates ± SEM.

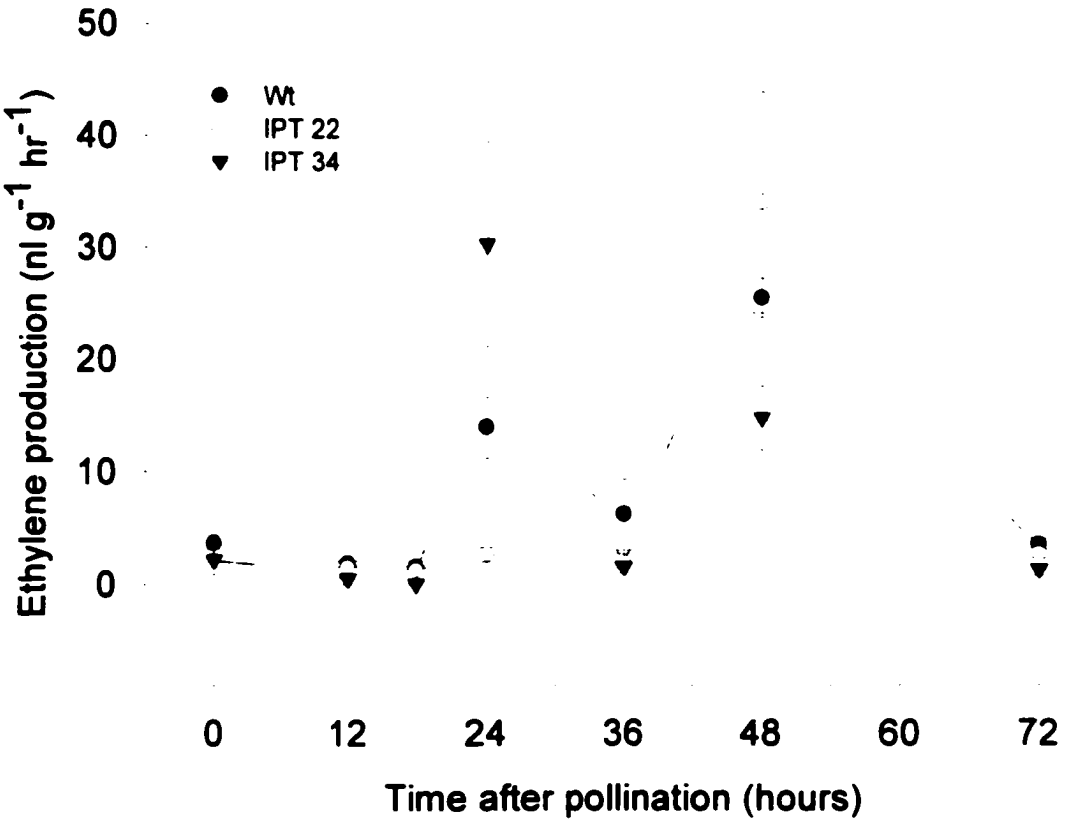

Fig 3.6 Ethylene production of WT, IPT22, and IPT34 corollas at various time points after pollination. Values are presented as the average of 4 replicates which included 3 corollas for each ± SEM.

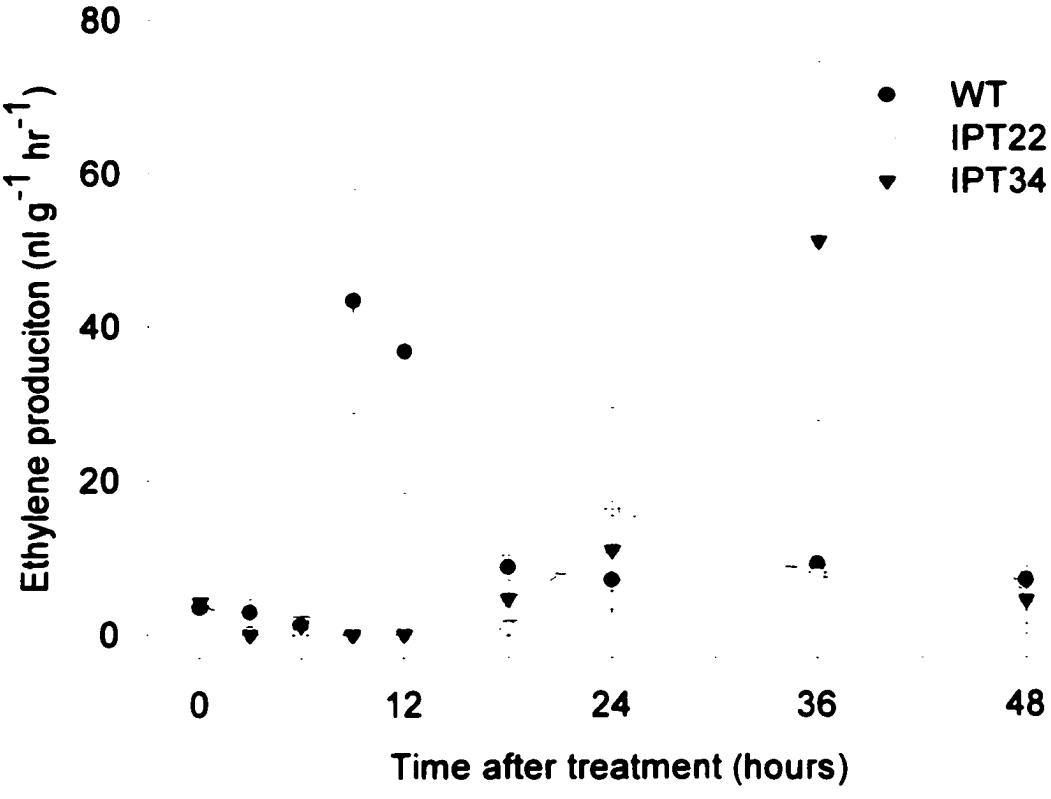

Fig 3.7 Ethylene production from WT, IPT22, and IPT34 corollas after treatment with 2 μL L^{-1} ethylene for 0. 3, 6, 9, 12, 24, 36, and 48 hours. Experiments were conducted three times with similar results. Data presented here is from one set. The average of four replicates of ethylene measurements ± SEM.

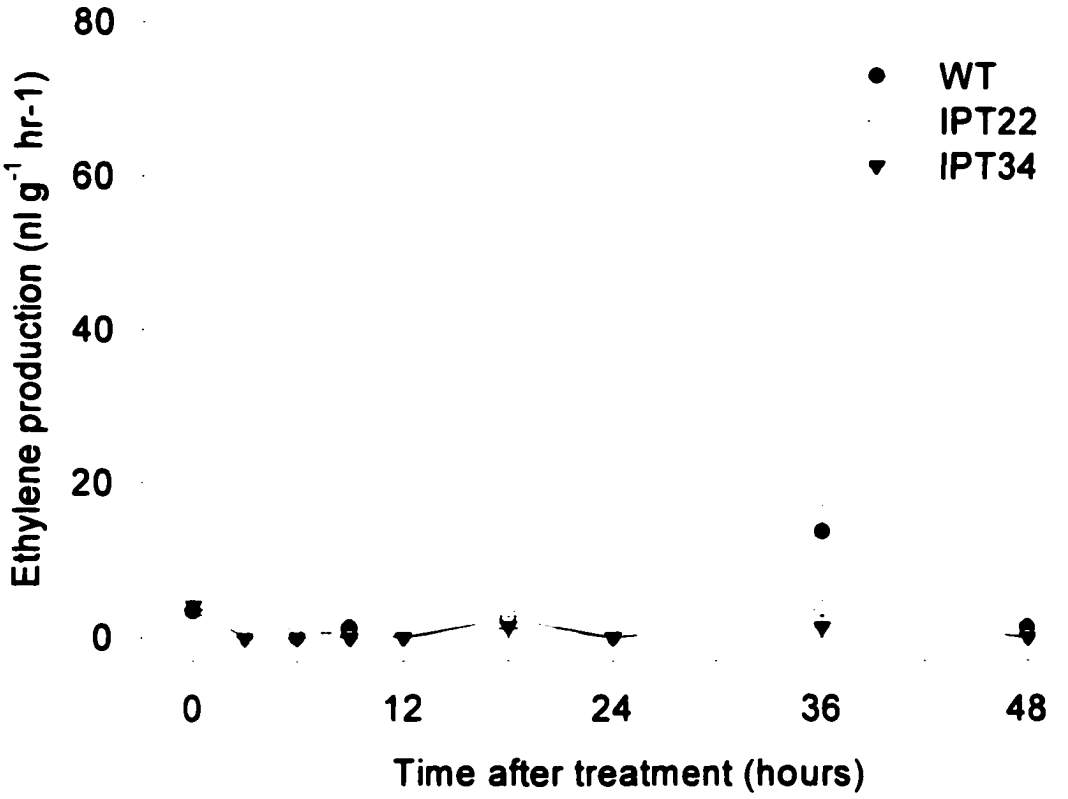

Fig 3.8 Ethylene production from WT, IPT22, and IPT34 corollas after treatment with air for 0. 3, 6, 9, 12, 24, 36, and 48 hours. Experiments were conducted three times with similar results. Data presented here is from one set. The average of four replicates of ethylene measurements ± SEM.

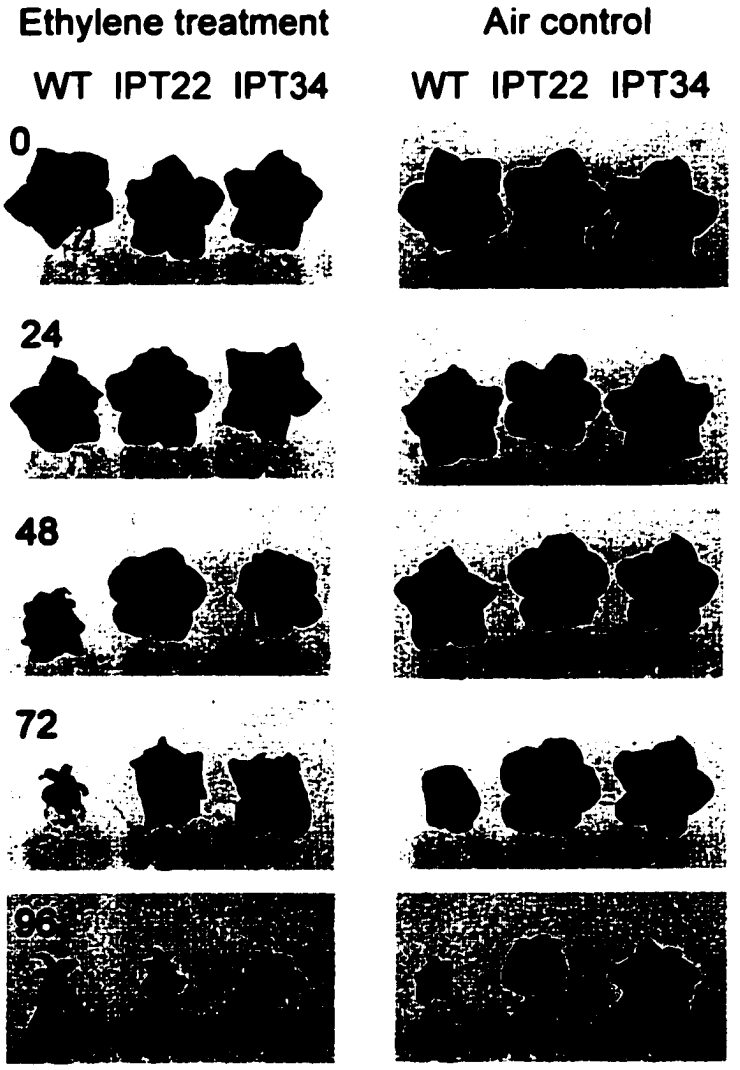

Fig 3. 9 Visual wilting symptom of flowers after removal from the 2 μL L^{-1} ethylene or air treatment. Numbers indicated here represented the time after 12 hours ethylene treatment in hours.

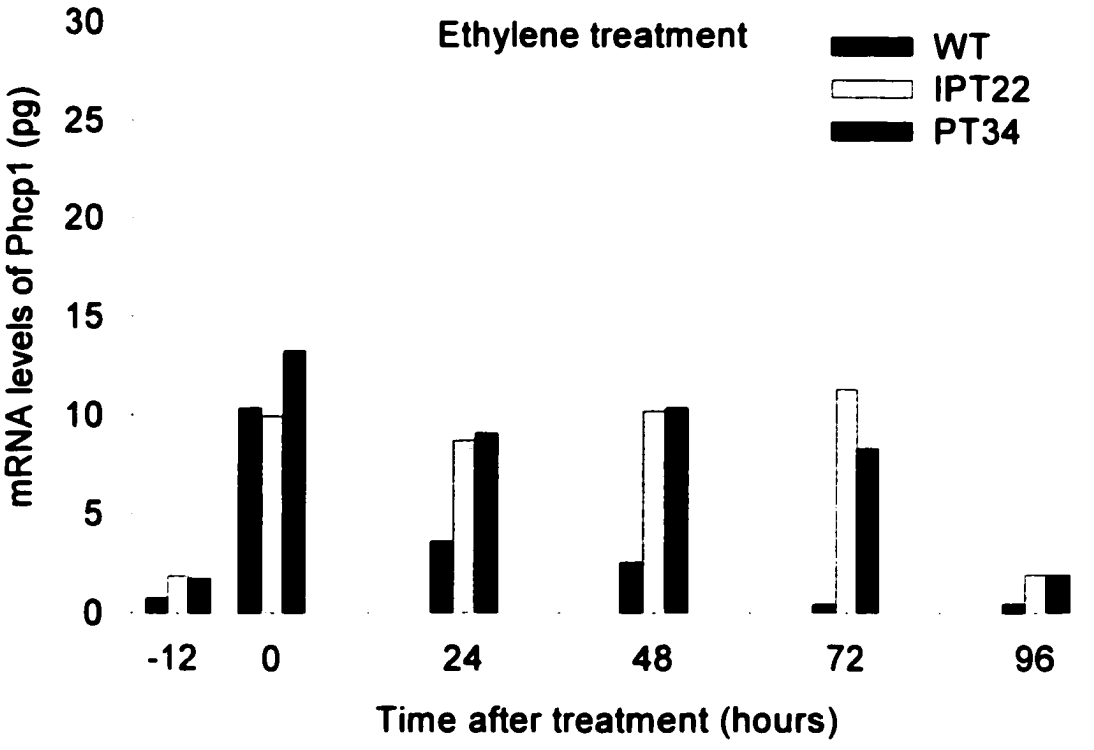

Fig 3.10 Phcp1 expression of corollas of WT, IPT22, and IPT34 after removal from the 2 μL L^{-1} ethylene treatment for 12 hours. PCR was conducted twice with similar results. Data presented here is from one set.

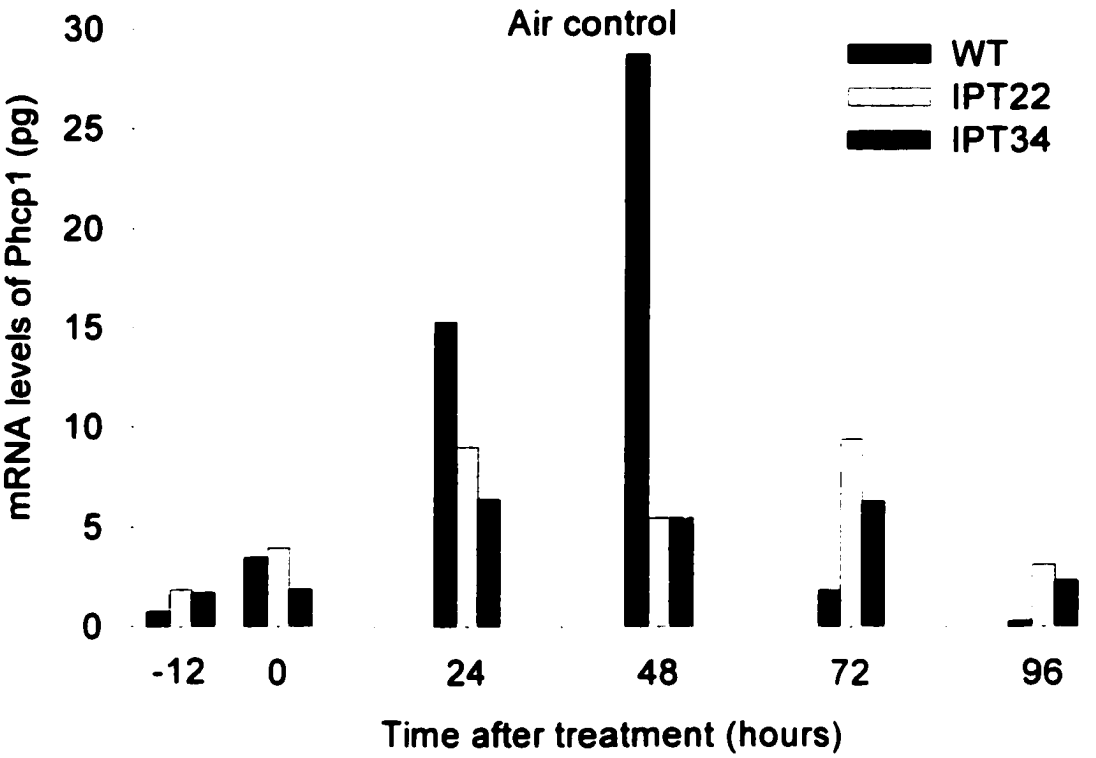

Fig 3.11 Phcp1 expression of corollas of WT, IPT22, and IPT34 after removal from the air control treatment for 12 hours. PCR was conducted twice with similar results. Data presented here is from one set.

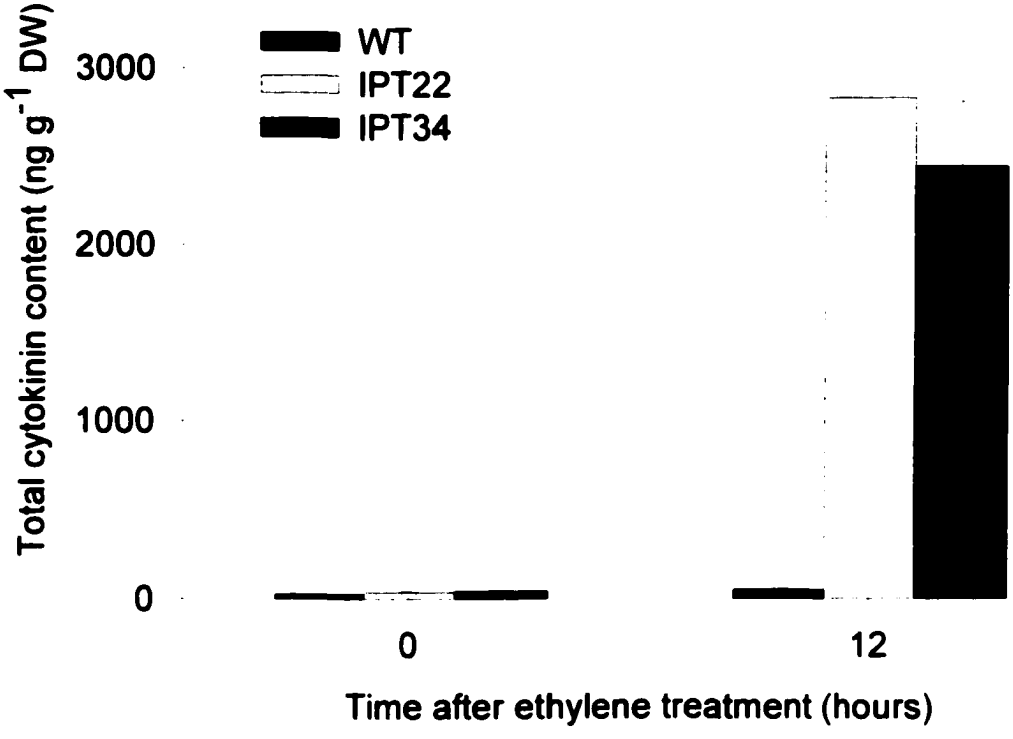

Fig 3.12 Total cytokinin content from the corollas of WT, IPT22, and IPT34 after exogenous application of 2 μL L^{-1} ethylene for 12 hours. Data presented here are the average of three replicates ± SEM.

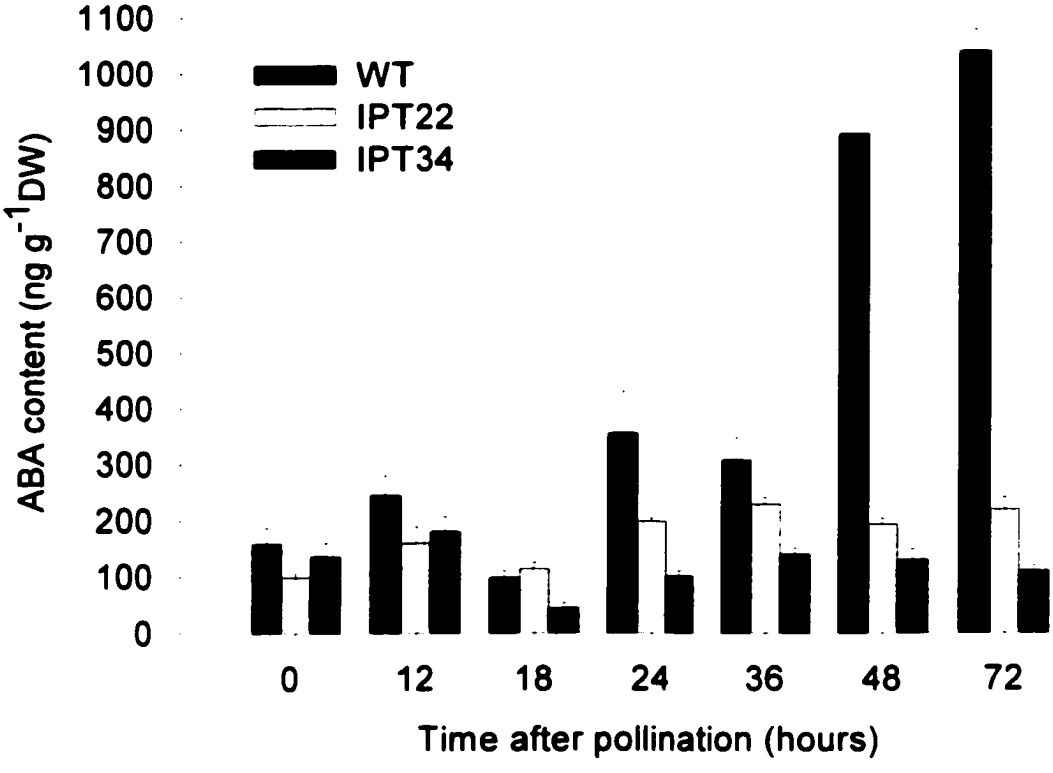

Fig 3.13 Endogenous ABA levels from the corollas of WT, IPT22, and IPT34 following the pollination. The data presented here are the average of three replicates ± SEM.

Literature cited

Abeles, F., P. Morgan, and M. Saltveit (1992). Ethylene in Plant Biology. (Academic, San Diego), 2nd Ed.

Adam, Z., A. Borochov, S. Mayak, and A. H. Halevy (1983). Correlative changes in sucrose uptake. ATPase activity and membrane fluidity in carnation petals during senescence. Physiol Plant 58: 257-262.

Addicott, F. T. (1982). Introductory comments: Abscisic acid in the physiology of plants. In Skoog, F. ed, Plant growth substances, 1997: proceedings of the 10th International Conference on Plant Growth Substances, Madison, Wisconsin. Berlin, New York, Springer-Verlag, p 241.

Aharoni, N., J. D. Anderson, and M. Lieberman (1979). Production and action of ethylene in senescing leaf discs: effect of indoleacetic acid, kinetin, silver ion, and carbon dioxide. Plant Physiol 64: 805-809.

Ainley, W. M., K. J. McNeil, J. W. Hill, W. L. Lingle, R. B. Simpson, M. L. Brenner, R. T. Nagao, and J. L. Key (1993). Regulatable endogenous production of cytokinins up to 'toxic' levels in transgenic plants and plant tissues. Plant Mol Bio 22: 13-23.

Akiyoshi, D. E., H. Kleee, R. Amasino, E. W. Nester, and M. Gordon (1984). T-DNA of *Agrobaterium tumerfaciens* encodes an enzyme of cytokinin biosynthesis. Proc Natl Acad Sci USA 81: 5994-5998.

Altvorst, A. C. van, A. G. Bovy, G. C. Angenent, and J. J. M. Dons (1997). Genetic modification of ethylene biosynthesis and ethylene sensitivity in carnation. *In* Biology and biotechnology of the plant hormone ethylene, Boston, Kluwer Academic Publishers. pp. 229-345.

Apelbaum, A. and M. Katchansky (1978). Effects of thiabendazole on ethylene production and sensitivity to ethylene of bud cut flowers. Hort Sci 13: 593-594.

Arditti, J. and H. Flick (1976). Post-pollination phenomena in orchid flowers. VI. Excised floral segments of Cymbidium. Amer J Bot 63: 201-211.

Axelos, M., J. Barbet, and C. Peaud-Lenoel (1984). Influence of cytokinins on the biosynthesis of light-harvesting chlorophyll a/b proteins in tobacco cell suspensions: detection by radioimmunological assay. Plant Sci Lett 33(2): 201-212.

Banowetz, G. M. (1992). The effects of endogenous cytokinin content on

benzyladenine induced nitrate reductase induction. Physiol Plant 86:341-348

Banowetz, G. M., J. R. Hess, and J. G. Carman (1994) A monoclonal antibody against the plant growth regulator, abscisic acid. Hybridoma 13:537-541.

Barden, L. E. and J. J. Hanan (1972). Effect of ethylene on carnation keeping life. J Am Soc Hort Sci 97: 785-788.

Barry, G. F., S. G. Roger, R. T. Fraley, and L. Brand (1984). Identification of a cloned cytokinin biosynthesis gene. Proc Natl Acad Sci USA 81: 4776-4780.

Bartoli, C. G., J. J. Guiamet, and E. D. Montaldi (1997). Ethylene production and responses to exogenous ethylene in senescing petals of *Chrysanthemum morifolium* RAM cv. Unsei. Plant Sci 124: 15-21.

Baumgartner, B., H. Kende, and P. Matile (1975). Ribonuclease in senescing morning glory. Purification and demonstration of *de novo* synthesis. Plant Physiol 55: 734-737.

Beinsberger, S. E., H. M. Clijsters, R. L. Valcke, and H. A. Onckelen (1992). Morphological characteristics and phytohormone content of *ipt*-transgenic tobacco. In Karssen, C. M., L. C. van Loon, and D. Vreugdenhil eds, Progress in plant growth regulation. Kluwer Academic Publisher, Dordrecht, pp 738-745.

Beyer Jr E. M. and Blomstrom D. C. (1980). Ethylene metabolism and its possible physiological role in plants. In Skoog, F ed, Plant growth substances, Springer-Verlag, Berlin, Heidelberg. pp 208-218.

Bianco, J., G. Garello, and M. T. Le Page-Degivry (1991). Gibberellins and abscisic acid in reproductive organs of Rosa hybrida. Acta Hort 298: 75-82.

Bichara, A.E. and J. Van Staden (1993). The effect of aminooxyacetic acid and cytokinin combinations on carnation flower longevity. Plant Growth Regul 13:161-167.

Bieleski, R. L. (1995). Onset of phloem export from senescent petals of daylily. Plant Physiol 109: 557-565.

Blackman, P. G. and W. J. Davies (1984). Modification of the CO_2 responses of maize stomata by abscisic acid and by naturally occurring and synthetic cytokinins. J Exp Bot 35: 174-179.

Bleecker, A. B., M. A. Estelle, C. Somerville, and H. Kende (1988). Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science 241: 1086-1089.

Borochov, A. and J. Faragher (1983). Comparison between ultraviolet irradiation and ethylene effects on senescence parameters in carnation flowers. Plant Physiol 71: 536-540.

Borochov, A., T. Tirosh, and A. H. Halevy (1976). Abscisic acid content of senescing petals on cut rose flowers as affected by sucrose and water stress. Plant Physiol 58: 175-178.

Borochov, A. and W. R. Woodson (1989). Physiology and Biochemistry of flower petal senescence. Hortic. Rev. 11:15-43.

Bossè, C. A. and J. Van Staden (1989). Cytokinins in cut carnation flowers V. Effects of cytokinin type, concentration and mode of application on flower longevity. J Plant Physiol 135: 155-159.

Botha, M. L. and C. S. Whitehead (1992). The effect of polyamines on ethylene synthesis during normal and pollination-induced senescence of *Petunia hybrida* L. flowers. Planta 188: 478-483.

Brown, J. H., R. L. Legge, E. C. Sisler, J. E. Baker, and J. E. Thompson (1986). Ethylene binding in senescing carnation petals. J Exp Bot 37: 526-534.

Brzobohartý, B., I. Moore and K. Palme (1994). Cytokinin metabolism: implications for regulation of plant growth and development. Plant Mol Biol 26: 1438-1497.

Callis, J. (1995). Regulation of protein degradation. Plant Cell 7:845-857.

Cercos, M., S. Santamaria, and J. Carbonell (1999). Cloning and characterization of TPE4A, a thiol-proteases gene induced during ovary senescence and seed germination in pea. Plant Physiol 119: 1341-1348.

Cervantes, E., A. Rodriguez, and G. Nicolas (1994). Ethylene regulated the expression of a cysteine gene during germination of chickpea (*Cicer arietinum* L.) Plant Mol Biol 25: 207-215.

Chang, C., S. F. Kwok, A. B. Bleecker, and E. M. Meyerowitz (1993). *Arabidopsis* ethylene-response gene *ETR1*: Similarity of product to two-component regulators. Science 262: 539-544.

Chatfiled, J. M. and D. J. Armstrong (1986). Regulation of cytokinin oxidase activity in callus tissues of *Phaseolus vulgaris* L. cv Great Northern. Plant Physiol 80: 493-499.

Chen, C. –M. and J. R. Ertl (1994). Cytokinin biosynthetic enzymes in plants and slime mold. In Mok, D. W. S. and M. C. Mok eds, Cytokinins: Chemistry, Activity and Function, CRC Press, Boca Raton, pp 81-85.

Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter (1979). Isolation of biological active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294-5299.

Clark, D. G, C. Dervinis, J. E. Barrett, and H. Klee (2002). Drought-induced leaf senescence and horticultural performance of transgenic P_{SAG12}-IPT *Petunias*. J Amer Soc Hort Sci In press

Clark, D. G., C. Richard, Z. Hilioti, S. Lind-Iversen, and K. Brown (1997). Effect of pollination on accumulation of ACC synthase and ACC oxidase transcripts, ethylene production and flower petal abscission in geranium (*Pelargonuim* x *hortorum* LH Bailey). Plant Mol Biol 34:855-865.

Cook, D., M. Rasche, and W. Eisinger (1985). Regulation of ethylene biosynthesis and action in cut carnation flower senescence by cytokinins. J Amer Soc Hort Sci 110: 24-27.

Coghlan, S. E. and D. R. Walters (1992). Photosynthesis in green-islands on powdery-infected barley leaves. Physiol Mol Plant Path 40(1): 31-38.

Cowan, A. K., A. L. P. Cairns, and B. Bartels-Rahm (1999). Regulation of abscisic acid metabolism: towards a metabolic basis for abscisic acid-cytokinin antagonism. J Exp Bot 50(334): 595-603.

Courtney, S. E., C. C. Rider, and A. D. Stead (1994). Changes in protein ubiquitination and the expression of ubiquitin-encoding transcripts in daylily petals during floral development and senescence. Physiol Plant 91: 196-204.

Crocker, W. and L. I. Knight (1908). Effects of illuminating gas and ethylene upon flowering carnations. Bot Gaz 46: 259-276.

Drake, R., I. John, A. Farrell, W. Copper, W. Schuch, and D. Grierson (1996). Isolation and analysis of cDNAs encoding tomato cysteine proteases expressed during leaf senescence, Plant Mol Biol 30: 755-767.

Dyer, T. A. and D. J. Osborne (1971). Leaf nucleic acids. II Metabolism during senescence and the effect of kinetin. J Experi Bot 22: 552-560.

Eisinger, W. (1977). Role of cytokinins in carnation flower senescence. Plant physiol 59: 707-709.

Eklof, S., C. Astot, T. Moritz, J. Blackwell, O. Olsson, and G. Sandberg (1996). Cytokinin metabolites and gradients in wild type and transgenic tobacco with moderate cytokinin overproduction. Physiol Plant 98: 333-344.

Evan-Chen, Z. and C. Itai (1975). The role of abscisic acid in senescence of detached tobacco leaves. Physiol Plantarum 34: 97-100.

Eze, J. M. O., S. Mayak, J. E. Thompson, and E. B. Dumbroff (1986). Senescence in cut carnation flowers: Temporal and physiological relationships among water statues. ethylene, abscisic acid and membrane permeability. Physiol Plant 68: 323-328.

Faiss M., J. Zalubilová,.M. Strnad, and T. Schmülling (1997) Conditional transgenic expression of the *ipt* gene indicates a function for cytokinins in paracrine signaling in whole tobacco plants. Plant J 12:401-415

Faragher, J. D. and S. Mayak (1984). Physiological responses of cut rose flowers to exposure to low temperature: changers in membrane permeability and ethylene production. J Expt Bot 35: 965-974.

Fjeld, T., N. A. Melberg, and W. R. Hogetveit (1995). Ethylene sensitivity and ethylene production in English holly (*Ilex aquifolium* L.) Acta Hortic 405: 306-313.

Fletcher, R. A., C. Teo, and A. Ali (1973). Stimulation of chlorophyll synthesis in cucumber cotyledons by benzyladenine. Can J Bot 51 (5): 937-939.

Fulton, T. M., J. Chuwongse, and S. D. Tanksley (1995). Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Mol Biol 13(3): 207-209.

Gamble, R. L., M. L. Coonfield, and G. E. Schaller (1998). Histidine kinase activity of the ETR1 ethylene receptor from Arabidopsis. Proc Natl Acad Sci 95(13): 7825-7829.

Gan, Susheng and R. M. Amasino (1995). Inhibition of leaf senescence by autoregulated production of cytokinin. Science 270: 1986-1988

Gan, Susheng and R. M. Amasino (1996). Cytokinins in plant senescence: from spray and pray to clone and play. BioEssays 18(7): 557-565.

Garello. G., C. D. B. Menard, and M. T. Le Page-Degivry (1995). The influence of light quality on rose flower senescence: involvement of abscisic acid. Plant growth Regul 16(2): 135-139.

Gaudin, V., T. Vrain, and L. Jouanin (1994). Bacterial genes modifying hormonal balances in plants. Plant Physiol Biochem 32: 11-29.

Goh, C. G., A. H. Halevy, R. Engel, and A. M. Kofranek (1985). Ethylene evolution and sensitivity in cut orchid flowers. Sci. Hortic. 26: 57-67.

Gilissen, L. J. W. and F. A. Hoekstra (1984). Pollination-induced corolla wilting in *Petunia hybrida*: rapid transfer through the style of a wilting substance. Plant Physiol 75: 496-498.

Granell, A., N. Harris, A. G. Pisabarro, and J. Carbonell (1992). Temporal and spatial expression of a thiolprotease gene during pea ovary senescence and its regulation by gibberellin, Plant J 2: 907-915.

Groot, S. P. C., R. Bouwer, M. Busscher, P. Lindhout, and H. J. Dons (1995). Increase of endogenous zeatin riboside by introduction of the *ipt* gene in wild type and the lateral suppressor mutant of tomato. Plant Growth Reg 16: 27-36.

Grossmann, K. and H. Hansen (2001). Ethylene –triggered abscisic acid: A principle in plant growth regulation? Physiol Plantarum 113: 9-14.

Gubrium, E. K., D. J. Clevenger, D. G. Clark, J. E. Barrett, and T. A. Neil (2000). Reproduction and horticultural performance of transgenic ethylene-insensitive petunias. J Amer Soc Hort Sci 125(3): 277-281.

Guerrero, C., M. S. Reid, and V. Valpuesta (1998). Analysis of the expression of two thiolprotease genes from daylily (*Hemerocallis* spp.) during flower senescence. Plant Mol Biol 36(4): 565-571.

Guzman, P. and J. R. Ecker (1990). Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. Plant Cell 2: 513-523.

Halevy, A. H. (1986a). Pollination-induced corolla senescence. Acta Hortic 181: 25-32.

Halevy, A. H. (1986b). Flower senescence. In Leshem, Y. Y., A. H. Halevy, and C. Frenkel eds, Processes and control of plant senescence. Elsevier, Amsterdam.

Halevy, A. H. (1995). The role of sensitivity to ethylene in pollination-induced corolla senescence syndrome. Acta Hortic 405: 210-215.

Halevy, A. H. and S. Mayak (1981) Senescence and postharvest physiology of cut flowers part 2, Hortic Rev 3:59-153.

Halevy, A. H., C. S. Whitehead, and A. M. Kofranek (1984). Does pollination induce cortolla abscission of cyclamen flowers by promoting ethylene production? Plant Physiology 75: 1090-1093.

Hanley, K. M. and W. J. Bramlage (1989). Endogenous levels of abscisic acid in aging carnation flower parts. J Plant Growth Regul 8: 225-236.

Hanson, A. D. and H. Kende (1975). Ethylene-enhanced ion and sucrose efflux in morning-glory flower tissue. Plant Physiol 55: 663-669.

Heide, O. M. and J. Qydvin (1969) Effects of 6-benzylaminopurine on the keeping quality and respiration of glasshouse carnations. Hortic Res 9:26-36

Hewelt A., E. Prinsen, J. Schell, H. Van Onckelen, and T. Schmülling (1994) Promoter tagging with a promoterless *ipt* gene leads to cytokinin-induced phenotypic variability in transgenic tobacco plants: implications of gene dosage effects. Plant J 6:879-891

Higgins, T. J. V. (1995). The effect of auxin on cytokinin levels and metabolism in transgenic tobacco tissue expression an *ipt* gene. Planta 196: 84-94.

Ivana, M., S. Lidiya, O. Miloš, Z. Oksana, K. Tatyana, E. Josef, O. Jaroslava, G. Svetlana, R. Yurii, and A. Nina (1997). Growth pattern, tuber formation and hormonal balance in *in vitro* potato plants carrying *ipt* gene. Plant Growth Regul 21: 27-36.

Jones, M. L., P. B. Larson, and W. R. Woodson (1995). Ethylene-regulated expression of a carnation cysteine proteinase during flower petal senescence. Plant Mol Biol 28: 505-512.

Jones, M. L. and W. R. Woodson (1997). Pollination-induced ethylene in carnation: Role of stylar ethylene in corolla senescence. Plant Physiol 115: 205-212.

Jordi, W., A. Schapendonk, E. Davelaar, G. M. Stoopen, C. S. Pot, R. De Visser, J. A. Van Rhijn, S. Gan, and R. M. Amasino (2000). Increased cytokinin levels in transgenic P_{SAG12}-*ipt* tobacco plants have large direct and indirect effects on leaf senescence, photosynthesis and N partitioning. Plant, Cell and Environment 23:279-289.

Jorgensen, R. A., P. D. Cluster, J. English, Q. D. Que, and C. A. Napoli (1996). Chalcon synthase consuppression phenotypes in petunia flowers: comparison of sense vs antisense constructs and single-copy vs complex T-DNA sequences. Plant Mol Bio 31: 957-973.

Kakimoto, T. (2001). Identification of plant cytokinin biosynthesis enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyltransferases. Plant and Cell Physiol 42: 677-685.

Kaminek, M. (1992). Progress in cytokinin research. Trends Biotechnol 10: 159-164.

Kelly, J. W., G. L. Staby, and G. W. Chism (1985). Translocation and metabolism of cytokinins in cut carnations. J Amer Soc Hort Sci 110: 856-859.

Kende, H (1964). Preservation of chlorophyll in leaf sections by substances obtained from root exudates. Science 145: 1066-1067. (cited in Gan and Amasino, 1996)

Kende, H (1993). Ethylene biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 44: 283-307.

Kende, H. and A. D. Hanson (1976). Relationship between ethylene evolution and senescence in morning-glory tissue. Plant Physiol 57: 523-527.

Ketsa, S. and A. Rugkong (2000). Ethylene production, senescence and ethylene sensitivity of Dendrobium 'pompadour' flowers following pollination. J Hortic Sci Biotech 75(2): 149-153.

Knee, M. (1995). Copper reverses silver inhibition of flower senescence in *Petunia hybrida*. Post Bio and Tech 6: 121-128.

Knee, M. (1996). Inhibition of *Petunia* flower senescence by 2,2'-bipyridyl. Post Bio and Tech 9: 351-360.

Koltunow, A. M., J. Truettner, K. H. Cox, M. Wallroth, and R. B. Goldberg (1990). Different temporal and spatial gene expression patterns occur during anther development. Plant Cell 2: 1201-1224.

Király, Z., M. El Hammady, and B. I. Pozsár (1967). Increased cytokinin activity of rest-infected bean and broad-bean leaves. Phytopathol 57: 93-94.

Kusnetsov, V., R. G. Herrmann, O. N. Kulaeva, and R. Oelmüller (1998). Vytokinin stimulates and abscisic acid inhibits greening of etiolated *Lupinus luteus* cotyledons by affecting the expression of the light-sensitive protochlorophyllide ocidoreductase. Mol Gen Genet 259: 21-28.

Larsen, P. B., E. N. Ashworth, M. L. Jones, and W. R. Woodson (1995). Pollination-induced ethylene in carnation: role of pollen tube growth and sexual compatibility. Plant Physiol 108: 1405-1412.

Lashbrook, C. C., D. M. Tieman, and H. J. Klee (1998). Differential regulation of the tomato ETR gene family throughout plant development. Plant J 15(2): 243-252.

Lawton, K. A., B. Huang, P. B. Goldsbrough, and W. R. Woodson (1989). Molecular cloning and characterization of senescence-related genes from carnation flower petals. Plant Physiol 90: 690-696.

Lawton, K. A., K. G. Raghothama, P. B. Glodsbrough, and W. R. Woodson (1990). Regulation of senescence-related gene expression in carnation flower petals by ethylene. Plant Physiol 93: 1370-1375.

LePage-Degivry, M. T., M. Orlandini, G. Garello, P. Barthe, and S. Gudin (1991). Regulation of ABA levels in senescing petals of rose flower. J of Plant growth Reg 10: 67-72.

Leshem, Y., A. H. Halevy, and C. Frenkel (1986). Process and control of plant senescence. Dev Crop Sci 8: 142-161.

Letham, D. S. (1994). Cytokinins as phtyohormones- sites of biosynthesis, translocation and function of cytokinins. In Mok, D.W.S. and Mok, M. C eds, Cytokinins: Chemistry, activity and function, Boca Raton, FL: CRC Press, pp 57-80.

Li, Y., G. Hagen, and T. J. Guilfoyle (1992) Altered morphology in transgenic tobacco plants that overproduce cytokinins in specific tissues and organs. Dev Biol 153: 386-395

Lieberman, M., S. Asen, and L. W. Mapson (1964). Ethylene oxide an antagonist of ethylene in metabolism. Nature 204: 756-758.

Lohman, K. N., S. Gan, M. C. John, and R. M. Amasino (1994). Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. Physiol Plant 92(2): 322-328.

MacLean, D. C. and R. R. Dedolph (1962) Effects of N6-benzylaminopurine on postharvest respiration of *Chrysanthemum morifolium* and *Dianthus caryophyllus*. Bot Gaz 124:20-21

Maxie, E. C., D. S. Farnham, F. G. Mitchell, N. F. Sommer, R. A. Parson, R. G. Synder, and H. L. Rae (1973). Temperature and ethylene effects on cut flowers of carnation (*Dianthus caryphyllus* L.) J Amer Soc Hort Sci 98: 568-572.

Mayak, S. and D. R. Dilley (1976a) Effect of sucrose on response of cut carnation to kinetin, ethylene and abscisic acid. J Am Soc Hortic Sci 101:583-585.

Mayak, S. and D. R. Dilley (1976b). Regulation of senescence in carnation (*Dianthus caryophyllus* L.) to ethylene. J Am Soc Hort Sci 101: 503-506.

Mayak, S. and D. R. Dilley (1976c). Regulation of senescence in carnation (*Dianthus caryophyllus* L.). Effect of abscisic acid and carbon dioxide on ethylene production. Plant Physiol. 58: 663-665.

Mayak, S. and A. H. Halevy (1970) Cytokinin activity in rose petals and its relation to senescence. Plant Physiol 46:497-499

Mayak, S. and A. H. Halevy (1972). Interrelationships of ethylene and abscisic acid in the control of rose petal senescence. Plant Physiol 50; 341-346.

Mayak, S. and A. H. Halevy (1974) The action of kinetin in improving the water balance and delaying senescence processes of cut rose flowers. Physiol Plant 32:330-336

Mayak, S. and A. M. Kofranek (1976) Altering the sensitivity of carnation flowers (*Dianthus caryophyllus* L.) to ethylene. J Am Soc Hortic Sci 101:203-506.

Mayak, S., A. T. Tirosh, J. E. Thompson, and S. Ghosh (1998). The fate of ribulose-1,5-bisphosphate carboxylase subunits during development of carnation petals. Plant Physiol Biochem 36: 835-841.

Mayak, S., Y. Vaadia, and D. R. Dilley (1977). Regulation of senescence in carnation (*Dianthus caryophyllus* L.) by ethylene. Mode of action. Plant Physiol 59: 591-593.

McKenzie, M. J., P. E. Jameson, and R. T. M. Poulter (1994). Cloning an *ipt* gene from *Agrobacterium tumerfaciences*: characterization of cytokinins in derivative transgenic plant tissue. Plant Growth Regul 14:217-228

McCabe, M. S., L. C. Garratt, F. Schepers, W. J. R. M. Jordi, G. M. stoopen, E, Davelaar, J. H. A. Van Rhijn, J. B. Power, and M. R. Davey (2001) effects of P-SAG12-IPT gene expression on development and senescence in transgenic lettuce. Plant Physiol 127: 505-512.

McKenzie, M. J., V. Mett, P. H. S. Reynolds, and E. Jameson (1998). Controlled cytokinin production in transgenic tobacco using a copper-inducible promoter. Plant Physiol 116: 969-977.

Medawar, P. B. (1957). An unsolved problem of biology. In The uniqueness of the individual. New York, USA: Basic Books, pp 44-70.

Medford, J. I., R. Horgan, Z. El-Sawi, and H. L. Klee (1989). Alterations of endogenous cytokinins in transgenic plants usig a chimeric isopentenyl transferase gene. Plant Cell 1: 403-413.

Mittler, R., and E. Lam (1995). Identification, characterization, and purification of a tobacco endonuclease activity induced upon hypersensitive response cell death. Plant Cell 7: 1951-1962.

Mor, Y. and M. S. Reid (1981). Isolated petals. A useful system for studying flower senescence. In Second International Symposium on Post Harvest Physiology of Cut Flowers, Davids, California. Eds Kofranek, A. M., M. S. Reid. P. 19-25.

Mor, Y., H. Spiegelstein, and A. H. Halevy (1983). Inhibition of ethylene biosynthesis in carnation petals by cytokinin. Plant Physiol 71: 541-546.

Mothes, K. and L. Engelbrecht (1961). Kinetin-induced directed transport of substances in excises leaves in the dark, Phytochem (Oxford) 1: 58-62.

Motyka, V., M. Faiss, S. Strnad, M. Kamínek, and T. Schmülling (1996). Changes in cytokinin content and cytoinin oxidase activity in response to derepression of *ipt* gene transcription in transgenic tobacco calli and plants. Plant Physiol 112: 1035-1043.

Müller, R., B. M. Stummann, A. S. Andersen, and M. Serek (1999). Involvement of ABA in postharvest life of miniature potted roses. Plant Growth Regul 29: 143-150.

Müller, R., B. M. Stummann, and M. Serek (2000). Characterization of an ethylene receptor family with differential expression in rose (*Rosa hybrida* L.) flowers. Plant Cell Rep 19(12): 1232-1239.

Nichols, R., G. Bufler, Y. Mor, D. W. Fujino, and M. S. Reid (1983). Changes in ethylene production and 1-aminocyclopropane-1-carboxylate content of pollinated carnation flowers. J Plant Growth Regul 2:1-8.

Nichols, R. (1966). Ethylene production during senescence of flowers. J Hort Sci 41: 279-290.

Nichols, R. (1968). The response of carnations (*Dianthus caryophyllus*) to ethylene. J Hort Sci 43: 335-349.

Nichols, R. (1977). Sites of ethylene production in the pollinated and unpollinated senescing carnation (*Dianthus caryophyllus*) inflorescence. Planta 135: 155-159.

Nichols, R., G. Bufler, Y. Mor, D. W. Fujino, and M. S. Reid (1983). Changes in ethylene production and 1-aminocyclopropane-1-carboxylic acid content of pollinated carnation flowers. J Plant Growth Regul 2: 1-8.

Noh, Y-S. and R. M. Amasino (1999). Identification of a promoter region responsible for the senescence-specific expression of SAG12. Plant Mol Biol 41: 181-194.

Nowak, J. and H. Veen (1982). Effects of silver thiosulfate on abscisic acid content in cut carnations as related flower senescence. J Plant Growth Regul 1: 153-159.

O'Brien, I. E. W., B. C. Baguley, B. G. Murray, B. A. M. Morris, and I. B. Ferguson (1998). Early stages of the apoptotic pathway in plant cells are reversible. Plant J 13:803-814.

Ooms, G. (1991). Phenotypic changes in T-*cyt*-transformed potato plants are consistent with enhanced sensitivity of specific cell types to normal regulation by root-derived cytokinin. Plant Mol. Biol. 17: 727-743.

Panavas, T., A. Pikula, P. D. Reid, B. Rubistein, and E. L. Walker (1999). Identification of senescence-associated genes from daylily petals. Plant Mol Biol 40: 237-248.

Panavas, T. and B. Rubinstein (1998). Oxidative events during programmed cell death of daylily (*Hemerocallis* hybrid) petals. Plant Sci 133: 125-138.

Panavas, T., E. L. Walker, and B. Rubinstein (1998). Possible involvement of abscisic acid in senescence of daylily petals. J Exp Bot 49 (329): 1987-1987.

Park, K. Y., A. Drory, and W. R. Woodson (1992). Molecular cloning of an 1-amnocycylopropane-1-carboxylate synthase from senescing carnation flower petals. Plant mol Biol 18: 377-386.

Paton, S., R. G. Fray, S. Brown, and D. Grierson (1996). Ethylene receptor expression is regulated during fruit ripening, flower senescence and abscission. Plant Mol Biol 31(6): 1227-1231.

Pech, J. C., A. Latche, C. Larrigaudiere, and M. S. Reid (1987). Control of early ethylene synthesis in pollinated petunia flowers. Plant Physiol Biochem 25: 431-437.

Philosoph-Hadas, S., E. Hadas, and N. Aharoni (1993). Characterization and use in ELISA of a new monoclonal antibody for quantitation of abscisic acid in senescing rice leaves. Plant Growth Reg 12: 71-78.

Porat, R., A. Borochov, and A. H. Halevy (1995). Factors affecting ethylene sensitivity in Phalaenopsis orchid flowers. Acta Hortic 420: 39-41.

Porat, R., Y. Reuveny, A. Borochov, and A. H. Halevy (1993). Petunia flower longevity: the role of sensitivity to ethylene. Physiol. Plant. 89: 291-294.

Rasche, M. and W. Eisinger (1984). Effects of benzyladenine and ethylene on the osmotic balance of cut carnation flowers during senescence. Plant Physiol 75: 126.

Redig, P., T. Schmülling, and H. Van Onckelen (1996). Analysis of cytokinin metabolism in *ipt* transgenic tobacco by liquid chromatograph-tandem mass spectrometry. Plant Physiol 112:141-148.

Reid, M. S. and M. Wu (1992). Ethylene and flower senescence. Plant Growth Regul. 11: 37-43.

Reutter, K., R. Atzorn, B. Hadeler, T. Schmülling, and R. Reski (1998). Expression of the bacterial *ipt* gene in *Physcomotrella* rescues mutations in *budding* and in *plastid division.* Planta 206: 196-203.

Richmond, A. E. and A. Lang (1957). Effect of kinetin on protein content and survival of detached *Xanthium* leaves. Science 125:650—651.

Roman, G., B. Lubarsky, J. J. Kieber, M. Rothenberg, and J. R. Ecker (1995). Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana:* five novel mutant loci integrated into a stress response pathway. Genetics 139: 1393-1409.

Ronen, M. and S. Mayak (1981). Interrelationship between abscisic acid and ethylene in the control of senescence processes in carnation flowers. J Exp Bot 32(129): 759-765.

Rubinstein, B. (2000). Regulation of cell death in flower petals. Plant Mol Bio 44:303-318.

Salisbury, F. B. (1994). The role of plant hormones. In Wilkinson, R. E. ed, Plant-environment interactions. Marcel Dekker, New York, pp 39-81.

Schaller, G. E. and A. B. Bleecker (1995). Ethylene-binding sites generated in yeast expressing the Arabidopsis ETR1 gene. Science 270: 1809-1811.

Schmulling, T., H-M. Rupp, M. Frank, and S. Schafer (1999). Recent advances in cytokinin research:receptor candidates, primary response genes, mutants and transgenic plants. In M Strnad, P Pec, E Beck eds, Advances in Regulation of Plant Growth and Development, Peres Publ, Prag, pp 85-96.

Schroeder, K. R., D. P. Stimart, and E. V. Nordheim (2001). Response of *Nicotiana alata* to insertion of an autoregulated senescence-inhibition gene. J Amer Soc Hort Sci 126: 523-530.

Serek, M., E. C. Sisler, and M. S. Reid (1995a). Effects of 1-MCP on the vase life and ethylene response of cut flowers. Plant Growth Regul 16: 93-97.

Serek, M., G. Tamari, E. C. Sisler, and A. Borochov (1995b). Inhibition of ethylene-induced cellular senescence symptoms by 1-methylcyclopropene, a new inhibitor of ethylene action. Physiol Plant 94: 229-232.

Serek, M. and M. S. Reid (2000). Ethylene and postharvest performance of potted kalanchoe. Postharvest Biol Technol 18(1): 43-38.

Singh, A., K. B. Evensen, and T. Kao (1992a). Ethylene synthesis and floral senescence following compatible and incompatible pollinations in *Petunnia inflata*. Plant Physiology 99:38-45.

Singh, S., D. S. Letham, and L. M. S. Palni (1992b). Cytokinin biochemistry in relation to leaf senescence. Translocation, metabolism and biosynthesis of cytokinins in relation to sequential leaf senescence of tobacco. Physiol Plantarum 86: 398-406.

Smart, C. M. (1994). Tansley review no. 64: gene expression during leaf senescence. New Phytol 126: 419-448.

Smart, C. M., S. R. Scofield, M. W. Bevan, and T. A. Dyer (1991) Delayed leaf senescence in tobacco plants transformed with *tmr*, a gene for cytokinin production in *Agrobacterium*. Plant Cell 3: 647-656

Smigocki, A. C. (1991). Cytokinin content and tissue distribution in plants transformed by a reconstructed isopentenyl transferase gene. Plant Molecular Bio 16: 105-115.

Smigocki, A. C. and L. D. Owens (1988). Cytokinin gene fused with a strong promoter enhances shoot organogenesis and zeatin levels in transformed plant cells. Proc. Natl Acad. Sci. USA 85: 5153-5135.

Solomon, M., B. Belenghi, M. Delledonne, E. Menachem, and A. Levine (1999). The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. Plant Cell 11: 431-443.

Stead, A. D. (1992). Pollination-induced flower senescence: a review. Plant Growth Reg 11: 13-20.

Stead, A. D. and K. G. Moore (1983). Studies on flower longevity in *Digitalis*. The role of ethylene in corolla abscission *Digitalis purpurea*, aging. Planta 157: 15-21.

Stead, A. D. and van Doorn (1994). Strategies of flower senescence-1 review. In Scott, R. J. and A. D. Stead eds. Molecular and cellular aspects of plant reproduction, Cambridge University Press, Cambridge, UK, pp. 215-238.

Stephenson, P. and B. Rubinstein (1998). Characterization of proteolytic activity during senescence in daylilies. Physiol Plant 104: 463-473.

Sylvestre, I. And A. Paulin (1987). Accelerate ethylene production as related to changes in lipids and electrolyte leakage during senescence of petals of cut carnations (*Dianthus caryophyllus*) Physiol Plant 70: 530-536.

Takei, K., H. Sakakibara, and T. Sugiyama (2001). Identification of genes encoding adenylate isopentenyl transferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. J Biol Chem 276:26405-26410

Taverner, E., D. S. Letham, J. Wang, E. Cornish, and D. A. Willcocks (1999). Influence of ethylene on cytokinin metabolism in relation to Petunia corolla senescence. Phytochem 51:341-347.

Tetley, R. M, and K. V. Thimann (1974). The metabolism of oat leaves during senescence. I. Respiration, carbohydrate metabolism, and the action of cytokinins. Plant Physiol 54: 294-303.

Teyssendier, de la S. B., M. Axelos, and C. Peaud-Lenoel (1985). Cytokinins modulate the expression of genes encoding the protein of the light-harvesting chlorophyll a/b complex. Plant Mol Bio 5(3): 155-163.

Thomas, T. H. (1992). Some reflections on the relationship between endogenous hormones and light-mediated seed dormancy. Plant Growth Regul 11: 239-248.

Thompson, J. E., C. D. Froese, Y. Hong, K. A. Hudak, and M. D. Smith (1997). Release of lipid catabolites from membranes by blebbing of lipid protein particles. In

Willam, J. P., M. U. Khan, and N. W. Lem eds, Physiology, biochemistry, and molecular biology of plant lipids. Kluwer Academic, Dordrecht, Boston, pp154-156.

Tieman, D. M. and H. J. Klee (1999). Differential expression of two novel members of the tomato ethylene-receptor family. Plant Physiol 120(1): 165-172.

Tieman, D. M., M. G. Taylor, J. A. Ciardi, and H. J. Klee (2000). The tomato ethylene receptors NR and LeETR4 are negative regulators of ethylene response and exhibit functional compensation within a multigene family. Proc Natl Acad Sci 97 (10):5663-5668.

Tournaire, C., S. Kushnir, G. Bauw, D. Inzé, B. T. de la Serve, and J.-P. Renaudin (1996). A thiol protease and an anionic peroxidase are induced by lowering cytokinins during callus growth in petunia. Plant Physiol 111: 159-168.

Trewavas, A. J. (1982). Growth substance sensitivity: the limiting factor in plant development. Physiol. Plant. 55: 60-77.

Trione, E. J., B. B. Krygier, G. M. Banowetz, and J. M. Kathrein (1985). The development of monoclonal antibodies against the cytokinin zeatin riboside. J Plant Growth Regul 4:101-109

Trione, E. J., B. B. Krygier, J. M. Kathrein, G. M. Banowetz, and L. A. Sayavedra-Soto (1987). Monoclonal antibodies against the cytokinin isopentenyl adenosine. Physiol Plant 70:467-472

Ueda, T., S. Seo, Y. Ohashi, and J. Hashimoto (2000). Circadian and senescence-enhanced expression of a tovacco cysteine protease gene. Plant Mol Bio 44: 649-657.

Uota, M. (1969). Carbon dioxide suppression of ethylene-induced sleepiness of carnation blooms. J Am Soc Hort Sci 94: 598-601.

Upfold, S. J. and J. Van Staden (1990) Cytokinins in cut carnation flowers. VII. The effect of zeatin and dihydrozeatin derivatives on flower longevity. Plant Growth Regul 9:77-81.

Van Der Straeten, D. and M. Van Montagu (1990). Biochemistry and molecular genetics of ethylene biosynthesis and signal transduction. In Flores, H. E., R. N. Arteca, and J. C. Shannon eds, Polyamines and Ethylene: Biochemistry, Physiology, and Interactions, American Society of Plant Physiologist, Rockville, pp 36-49.

Van Doorn, W. G. and A. D. Stead (1994). The physiology of petal senescence which is not initiated by ethylene. In Scott, R. J. and A. D. Stead eds, Molecular and Cellular Aspects of Plant Reproduction, Cambridge University Press, Cambridge, UK, pp 239-254.

Van Loven, K., S. E. I. Beinsberger, R. L. M. Valcke, H. A. Onckelen, and H. M. M. Clijsters (1993). Morphometric analysis of the growth of the Phsp70-*ipt* transgenic tobacco plants. J. Experi. Bot. 44: 1671-1678.

Van Meeteren, U. (1979) Water relations and keeping quality of cut Gerbera flowers. III. Water content, permeability and dry weight of aging petals. Sci Hortic 10:261-269.

Van Staden, J., E. Cook, and L. D. Noodén (1968). Cytokinins and senescence. In Noodén, L. D. and A. C. Leopold eds, Senescence and Aging in Plants, Academic Press, San Diego, pp 281-328. (cited in Gan and Amasino, 1996)

Van Staden, J. and G. G. Dimalla (1980). The effect of silver thiosulfate on the physiology of cut carnations. II Influence on endogenous cytokinins. Z Pflanzenphysiol 99: 19-26.

Van Staden, J., B. C. Featonby-Smith, S. Mayak, H. Spiegelstein, and A. H. Halevy (1987). Cytokinins in cut carnation flowers. II. Relationship between endogenous ethylene and cytokinin levels in the petals. Plant Growth Regul 5: 75-86.

Vardi, Y. and S. Mayak (1989). Involvement of abscisic acid during water stress and recovery in petunia flowers. Acta Hort 261: 107-112.

Venkatarayappa, T., R. A. Fletcher, and J. E. Thompson (1984). Retardation and reversal of senescence in bean leaves by benzyladenine and decapitation. Plant and Cell Physiol 25: 407-418.

Wagstaff, C., M. K. Leverentz, G. Griffiths, B. Thomas, U. Chanasut, A. D. Stead, and H. J. Rogers (2002). Cystein protein gene expression and proteolytic activity during senescence of *Alstroemeria* petals. J Exp Bot 53 (367): 1-8.

Wallner, S., R. Kassalen, J. Burgood, and R. Graig (1979). Pollination, ethylene production and shattering in geraniums. HortSci 14: 446.

Wang, J., D. S. Letham, E. Cornish, K. Wei, C. H. Hocart, M. Michael, and K. R. Stevenson (1997). Studies of cytokinin action and metabolism using tobacco plants expressing either the *ipt* or the *GUS* gene controlled by a chalcone synthase promoter. II. *ipt* and *GUS* gene expression, cytokinin levels and metabolism. Aust J Plant Pysiol 24:673-683.

Wang, H. and W. R. Woodson (1989). Reversible inhibition of ethylene action and interruption of petal senescence in carnation flowers by norbornadiene. Plant Physiol 89: 434- 438.

Watada, A. E., R. C. Herner, A. A. Kader, R. J. Romani, and G. L. Staby (1984). Terminology for the description of developmental stages of horticultural crops. HortScience 19: 20-21.

Whitehead, C. S. and C. A. Bossè (1991). The effect of ethylene and short-chain saturated fatty acids on ethylene sensitivity and binding in ripening bananas. J Plant Physiol 137: 358-362.

Whitehead, C. S. and A. H. Halevy (1989). Ethylene sensitivity: the role of short chain saturated fatty acids in pollination-induced senescence of *Petunia hybrida* flowers. Plant Growth Regul 8: 41-54.

Whitehead, C. S., A. H. Halevy, and M. S. Reid (1984). Roles of ethylene and 1-aminocyclopropane-1-carboxylic acid in pollination and wound-induced senescence of *Petunia hybrida* flowers. Physiol Plant 61: 643-648.

Whitehead, C. S. and D. Vasiljevic (1993). Role of short-chain saturated fatty-acids in the control of ethylene sensitivity in senescing carnation flowers. Physiol Plantarum 88: 243-250.

Wilkinson, J. Q., M. B. Lanahan, D. G. Clark, A. B. Bleecker, C. Chang, E. M. Meyerowitz, and H. J. Klee (1997). A dominant mutant receptor from *Arabidopsis* confers ethylene insensitivity in heterologous plants. Nature Biotechnol 15:444-447.

Wilkinson, J. Q., M. B. Lanahan, H. –C. Yen, J. J. Giovannoni J. J., and H. J. Klee (1995). An ethylene-inducible component of signal transduction encoded by *Never-ripe*. Science 270: 1807-1809.

Woltering, E. J. and W. G. Van Doorn (1988). Role of ethylene and senescence of petals: morphological and taxonomical relationships. J Exp Bot 39: 1605-1616.

Woodson, W. R. (1994). Molecular biology of flower senescence in carnation. In Scott, R. J. and A. D. Stead eds, Society of Experimental Biology Seminar Series 55: Molecular and Cellular Aspects of Plant Reproduction, Cambridge University Press, pp 255-267.

Woodson, W. R., A. S. Brandt, H. Itzhaki, J. M. Maxson, H. Wang, K. Y. Park, and P. B. Larsen (1993). Ethylene regulation and function of flower senescencerelated genes. In Pech, J. C., A. Latché, and C. Balagué eds, Cellular and molecular aspects of the plant hormone ethylene, Kluwer Academic Publishers, Dordrecht, pp 291-297.

Woodson, W. R., S. H. Hanchey, and D. N. Chisholm (1985). Role of ethylene in the senescence of isolated hibiscus petals. Plant Physiol 79: 679-683.

Woodson, W. R. and A. K. Handa (1987). Changes in protein patterns and *in vivo* protein synthesis during presence and senescence of *Hibiscus* petals. J Plant Physiol 128: 67-75.

Woodson, W. R., K. Y. Park, A. Drory, P. B. Larsen, and H. Wang (1992). Expression of ethylene biosynthetic pathway transcripts in senescing carnation flowers. Plant Physiol 99: 526-532.

Wu, M., L. Zacarias, and M. S. Reid (1991). Variation in the senescence of carnation (*Dianthus caryophyllus* L.) cultivars. II Comparison of sensitivity to exogenous ethylene and to ethylene binding. Sci. Hortic. 48: 109-116.

Xu, F. X. and M. L. Chye (1999). Expression of cysteine proteinase during developmental events associated with programmed cell death in brinjal. Plant J 17: 321-327.

Xu, Y. and R. Hanson (2000). Programmed cell death during pollination-induced petal senescence in petunia. Plant Physiology 122: 1323-1333.

Yang, S. F. and N. E. Hoffman (1984). Ethylene biosynthesis and its regulation in higher plants. Annual Rev Plant Physiol 35: 155-189.

Ye, Z. -H. and J. E. Varner (1996). Induction of cysteine and serine protease during xylogenesis in *Zinnia elegans*. Plant Mol Biol 30: 1233-1246.

Zhang, J., T. Van Toai, L. Huynh, and J. Preiszner (2000). Development of flooding-tolerant *Arabidopsis thaliana* by autoregulated cytokinin production. Mol Breeding 6: 135-144.

Zimmerman, P. W., A. E. Hitchcock, and W. Crocker (1931). The effect of ethylene and illuminating gas on roses. Contrib Boyce Thomp Inst 3:459-481.

Zubko, E., C. J. Adams, I. Machaekova, J. Malbeck, C. Scollan, and P. Meyer (2002) Activation tagging identifies a gene from *Petunia hybrida* responsible for the production of active cytokinins in plants. Plant J 29:797-808

List of Abbreviations

1-MCP, 1-methylcyclopropene (also SIS-X)
ABA, abscisic acid
ACC, 1-aminocyclopropane-1-carboxylic acid
BA, benzyladenine
Ck, cytokinin
Ct, threshold cycle
DAA, day after anthesis
DEPC, Diethyl pyrocarbonate
Hap, hours after pollination
Hacp, hours after compatible pollination
Het, hours after ethylene treatment
iP, isopentenyl adenine
iP-9G, isopentenyl-9-glucoside
iPA, isopentenyl adenosine
ipt, isopentenyl transferase
diHZ, dihydrozeatin
diHZ-9G, dihydrozeatin-9-glucoside
diHZR, dihydrozeatin riboside
DW, dry weight
IPT22, one of the petunia transgenic lines with SAG_{12}-*ipt*
IPT34, one of the petunia transgenic lines with SAG_{12}-*ipt*
LHCP II, light-harvesting chlorophyll *a/b* binding protein
PCD, programmed cell death
PCR, Polymerase chain reaction
Phcp1, Petunia hybrida cysteine protease 1
ROS, reactive oxygen species
SAGs, senescence-associated genes
Sho, an isopentenyltransferase homologue cloned from petunia
tZ, trans zeatin
tZR, trans zeatin riboside
WT, wild type 'V26' petunias
Z, zeatin
Z-9-G, zeatin-9-glucoside
ZR, zeatin riboside