Leaf senescence in alstroemeria: regulation by phytochrome, gibberellins and cytokinins

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Leaf senescence in alstroemeria: regulation by phytochrome, gibberellins and cytokinins

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Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de landbouwuniversiteit Wageningen,
Dr C.M. Karssen,
in het openbaar te verdedigen
op 30 september 1998
des namiddags te vier uur in de Aula

This research was supported by a grant from the Foundation for Life Sciences (SLW), which is subsidised by the Netherlands Organisation for Scientific Research (NWO).

Cover design: Dieuwke Parlevliet

CIP-data Koninklijke Bibliotheek, Den Haag

Kappers, I.F.

Leaf senescence in alstroemeria: regulation by phytochrome, gibberellins and cytokinins / I.F. Kappers. - [S.l.: s.n]
Thesis Wageningen. - With ref. - with summaries in Dutch and English
ISBN 90-5485-919-9

Stellingen

1. Activatie van fytochroom leidt tot een tijdelijke toename in *meta*-topoline in bladeren.
   Dit proefschrift.

2. Voor het behoud van chlorofyl in bladeren van alstroemeria is GA_4 effectiever dan GA_1.
   Dit proefschrift.

3. Het groen houden van alstroemeria-bladeren door gibberellinen dient voornamelijk een cosmetisch doel en leidt niet tot het behoud van de fysiologische functie van chlorofyl.
   Dit proefschrift
   Jordi et al. 1994. Physiologia Plantarum 90: 293-298.

4. Gezien het grote aantal verschillende gibberellinen die voorkomen in planten en het beperkte aantal hiervan dat daadwerkelijk een fysiologische rol speelt, zouden publicaties die slechts nieuwe gibberellinen identificeren, geweigerd moeten worden.

5. Als mensen net als planten, verrood licht zouden kunnen waarnemen waren bladeren niet groen maar donkerrood.

6. Het gebruik van spaarlampen spaart niet alleen energie maar ook het bladgroen in alstroemeria's.

7. GA_3-toevoeging aan het vaaswater van rozen leidt tot minder zwakte-pathogenen zoals Botrytis. Dit berust niet op een fungicide werking van GA_3 maar op een hogere weerstand van de GA_3-behandelde rozen.
   Shaul et al. 1995. Postharvest Biology and Technology 6: 3-4.

8. Statistische significantie impliceert niet automatisch biologische relevantie.

9. Handleidingen worden pas dan handreikingen als de werking van het apparaat doorgrond is.

10. Het feit dat steeds meer geld voor onderzoek uit de markt gehaald moet worden beperkt de wetenschappelijke vrijheid en bijbehorende creativiteit.

11. Communicatie is van wezenlijk belang voor menszijn.

12. Ieder conflict tussen Genesis en de evolutietheorie bestaat dankzij het in meer of mindere mate letterlijk nemen van het Genesisverhaal.

13. Een eerste voorwaarde voor een multiraciale samenleving is niet integratie maar acceptatie.

14. Zappen is als een modern gezelschapsspelletje: voor de één monopoly, voor de ander een potje mens-erger-je-nieten.

Stellingen behorende bij het proefschrift 'Leaf senescence in alstroemeria: regulation by phytochrome, gibberellins and cytokinins' door I.F. Kappers. Wageningen, 30 september 1998.

Abstract

Kappers, IF, 1998. Leaf senescence in alstroemeria: regulation by phytochrome, gibberellins and cytokinins. Thesis Wageningen Agricultural University, Wageningen, The Netherlands, 143 pp; English and Dutch summaries.

Leaf senescence in plants is a regulated process influenced by light as well as phytohormones. In the present study the putative role of the phytohormones cytokinins and gibberellins as mediators for the light signal on leaf senescence in alstroemeria was studied. It was found that low photon fluences of red light ensured maximal delay of chlorophyll and protein breakdown. This effect of red light could be completely counteracted by a subsequent far red irradiation, indicating phytochrome involvement.

Application studies with gibberellins showed that GA_4 was most effective in delaying leaf senescence and it was proven that GA_4 is not converted into GA_1 but is biologically active by itself. A total of 11 gibberellins was detected to be endogenous in alstroemeria leaves. During senescence the relative concentration of precursors and active gibberellins decreased whereas that of inactivated gibberellins increased strongly. Although irradiation of the leaves with red light resulted in delayed senescence and a higher GA_4 concentration compared to dark-incubated leaves, based on the obtained results, GAs are not considered to act as mediators for the transduction of the light signal.

Alstroemeria leaves were found to contain isoprenoid-derived cytokinins and aromatic cytokinins. Irradiation of leaves with red light resulted in a transient increase in *meta*-topolin and *meta*-topolin riboside approximately one hour after the start of illumination. No light related changes in concentration were found for other cytokinins in these leaves.

Although the visual effect of red light, cytokinins and gibberellins is similar, the mode of action of the regulators may be different. It was found that both red light and *meta*-topolin had a positive effect on chlorophyll biosynthetic reactions as well as on the rate of photosynthesis and expression of genes encoding for chlorophyll binding proteins (*cab*). GA_4 did not positively affect these parameters. The chlorophyll catabolic reaction, determined as Mg-dechelatase activity was not differentially affected by either *meta*-topolin, GA_4 or red light. From the results, it is suggested that aromatic cytokinins are primarily involved in regulation of leaf senescence and can function as a mediator for the transduction of the phytochrome signal.

Dit is mijn geheim, zei de vos, het is heel eenvoudig:
alleen met het hart kun je goed zien. Het wezenlijke
is voor de ogen onzichtbaar.

Vertaald uit: Le Petit Prince
van Alexander de Saint-Exupéry

Contents

Abbreviations

A_n nm	absorbance at n nm
BA	benzyladenine
BAPR	benzyladenine riboside
BAP9G	benzyladenine 9-glucoside
BSTFA	N,O-bistrimethylsilyltrifluoroacetamide
cab	gene coding for chlorophyll a/b binding protein
Chlide	chlorophyllide
CK	cytokinin
$[CK]_{50}$	CK concentration required for half-maximum response
DHZ	dihydro zeatin
DHZR	dihydro zeatin riboside
DHZ9G	dihydro zeatin 9-glucoside
DHZOG	dihydro zeatin O-glucoside
DHZROG	dihydro zeatin riboside O-glucoside
DMF	dimethylformamide
EC	enzyme commission number
ELISA	enzyme-linked immunosorbent assay
GA, GA_n	gibberellin, gibberellin n
$[GA]_{50}$	GA concentration required for half-maximum response
GC-MS	gas chromatography-mass spectrometry
GC-SIM	gas chromatography-selected ion monitoring
iP	isopentenyl adenine
iPR	isopentenyl adenosine
iPRMP	isopentenyl adenosine-5'-monophosphate
iP9G	isopentenyl adenosine 9 glucoside
KRI	Kováts retention index
$[M]^+$	molecular ion, parent ion
*m*T	*meta*-topolin (*meta*-OH-6-benzyladenine)
*m*TR	*meta*-topolin riboside
*m*TRMP	*meta*-topolin riboside-5'monophosphate

*m*T9G	*meta*-topolin 9-glucoside
*m*TOG	*meta*-topolin O-glucoside
*m*TROG	*meta*-topolin riboside O-glucoside
Me	methyl derivative
MeTMSi	methyl trimethylsilyl derivative
m/z	mass/charge ratio
ND	not determined
oT	*ortho*-topolin (*ortho*-OH-6-benzyladenine)
oTR	*ortho*-topolin riboside
oT9G	*ortho*-topolin 9-glucoside
oTOG	*ortho*-topolin O-glucoside
oTROG	*ortho*-topolin riboside O-glucoside
p	Hill-coefficient
P_{fr}	FR-absorbing form of phytochrome
P_r	R-absorbing form of phytochrome
P_{tot}	total phytochrome ($P_r + P_{fr}$)
Pchlide	protochlorophyllide
φ	phytochrome photoequilibrium (P_{fr} / P_{tot})
QAE	quaternary ammonium ether
R_{max}	maximum response
R_{min}	minimum response in the absence of exogenous hormone
SD	standard deviation of series
SE	standard error
TIC	total ion current
TMCS	trimethylchlorosilane
$^{v}/_{v}$	volume/volume
$^{w}/_{v}$	weight/volume
Z	zeatin
ZR	zeatin riboside
ZRMP	zeatin riboside-5'monophosphate
Z9G	zeatin 9-glucoside
ZOG	zeatin O-glucoside
ZROG	zeatin riboside O-glucoside

1 Introduction

Light controls almost every step in the life cycle of a plant. Next to photosynthesis, for which light is essential, a number of photoreceptors is involved in photomorphogenisis. Although the structure and function of a number of photoreceptors are well characterised, the sequence of events that occurs after light perception and the mechanisms by which the initial signal is transduced into a physiological effect are largely unknown. Phytohormones have been proposed to be involved in this transduction pathway (Furuya 1993). Indeed, as discussed by Chory *et al.* (1994), a plethora of data shows that light and hormones control many overlapping aspects of plant development. Depending on the species considered and the experimental conditions, all kinds of interactions, additivity, synergism and antagonism, have been observed.
Although the individual effects of light and phytohormones on the process of senescence have been thoroughly described, not much is known about possible interactions of light and phytohormones in this developmental process. The putative role of phytohormones as mediators for the light signal on leaf senescence is the objective of the studies described in this thesis.

1.1 Leaf senescence

Senescence is an inextricable part of the development of any organism. It is the sequence of biochemical and physiological events from the mature, fully expanded state until death. Since long, senescence is considered as internally regulated and programmed, because it is specific and orderly in terms of when, where and how it occurs (Noodén 1988). Studies with enucleation, selective inhibitors, mutations, and more recently, genetic engineering have supported the idea that senescence is an active process and is controlled by the nucleus (Noodén 1988, Smart 1994, Buchanan-Wollaston 1997, Gan and

Amasino 1997). Whatever the internal or external trigger, it is possible that the control steps of leaf senescence at the molecular level are the same. Recently, Weaver *et al.* (1998) found that mostly senescence-associated genes respond in a similar fashion to various senescence-inducing treatments. The identification of such steps is a major objective for current senescence research.

A major environmental factor influencing senescence is the light that can have an effect via the photoperiod (day length), irradiance level or the spectral distribution via phytochrome. Day length appears to influence the onset of senescence in many species (Smart 1994). However, day length may act through its effect on the switch to flowering, which can trigger leaf senescence. The relationship between the irradiance level and leaf longevity is far from simple. Leaves generally remain green longer in full sunlight than when they are shaded, but plants of the forest undercanopy exhibit leaf longevity although they live in deep shade for most of the year (Harrington *et al.* 1989). Under excessive irradiation, leaves turn yellow due to photoinhibition and chlorophyll bleaching, especially at periods of low temperature. Light can reverse senescence induced by a short dark treatment, but it becomes progressively less effective after longer dark periods (Wittenbach 1977). It is only possible to reverse senescence in its early stages, which may be because of the proposed 'point of no return' in the process.

Like many correlative controls, those regulating (monocarpic) senescence appear to be mediated by hormones (Noodén 1988). Next to cytokinins the best known senescence-inhibiting plant hormones, gibberellins appear to be important in a number of genera like *Rumex* (Whyte and Luckwill 1966), *Taraxacum* (Fletcher and Osborne 1966) and *Lilium* (Han 1995). Auxines can either enhance or inhibite senescence, depending on the species involved. Ethylene and abscisic acid accelerate senescence in the leaves of many, but not all species (Noodén 1988). Recently, genes which direct hormone-biosynthesis and –perception have been cloned which enabled direct manipulation of hormone level and action in transgenic plants. Such experiments directly demonstrated retarded leaf senescence in tomato plants which have low levels of ethylene biosynthesis (John *et al.* 1995) or a disrupted ethylene perception (Gebic and Bleecker 1995). Senescence is primarily characterised by the loss of chlorophyll but also by loss of total protein, total RNA, chloroplast RNA as well as activation of specific proteins, peroxidases and some RNA's (Thomas and Stoddart 1980). These characteristics have been confirmed by the expression patterns of senescence-involved genes (Smart 1994).

1.2 Chlorophyll

In chlorophyll (from the Greek words for 'green' and 'leaf'), four pyrrole rings are ligated into a tetrapyrrole ring with magnesium in the center. Ring IV is esterified with phytol. Chlorophyll *a* has a methyl group at carbon-3, but higher plants and algae use for light harvesting an additional form of chlorophyll, chlorophyll *b*, that has a formyl group instead of a methyl group at this position (Von Wettstein *et al.* 1995).

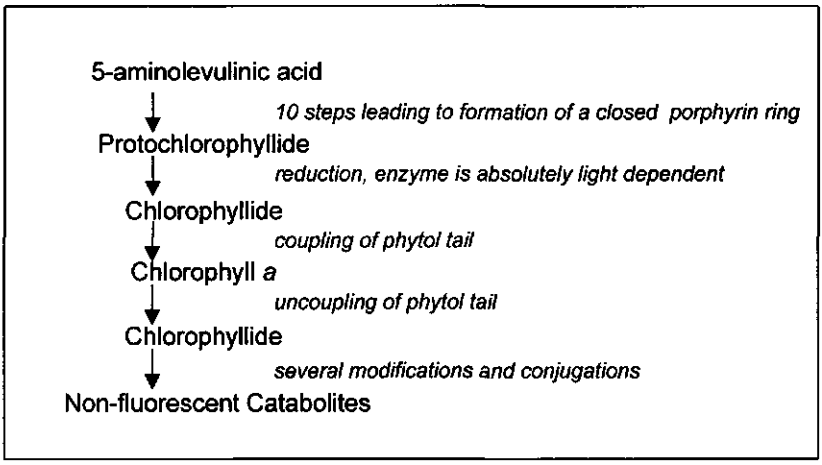

Figure 1.1 *Schematic representation of the major biosynthetic reactions leading to chlorophyll and the subsequent catabolic reactions.*

The porphyrin ring with its conjugated double bonds is assembled in the chloroplast from eight molecules of 5-aminolevulinic acid. The biosynthetic pathway of chlorophyll from 5-aminolevulinic acid to protochlorophyllide consists of 10 steps that can all occur without the presence of light. However, the conversion of protochlorophyllide, the next-to-last precursor of chlorophyll to chlorophyllide is absolutely light dependent (Von Wettstein *et al.* 1995). Finally, chlorophyll is formed by esterification with phytol.

All chlorophyll molecules in the chloroplast are bound non-covalently to proteins in the photosynthetic membrane. Chlorophyll *a* and *b* binding (*cab*) proteins are encoded by nuclear genes, synthesised on cytoplasmic ribosomes, imported across the two membranes of the chloroplast and inserted into the thylakoid membrane. About 50% of the total chlorophyll in mature leaves forms a protein complex, LHCII, light-harvesting complex II,

engaged in transferring light energy to photochemical reaction centers (Von Wettstein *et al.* 1995).
Chlorophyll catabolism starts with the removal of the phytol tail by the same enzyme that binds the phytol tail, chlorophyll synthetase. Whereas the porphyrin moieties of chlorophyll are further broken down, phytol appears to be rather stable (Matile *et al.* 1989). The subsequent breakdown steps of chlorophyllide involves dechelatase of the magnesium and cleavage of the porphyrin leading to non-fluorescent catabolites. These catabolites of chlorophyll are localised in the vaculole (Matile *et al.* 1988).

1.3 Phytochrome

In 1952 it was first proposed by Borthwick *et al.* that a photoreversible pigment acted as a switch in the control of germination of lettuce seeds. The pigment could be turned on by red light (maximum response at 660 nm) or off by far-red light (maximum response at 730 nm); after successive alternating red and far-red irradiation the final light quality determined the response. Since then, hundreds of these classical phytochrome-mediated responses have been described. In 1960 Borthwick and Hendricks proposed the term phytochrome from the Greek words for 'plant' and 'colour'.
Phytochrome is a Y-shaped molecule and consists of two identical monomers composed of an apoprotein and a chromophore (Jones and Erickson 1989). Phytochrome is a photochromic pigment, *i.e.* can be repeatedly interconverted by light between two photo-isomers.

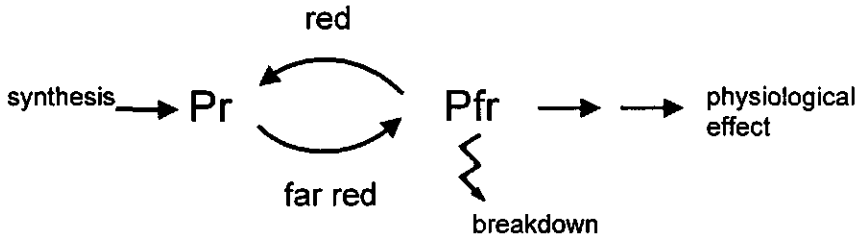

Figure 1.2 *Schematic representation of photoconversion of phytochrome*

The absorption bands of both forms overlap. Because of this a photoequilibrium (φ) is being established, representing the proportion of

total phytochrome molecules in the Pfr form. The precise value of φ depends on the spectral distribution of the light. Using the formulae of Mancinelli (1994) the minimum and maximum values of φ at equilibrium for monochromatic FR (730 nm) and R (660 nm) are about 0.02 and 0.87, respectively.

Phytochrome is synthesised as Pr, the chromophore being exported from the plastids and assembled with apoprotein in the cytoplasm. Upon photoconversion to Pfr (Hendricks 1964) it becomes active and initiates the diverse array of responses under its control. One of the most rapid effects triggered by Pfr is an alteration of gene expression (Quail 1991). Red light is not only necessary for converting phytochrome to its physiologically active form Pfr, but also for initiating the rapid breakdown of the Pfr pool (Vierstra 1994). Moreover, Pfr down regulates the *de-novo* synthesis of Pr (Colbert 1991).

Molecular evidence that plants contain several types of phytochrome encoded by a small multigene family was provided initially from studies with *Arabidopsis*. These phytochrome (*PHY*) genes were designated *PHYA* through *PHYE* (Clack *et al.* 1994). Many other complete *PHY* clones have been obtained from different species, indicating that the *PHY* family is widespread throughout the plant kingdom (Pratt 1995).

Phytochromes function via different response modes depending on the fluence rate and duration of the light (Mancinelli 1994). Three main response modes can be distinguished:

1. Very low fluence responses (VLFR), are saturated at extremely low fluences of less than 100 nmol m^{-2}. VLFR reactions are not reversible by far-red light, in fact far-red light can induce VLFR responses. VLFR responses have been observed in dark-imbibed seeds or seedlings grown in absolute darkness (Smith and Whitelam 1990).
2. Low fluence responses (LFR), are the classical phytochrome mediated responses. They are induced by a single pulse of red light and counteracted by subsequent far-red irradiation. LFR responses follow the Bunsen-Roscoe law, *i.e.* reciprocal relationship between the fluence rate and the duration of the light. The photon fluences required for saturation of the response by red light vary from 1 to 1000 μmol m^{-2}.
3. High irradiance responses (HIR) require continuous long term exposures to relatively high fluence rates (Mancinelli 1994) and show no red / far red reversibility. The extent of the HIR is a function of wavelength, irradiance and duration of the light treatment.

The transduction chain of the signal from the photoreceptor triggered towards the final response induced is still largely a black box. The signal transduction involves phosphorylation of proteins, activation of GTP-binding proteins and changes in the phosphatidylinositol and calcium metabolism (Roux 1994, Bowler and Chua 1994). Using micro-injection techniques Neuhaus and co-workers (1993) elucidated two signalling pathways resulting from activation of phytochrome leading to anthocyanin biosynthesis which requires cyclic GMP and inducing gene expression of cab proteins which requires both calcium and calmodulin. However, both pathways have 'cross-talk' and are both required for full development of the functional plastids (Bowler *et al.* 1994).

1.4 Gibberellins

In 1926, Kurosawa demonstrated that rice plants treated with a sterile filtrate of the fungus *Gibberella fujikuroi* showed the same symptoms as plants infected by this fungus. In 1938 the active compound of this filtrate was isolated and named gibberellin by Yabuta and Sumiki. Gibberellic acid (now GA_3) was the first GA to have its structure completely determined (Cross *et al.* 1959). The first identification of GAs in higher plants was made in immature seeds of *Phaseolus multiflorus*, and the isolated GA was found to be identical with the fungal GA_1 (MacMillan and Suter 1958). To date, over 115 different GA structures have been characterised in higher plants and fungi.

1.4.1 Gibberellin chemistry

Gibberellins are tetracyclic diterpenoid acids and can be divided into two main groups, the C_{20}-gibberellins with twenty carbon atoms, and the C_{19}-gibberellins which have lost the carbon-20. The systematic nomenclature is based on the name gibberellane, which was given to the tetracyclic ring system as in Fig 1.3, with four different rings named A, B, C and D (Rowe 1968).
Because the systematic nomenclature is rather confusing, MacMillan and Takahashi (1968) proposed a trivial nomenclature. According to their suggestions, each new naturally occurring gibberellin is given an A number

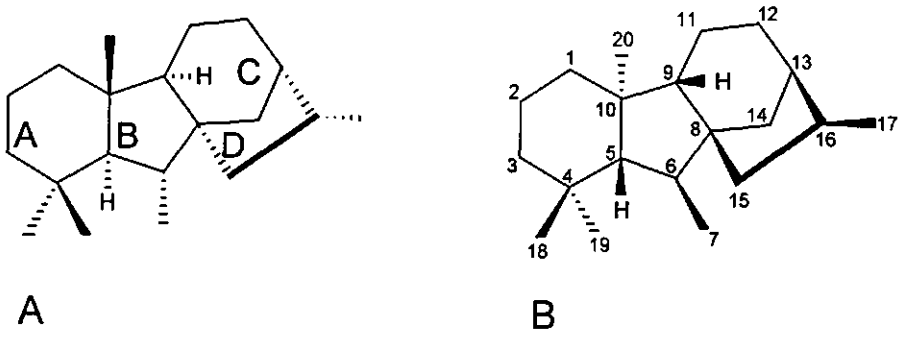

Figure 1.3 *Structural formula and numbering system of gibberellane (A) and ent-gibberellane (C_{20}-gibberellins) (B)*

after it has been suffisciently characterised. In contrast, the gibberellin precursors are named by their systematic names, like e.*g. ent*-kaurene.

Although there are over 115 fully characterised GAs, they can easily be described according to their oxidation patterns. The C_{20}-GAs can be characterised by the carbon-20, which can be present as CH_3, CH_2OH, CHO or COOH (Pearce *et al.* 1994). The C_{20}-GAs with the carbon-20 as CH_2OH can form a δ-lactone with the carbon-19. Except for GA_{11}, the C_{19}-GAs possess a 19-10 γ-lactone bridge. The major positions of hydroxylations are at the 2β-, 3β- and 13-positions, but hydroxylation can also occur at other positions. The position of the hydroxylations, carboxylations and lacton bridges affect the degree of biological activity in bioassays (Crozier *et al.* 1970). The C_{19}-GAs are more active than the C_{20}-GAs. The C_{19}-GAs with a 3β-hydroxylation, 3β,13-dihydroxylation or 1,2-unsaturation are highly active, but the GAs with 2β-hydroxylation are without activity (Sponsel 1995).
There are a number of GA conjugates identified in plants (Sembdner and Schneider 1988). Next to glucosides and glucosyl esters, acetyl derivatives as well as alkyl esters have been isolated from various species and tissues. GA conjugates have been considered to act as storage forms, and are thought to be favoured for long distance transport or compartmentalisation.

1.4.2 Gibberellin metabolism

The metabolism of GAs can be divided into three steps: firstly the biosynthesis of *ent*-kaurene from mevalonic acid, secondly the biosynthesis of GA_{12}-aldehyde from *ent*-kaurene and lastly the biosynthesis after GA_{12}-aldehyde. The first two steps are thought to be identical in both fungi and higher plants whereas the third step can vary between species and tissues (Graebe 1987).
GA biosynthesis branches from the general terpene biosynthetic pathway at geranylgeranyl diphosphate, which is a common precursor for carotenoids, abscisic acid and the phytol chain of chlorophyll as well as for GAs (Graebe 1987, Hedden and Kamiya 1997, Sponsel 1995). Because *ent*-kaurene is the first commited intermediate in the GA-pathway, its synthesis is thought to be rate-limiting for endogenous GA-biosynthesis (Graebe 1987). A strong correlation between the rate of *ent*-kaurene synthesis and growth potential of tissues suggests that the rate of precursor synthesis may indeed control the rate of GA biosynthesis (Sun and Kamiya 1997).

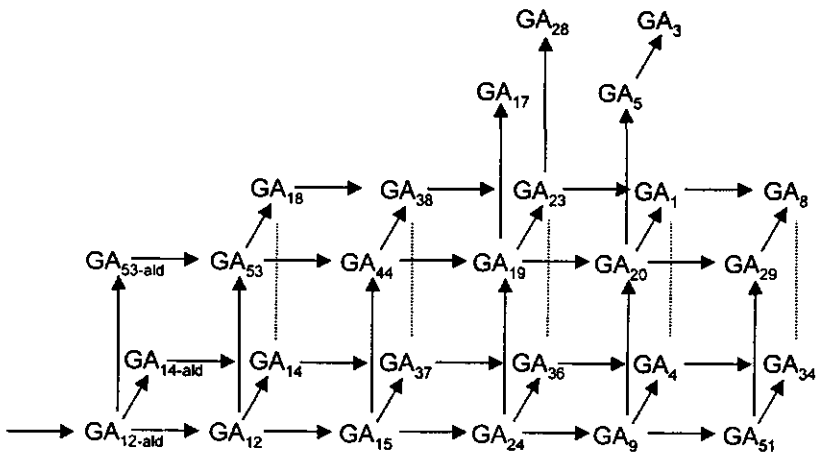

Figure 1.4 *Schematic representation of metabolic conversions of gibberellins in higher plants, showing known conversions. Dotted lines indicate hypothetical conversions. Adapted from Sponsel (1995).*

The site of ent-kaurene synthesis has been suggested to be located in the plastids and copalyl diphosphate synthase and *ent*-kaurene synthase activity were shown present only in the stromal fraction of proplastids and developing chloroplasts, but not in the mature choroplasts isolated from leaves (Aach *et al.* 1995).

Interestingly, the enzymes involved in the conversion of ent-kaurene to GA_{12}-aldehyde are associated with the endoplasmic reticulum, and the pathway after the formation of GA_{12}-aldehyde is in the cytoplasm (reviewed in Hedden and Kamiya 1997). Compartmentalisation of GA biosynthesis in different organelles may play an important role in the regulation of the pathway.
The biosynthetic pathway up to GA_{12}-aldehyde appears to be the same in all plants. In contrast, the conversion of GA_{12}-aldehyde into other GAs can vary from genus to genus. However, there is a basic sequence of reactions starting from GA_{12}-aldehyde which is common to all pathways (Fig. 1.4). This sequence involves the successive oxidation of carbon-20 and the formation of C_{19}-GAs. In vegetative tissues the native GAs are structurally less diverse than in reproductive tissues, and are formed through a number of highly conserved pathways (Sponsel 1995).

1.5 Cytokinins

Although the idea that specific endogenous substances are required for cell division dates from the previous century (Wiesner 1892), cytokinin research raised interest from the 1950's, when Skoog and co-workers started their investigations on tissue culture. The active compound needed for cell division in these cultures was isolated from herring sperm and named 'kinetin' (Miller *et al.* 1955). This component is an artefact resulting from the heating of DNA and not endogenous to plants. In 1963, Letham was the first to purify the natural occurring plant cytokinin from *Zea mays*. Although cytokinins were first discovered by their ability to induce cell division, they are now known to act in combination with other phytohormones to regulate diverse responses in plants, including seed germination, *de novo* bud formation, leaf expansion, and senescence.

1.5.1 Cytokinin chemistry

To date, cytokinins are defined as compounds that have a purine structure with a 5-carbon N^6-substituent, regardless of their activity on cell division. Figure 1.5 shows the basic structure of cytokinins. The nature of the side chain on the R_1-position determines the type of cytokinin, whereas modifications on N7 or N9 determine the form of which the free base, riboside, monophosphate, glucoside and O-glucoside are most occurring in plants (Wagner 1991). Isoprenoid cytokinins possess a side-chain originating from an isoprenoid molecule resulting in iP, Z and DHZ, depending on a hydroxyl group and unsaturated bond. Aromatic cytokinins posses a benzylic side chain that can be hydroxylated at different positions (Fig. 1.5).

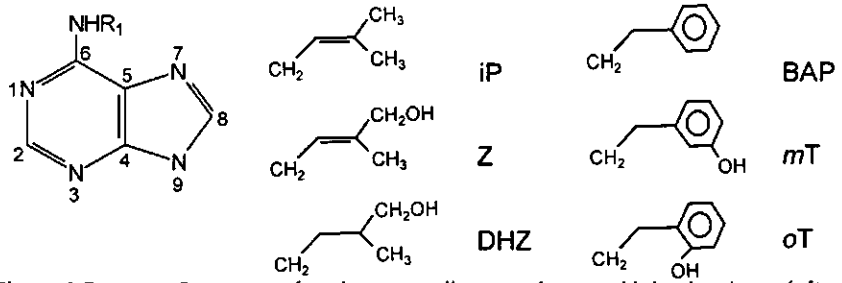

Figure 1.5 *Structure of major naturally occurring cytokinins in plants (after Wagner 1991).*

1.5.2 Cytokinin metabolism

Cytokinin biosynthesis in mature plants takes place mainly in the roots, although smaller amounts can be synthesised by the shoot apex and some other plant tissues (Henson and Wareing 1976). Two biosynthetic pathways have been reported for isoprenoid cytokinins: the *de novo* biosynthetic pathway and the tRNA pathway (Prinsen *et al.* 1997). It is very likely that the *de novo* biosynthetic pathway accounts for the majority of newly synthesised cytokinin. The key step in this pathway is the formation of N^6-(Δ^2-isopentenyl) adenosine-5'-monphosphate from Δ^2-isopentenyl pyrophosphate and adenosine-5'-monophosphate catalysed by isopentenyl-transferase. By dephosphorylation, reduction and glucosylation the ribosides, free bases and glucosides are formed (Wagner 1991; Jameson 1994). The biosynthetic pathway of aromatic cytokinins is still largely unknown. Based on the

structure of the molecule, aromatic cytokinins are unlikely to diverge from isoprenoid cytokinins but have their own biosynthetic pathway (Strnad 1997). To date, no (putative) cytokinin receptor has been described but it is not likely that this receptor will recognises both isoprenoid and aromatic cytokinins, because most likely recognition probably will be on the side-chain Matsubara (1990) reviewed the extensive studies on structure/activity relationships of different cytokinins in several bio-assays. Aromatic and isoprenoid cytokinins have different action spectra: It is hypothesised that isoprenoid cytokinins are highly involved in processes in young, developing plants such as cell division and induction of new buds, while aromatic cytokinins have more affinity with processes in older plants such as senescence (Strnad 1997).

1.6 Interaction

Several types of interaction between phytochrome and gibberellins or cytokinins are described for various species. Photoregulation of hormone metabolism can be by the presence or absence of light, the transition between light and darkness, the duration or alteration of the photoperiod, the quality of the light (*e.g.* the ratio of red and far-red) and the quantity of light. An overview is given in Table 1.1. The best described physiological processes involving regulation by light and gibberellins are the induction of seed germination and the control of stem elongation. Several studies provided strong but somewhat contrasting arguments for an involvement of GAs in the light-signalling pathway.
Also cytokinins and light cause similar effects on plant development, for example the opening and development of cotelydons and the differentiation of chloroplasts. The transcription of numerous chloroplastic genes is induced by light and cytokinins (Cohen *et al.* 1988; Bracale *et al.* 1998). The level of interaction between light and cytokinins is still unclear. Work with *Arabidopsis* indicates that phytochromes and cytokinins act independently to regulate many growth and development processes (Su and Howell 1995). However, it is still possible that light does modify the metabolism of cytokinins or regulates signal transduction steps.

Table 1.1 *Interactions between light, gibberellins and cytokinins known to occur in plants.*

Type of photoregulation	Hormonal reaction	Plant model system	Reference
Presence or absence of light	Dark-grown plants had increased responsiveness to GA_1	Pea	Weller *et al.* 1994
	Darkness decreased CK concentration	Iris	Mae and Vonk 1974
Transition between light and dark	Light affected GA metabolism in dark-grown plants	Pea	Sponsel 1986
	Light affected GA metabolism in dark-grown plants	Lettuce	Toyomasu *et al.*.1992
	Increase in CK concentration at daybreak	Poplar	Hewett and Wareing 1973 Strnad, unpublished results
	Light decreased CK concentration in dark-stressed shoots	Rose	Van Staden et al. 1981
Duration or alteration of photoperiod	Short days increased GA_{53} and GA_{19}; GAs of the 13-OH pathway are synthesised in a rhythmic pattern in short day conditions.	Spinach	Talon *et al.* 1991
	GAs do not mediate the effect of extented photoperiod, both factors act through independent mechanisms	*Thlaspi arvense*	Metzger 1988
	Single long night transiently increased CKs in xylem and phloem	*Xanthium strumarium*	Kinet *et al.* 1994
	Short days increased CK concentration in leaves	Begonia	Hansen *et al.* 1988
	Transition to long days increased CK concentration	Sinapis	Lejeune *et al.* 1988

continuation of Table 1.1

Quality of the light (R or FR)	FR enhanced response to GA_1 and GA_{20} and decreased 2β-hydroxylase activity	Cowpea	Martínez-García and García-Martínez 1992
	R reduced sensitivity to GAs	Rice	Nick and Furuya 1993
		Arabidopsis	Reed *et al.* 1996
	Response to R not mediated through GAs but through IAA	Pea	Behringer *et al.* 1990
	R decreased CKs in root exudate of dark-grown plants, reversibility by FR	*Chenopodium*	Machackova *et al.* 1996
	CK concentration increased after 30 min R	*Picea*	Taylor and Wareing 1979
Quantity of the light	Low irradiance increased GA_1 and GA_{20} biosynthesis and GA_1 responsiveness	Pea	Gawronska *et al.* 1995
Independent action	GA_4 and phytochrome act primarily through separate mechanisms that interact at a step close to the terminal response	*Cucumis*	López-Juez *et al.* 1995
	de-etiolated mutants grown in darkness show many characteristics of light-grown plants and have altered response to CKs	*Arabidopsis*	Chory *et al.* 1994
	Differential abilities of Ca^{++} to block effects of R and CKs suggest different classes of Ca^{++} channels	*Cucumis*	Reiss and Beale 1995
	Light inhibited hypocotyl elongation in *cytokinin-resistant/ethylene-insensitive* mutants	*Arabidopsis*	Su and Howell 1995

1.7 Aim and outline of the thesis

The aim of this study is to obtain information on the role of phytochrome, endogenous gibberellins and cytokinins in the regulation of leaf senescence of alstroemeria cut flowers. The approach used in this study was to monitor endogenous gibberellins and cytokinins during the course of senescence. Also the relation between light conditions and the activation of phytochrome and the endogenous gibberellin and cytokinin contents was studied. Finally, an attempt was made to get insight in the regulation points for control of chlorophyll breakdown.

In Chapter 2, the model system of phytochrome-regulated leaf senescence of alstroemeria leaves is described. Chapter 3 descibes the method for identification of gibberellins in leaves and gives quantitative data on gibberellin concentrations in leaves of different age. In Chapter 4, the possible interaction of red light and gibberellins in their regulation of senescence is discussed. A comparison of two active gibberellins is made in Chapter 5 based on the information given in Chapter 4. The possible interaction of red light and cytokinins is studied in Chapter 6. Chapter 7 deals with the possible points of chlorophyll biosynthesis and degradation and chlorophyll function, where red light, gibberellins and cytokinins can intervene. In the general discussion (Chapter 8), the results of the previous chapters are integrated.

2 Characterisation of light-regulated leaf senescence in alstroemeria

2.1 Abstract

A model system is described to study the role of gibberellins and cytokinins in light-regulated senescence. The plant species alstroemeria is shortly introduced. Leaves of *Alstroemeria hybrida* cultivar Cinderella and Westland were characterised and compared in their senescence pattern. Photon fluence rate studies were performed to determine the irradiance level and duration needed for maximum effects on chlorophyll retention. It is concluded that the effect of red light on delay of senescence is not via maintenance of photosynthesis but by activation of phytochrome as the effect of red light on delay of chlorophyll and protein breakdown is far-red reversible.

2.2 Introduction

Interaction of light and phytohormones is described for developmental processes like seed germination, stem elongation (gibberellins and light), betacyanin synthesis and chloroplast development (cytokinins and light). To study the role of phytohormones in light-regulated senescence, a model system is desired with sufficient sensitivity to detect small differences in treatments. For the study described in this thesis, leaves of alstroemeria were used to investigate possible interactions of cytokinins, gibberellins and light on senescence. In alstroemeria, leaf senescence is delayed by low concentrations of exogenously applied gibberellins and, although at higher concentrations, cytokinins (Jordi *et al.* 1995). The concentration of gibberellin

needed for delaying senescence in alstroemeria is of a similar order of magnitude as reported for *Rumex obtusifolius*, a plant species of which senescence was strongly delayed by gibberellins (Whyte and Luckwill 1966). The delay of senescence in alstroemeria leaves by low fluences of light was first reported by Van Doorn and Van Lieburg (1993) and they suggested an involvement of phytochrome. Therefore, alstroemeria leaves are very suitable as a modelsystem to unravel possible interactions of gibberellins, cytokinins, and light on the process of senescence. In this chapter the species alstroemeria is shortly introduced and the effects of light on senescence characteristics are described.

2.3 Alstroemeria

The genus *Alstroemeria* was discovered in 1714 when Louis Feuillet noticed and first described a species of alstroemeria growing along a stream running through the city of Conceptión in Chile (Aker and Healy 1990). He classified this plant as a species of *Hemerocallis*. Later, Linnaeus noted the lack of similarity and named it *Alstroemeria pelegrina* in honor of his friend and student, Claes Alstroemer (1736-1794) from Sweden who brought the first seed from South America to Europe.
The natural habitat of alstroemeria is mainly in Chile and Brazil wheras some species are found in Bolivia, Peru, Venezuela, Paraquay and Argentina as well (Aker and Healy 1990). Alstroemeria plants consist of a sympodial, fleshy, multistemmed rhizome from which aerial shoots and fibrous roots arise. The shoots can be either reproductive or vegetative depending on the environmental conditons (Anonymus 1981). Nearly all members of the genus have leaves that are resupinate (turned upside down) due to the twisting of the leaf base. The leaves are gray-green to dark green, and mostly hairless on both sides. The genus *Alstroemeria* consists of approximately 60 species. Most cultivars of commercial interest are allelotetraploïd crossings of Brasilian and Chilene species named Hybrid-types. The cultivars Cinderella and Westland used in this study, although from different breeding compagnies, both originated from similar parent species of *Alstroemeria*. Both cultivars have large, open pink flowers with lilac-rose fingerprints in the center of the outer petals (Bridgen 1993).
Alstroemeria has gained world-wide importance as a cut flower due to its low-energy growing conditions (Anonymus 1981), high productivity in a year-round culture, and excellent vase life of its attractive flowers, which are

available in a wide range of colours (Blom and Piott 1990). In The Netherlands, cut flowers of alstroemeria rank tenth in the auction when compared to other cut flowers. In 1997 110 ha. alstroemeria's were produced with a commercial value of Dfl 89 million (Anonymous 1997). Primary objectives for the breeders include vigorous growth forms, continuous year-round production and new flower colours. A number of cultivars exhibit a rapid senescence of the leaves during the vase life before the quality of the flower declines resulting in a lower keeping quality.

2.4 Material and methods

Plant material

In this study two cultivars of *Alstroemeria pelegrina* were used, *i.e.* cv. Westland and cv. Cinderella. Both culivars were grown at a commercial nursery under greenhouse conditions usual for the growth of alstroemeria (Anonymus 1981). Unless otherwise mentioned (as in Chapter 3), flower stalks were cut at the developmental stage when the leaves of the upper whorl were fully expanded and dark-green and the petals of the flower buds of the inflorescence were still closed but already coloured red. This developmental stage is the normal stage for auction selling.
Stalks were cut at approximately 60 cm below the inflorescence, transported to the laboratory and handled within 3 hours after cutting. The five uppermost leaves, positioned in a whorl directly below the flowers, were used for the experiments. Two model systems were used: (A) excised leaf tips and (B) deflowered stems. For system A leaf tips, ± 3 cm^2 and circa 150 mg fresh weight, were excised with a sharp knife and placed with the cut end in demineralised water. For deflowered stems (system B), flowers were removed and the stem was cut at 15 cm below the whorl leaves. The stems were placed in demineralized water. Removal of the flowers had no effect on senescence characteristics (Jordi *et al.* 1993) and ensured a uniform irradiation of the leaves. Both leaf tips and deflowered stems were kept in a climate room at 20 ± 1°C and *ca* 65% relative humidity during the duration of the experiment.

Light conditions

Irradiance levels and spectral photon distribution were measured by a Li-Cor Li-1800 spectroradiometer (Li-Cor inc., Lincoln, USA). Green safe-light was obtained by filtering the light of Philips TL 40W/84 fluorescent tubes through 2 layers of green Strand cinelux (nr. 439) filter (Strand Lighting Ltd., Middlesex, UK). The light irradiance level at the level of the leaves was less than 0.5 µmol m^{-2} s^{-1}.

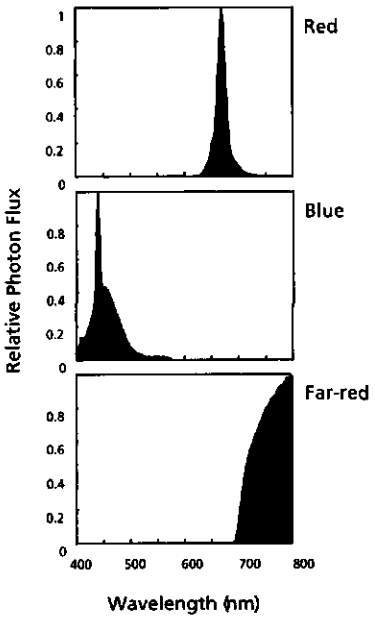

Figure 2.1 *Spectral photon distribution of red, blue and far-red light.*

Philips TL 40W/15 fluorescent tubes were used as light sources for red light (Fig. 2.1). The λ_{max} was at 660 nm. The phytochrome equilibrium (φ), P_{fr}/P_{tot} was 0.87 calculated from extinction coefficients of rye phytochrome according to Lagarias *et al.* (1987). The photon fluence rate was varied by placing the leaves at various distances to the lamp. Blue light was obtained from Philips TL 40W/18 fluorescent tubes in combination with two layers of a blue filter (Strand cinelux nr. 419) with λ_{max} at 442 nm and a φ of 0.54 (Fig. 2.1). The photon fluence rate was 6.2 µmol m^{-2} s^{-1} at the level of the leaves. Broad-band far-red light (φ = 0.054) was obtained by filtering the light of Philips 40W incandescent light bulbs through one layer of orange (nr. 405)

and one layer of blue filter respectively (Fig. 2.1). The photon fluence rate was 5.4 μmol m^{-2} s^{-1} at the level of the leaves.

Chlorophyll analysis

To determine the chlorophyll content of the leaves, a sample of 100 mg fresh weight was extracted in 5 ml dimethylformamide (DMF) during 24 hours at 4°C in darkness. The optical density was determined spectrophotometrically at 647 and 664.5 nm. Using the molar extinction coefficient in dimethylformamide (Inskeep and Bloom 1985), chlorophyll a and b were calculated with the formula's:

$(12.7\ A_{664.5} - 2.79\ A_{647}) \times (0.005 / FW)$	Chlorophyll a	mg $(g\ FW)^{-1}$
$(20.7\ A_{647} - 4.62\ A_{664.5}) \times (0.005 / FW)$	Chlorophyll b	mg $(g\ FW)^{-1}$
$(17.9\ A_{647} + 8.08\ A_{664.5}) \times (0.005 / FW)$	Chlorophyll a + b	mg $(g\ FW)^{-1}$

Molecular weight of chlorophyll a: 893.5 and of chlorophyll b: 907.5

Protein analysis

For analysis of soluble protein, leaves were frozen and homogenized in liquid nitrogen. Ice-cold extraction buffer (60 mM TRIS/HCl pH8.0, 10 mM EDTA, 30 mM β-mercapto ethanol, 0.5 M NaCl and 0.1 mM PMSF) was added (5 ml buffer $(g\ FW^{-1})$) and extraction was performed by intensive mixing. The extract was centrifuged (10 min at 5000 rpm at 0°C) and washed once. The total soluble protein content was determined spectrophotometrically at 595 nm after adding Coomassie Brilliant Blue R250 (Pierce, Rockford, USA) using bovine serum albumin as a standard.
Soluble proteins were fractionated and visualised by applying an aliquot of 10 μl on a 10% SDS-polyacrylamide gel and after electroforesis subsequently stained with Coomassie Brilliant Blue (R250).

Photosynthesis

Leaf photosynthesis was measured by using a infra red gas analyzer. The air led into the leaf chamber had a constant CO_2 pressure of 33 Pa. The average conditions within the leaf chamber were 19.8°C and a vapour pressure deficit of 0.57 kPa. Different photon fluence rates were obtained by fractional

filtering with neutral grey filters. Rates of photosynthesis were calculated from the flow rates and the measured concentrations of CO_2 and vapour in the ingoing and outgoing air stream according to Von Caemmerer and Farquhar (1981).

2.5 Results and discussion

Senescence in darkness

Data are presented as percentage of the initial amount of chlorophyll or protein in order to correct for the small differences between experiments.
The changes in chlorophyll content of the leaves of cvs Westland and Cinderella are shown during 10 days of incubation in darkness (Fig. 2.2). During the first 3 (Westland) to 4 (Cinderella) days, chlorophyll contents remained approximately stable or only slightly decreased. Thereafter, chlorophyll content decreased rapidly in darkness from day 3 - 4 onwards. Leaves of cv. Westland were homogeneously yellow after 7 to 8 days of darkness, while those of cv. Cinderella were homogeneously yellow after 10 days. For both cultivars no differences in chlorophyll pattern in time were found between detached leaf tips and the deflowered stems (Fig. 2.2).
At the start of the experiments the absolute soluble protein concentration in the leaves was 12.4 ± 0.8 mg $(g\ FW)^{-1}$ for cv. Cinderella and 10.7 ± 0.6 mg $(g\ FW)^{-1}$ for cv. Cinderella, respectively. In darkness the leaves showed a gradual decrease in protein concentration starting immediately after the beginning of the experiment resulting in less than 30% of the initial content after 6 (Westland) and 10 (Cinderella) days (Fig. 2.3).
To visualise changes in polypeptides during leaf senescence, proteins were subjected to SDS-PAGE electroforesis (Fig. 2.4). The protein patterns in mature leaves of cv. Cinderella and cv. Westland are very comparable (data not shown). The most obvious change in the composition of polypeptides during senescence was the decrease in Rubisco large sub unit content, the most abundant protein in the leaves at the start of the experiments. When leaves received a daily red light irradiation, this decrease was significant less (Fig. 2.4).

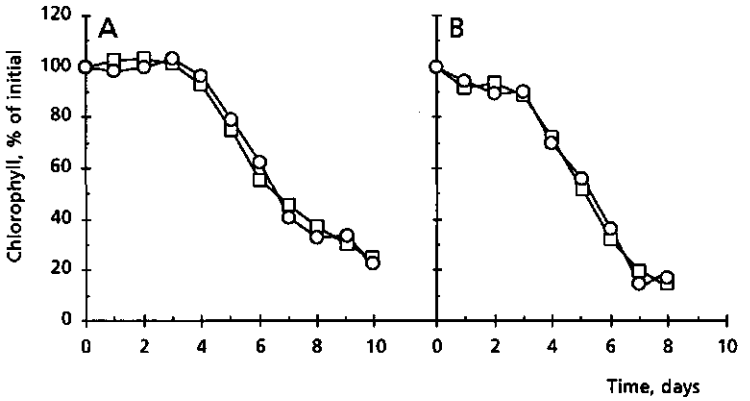

Figure 2.2 *Chlorophyll content in leaf tips (□) and leaves of deflowered stems (○) of cv. Cinderella (A) and Westland (B). Leaves were placed in darkness for 10 days. Initial chlorophyll concentration was 2.05 ± 0.16 mg (g FW)$^{-1}$ for cv. Cinderella and 1.74 ± 0.09 mg (g FW)$^{-1}$ for cv. Westland. Data are means of 5 replications.*

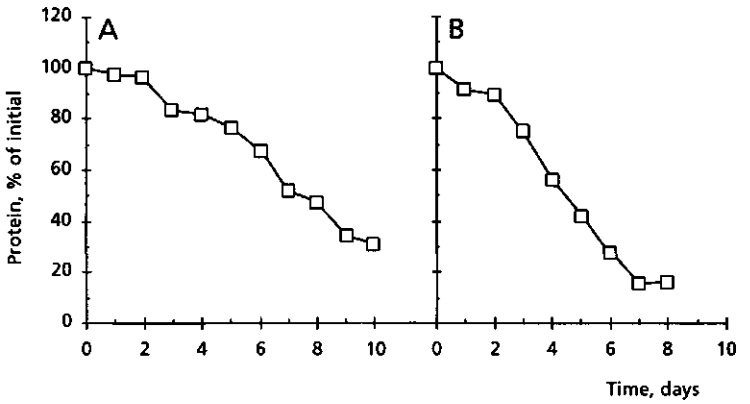

Figure 2.3 *Soluble protein content in leaf tips (□)of cv. Cinderella (A) and Westland (B). Leaves were placed in darkness for 10 days. Initial protein concentration was 12.4 ± 0.8 mg (g FW)$^{-1}$ for cv. Cinderella and 10.7 ± 0.6 mg (g FW)$^{-1}$ for cv. Westland. Data are means of 5 replications.*

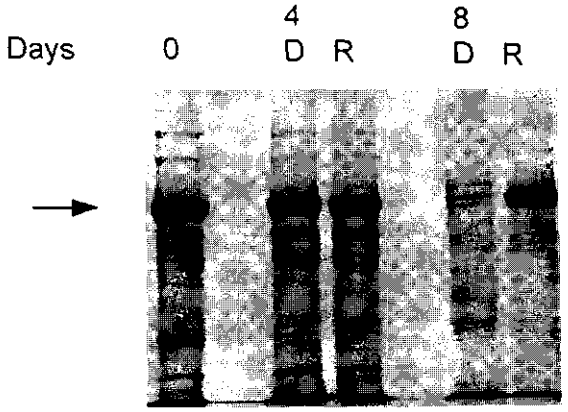

Figure 2.4 *Soluble protein composition of alstroemeria cv. Westland leaves at different time intervals placed in either darkness or subjected to a daily red irradiation. The arrow indicates the large subunit of Rubisco.*

Effect of light on chlorophyll concentration

Figure 2.5 presents the effects of various durations and photon fluxes of red light on the chlorophyll concentration of leaves of cv. Westland after 8 days. These light treatments were given daily to the leaves. Degradation of chlorophyll was suppressed at photon fluence rates higher than 0.5 μmol m^{-2} s^{-1}. Increasing photon fluence rates remained the chlorophyll concentration in the leaves at higher levels after 8 days. The maximum effect was reached at 2.9 μmol m^{-2} s^{-1}. An extended exposure to the light source remained the chlorophyll concentration in the leaves as well, with maximum effect on chlorophyll retention reached after 5 min with a photon fluence rate of 5.1 μmol m^{-2} s^{-1}. This response can be classified as a low fluence response obeying the Bunsen-Roscoe law (Chapter 1.3). The maximum effect of red light was a chlorophyll concentration of approximately 60% of the initial amount after 8 days (cv. Westland). To ensure sufficient irradiation of the leaves the standard light treatment of 5 μmol m^{-2} s^{-1} red light for 10 min day^{-1} was used in all experiments described in this thesis unless otherwise mentioned (in Chapter 4 and 6). Leaves irradiated with this amount of light showed a delay of the onset of leaf senescence as well as a decreased rate of chlorophyll loss during the following 4 days (Fig. 2.6). This behaviour is comparable to that of leaves kept under day light conditions (Fig. 2.6).

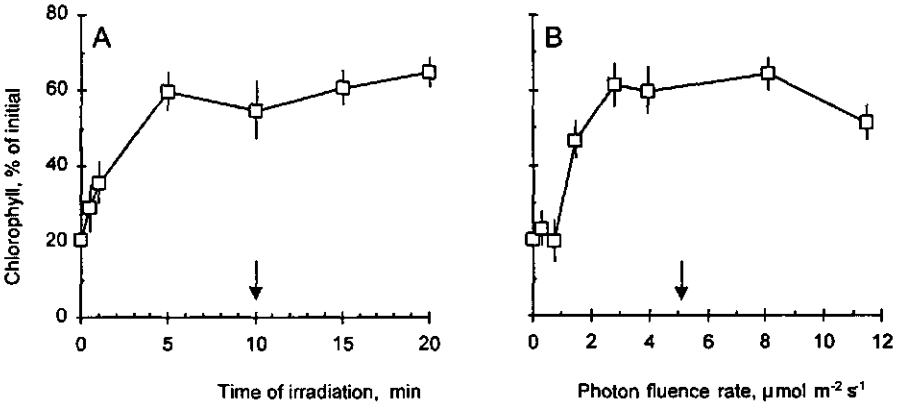

Figure 2.5 *Chlorophyll content in leaves of deflowered stems of cv. Westland after 8 days. Leaves received daily light treatments of various durations and photon fluence rates. A: Increasing light durations at 5.1 ± 0.8 µmol m^{-2} s^{-1} and B: Increasing photon fluence rates during 5 min daily irradiation. Arrows show the time and photon fluence rate that was used as a standard treatment in the experiments described in this thesis. Data are means of 5 replications ± SD.*

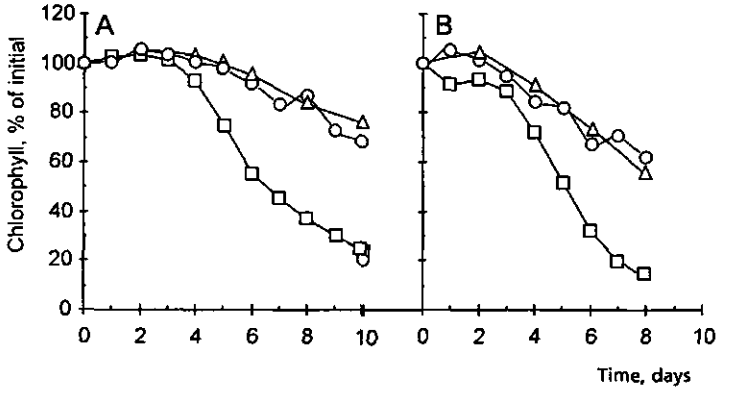

Figure 2.6 *Chlorophyll content in leaves of deflowered stems of cv. Cinderella (A) and Westland (B). Leaves were placed either in darkness (□); under day light conditions (Δ) or received daily 10 min 5.0 µmol m^{-2} s^{-1} red light (○). Initial chlorophyll concentration was 2.05 ± 0.16 mg (g FW)$^{-1}$ for cv. Cinderella and 1.74 ± 0.09 mg (g FW)$^{-1}$ for cv. Westland. Data are means of 5 replications.*

At the start of the experiment the molar ratio chlorophyll *a* : chlorophyll *b* was 2.9 for both cultivars. No significant changes in chlorophyll *a/b* ratio were determined during the course of the experiments in darkness nor in the light (data not shown). The fresh weight of the leaves changed less than 2% during the course of the experiments independent of the treatment. During the first 2 days sucrose, and hexose concentrations of the leaves decreased substantially and a photon fluence of 5 µmol m^{-2} s^{-1} red light had no effect on these concentrations (data not shown).
Light response curves of photosynthetic activity of leaves of cv. Cinderella and cv. Westland are shown in Figure 2.7. The light compensation points of photosynthesis in mature leaves were at 4.65 µmol m^{-2} s^{-1} (cv. Cinderella) and 3.72 µmol m^{-2} s^{-1} (cv. Westland) red light. The photosynthesis at the given red light photon fluence rate during 10 min per day will be neglectable and therefore, an effect of red light irradiation via maintenance of photosynthesis on delay of senescence can be excluded.

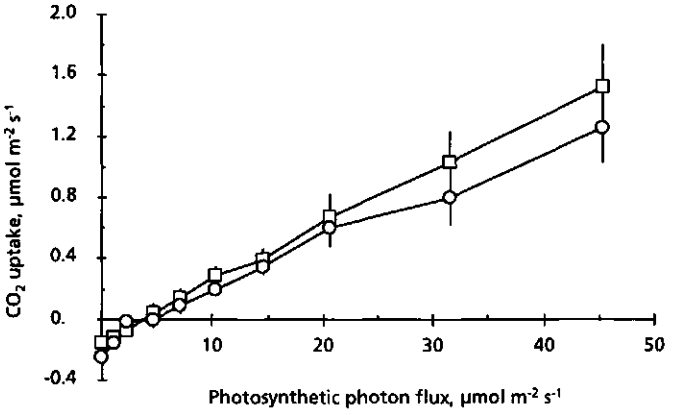

Figure 2.7 *Photosynthesis as CO_2 uptake (µmol m^{-2} s^{-1}) of fresh mature leaves of cv. Cinderella (○) and cv. Westland (□) at increasing red light intensities. Data are means of 5 replications ± SD.*

Table 2.1 presents chlorophyll and soluble protein concentrations after 8 days of different light treatments. Red light effectively delayed both chlorophyll and soluble protein breakdown in both cultivars. Far-red light resulted in similar chlorophyll and protein concentrations after 8 days as

leaves placed in darkness (data not shown). Daily irradiation of leaves with red light immediately followed by far-red light did not prevent chlorophyll loss and resulted in similar kinetics as the dark or far-red light treatment. These results show that phytochrome is the photoreceptor involved in the senescence process of these leaves.
Blue light significantly inhibit chlorophyll and protein degadation but to a far les extent than did red light. Furthermore, as with red light, far-red light also counteracted the effect of blue light completely, indicating that the effect of blue light was due to phytochrome activation also.

Table 2.1 *Chlorophyll and soluble protein concentration in leaves of alstroemeria cv. Cinderella and cv. Westland affected by various light treatments during 8 days. Photon fluence rates of the light sources were 5.0 µmol m^{-2} s^{-1} for red light, 5.4 µmol m^{-2} s^{-1} for far red light and 6.2 µmol m^{-2} s^{-1} for blue light. ND: not determined. Data are means of 5 replications ± SD.*

Treatment	Chlorophyll mg (g FW)$^{-1}$		Soluble protein mg (g FW)$^{-1}$	
	Cinderella	Westland	Cinderella	Westland
Initial concentration (day 0)	1.98 ± 0.11	1.56 ± 0.03	12.47 ± 0.8	10.71 ± 0.6
Concentration after 8 days of:				
Darkness	0.72 ± 0.06	0.28 ± 0.08	5.48 ± 1.5	1.20 ± 1.2
Red light for 10 min day^{-1}	1.54 ± 0.10	1.16 ± 0.12	8.60 ± 1.9	6.35 ± 0.9
Far red light for 10 min day^{-1}	0.75 ± 0.09	0.26 ± 0.15	4.88 ± 0.8	1.23 ± 0.6
Red followed by far-red light	0.82 ± 0.11	0.30 ± 0.04	5.03 ± 1.3	1.15 ± 0.8
Blue light for 10 min day^{-1}	ND	0.84 ± 0.11	ND	4.63 ± 1.3
Blue followed by far-red light	ND	0.27 ± 0.13	ND	1.56 ± 1.6

In conclusion, both cultivars of alstroemeria tested in this study show comparable senescence kinetics. The chlorophyll and protein concentrations after 8 (cv. Westland) or 10 days (cv. Cinderella) are less than 20% of the initial concentrations. An irradiation with 5 µmol m^{-2} s^{-1} red light during 10 min per day results in retention of approximately 60% of the initial concentration of chlorophyll. This effect is due to a phytochrome activation as it could be completly counteracted by a far-red irradiation.

Chapter 3 is based on the publication:

Kappers IF, Jordi W, Maas FM and Van der Plas LHW (1997) Gibberellins in leaves of *Alstroemeria hybrida*: identification and quantification in relation to leaf age. Journal of Plant Growth regulation 16: 219-225.

3 Identification and quantification of gibberellins in relation to leaf age

3.1 Abstract

In alstroemeria (*Alstroemeria hybrida*), leaf senescence is retarded effectively by the application of gibberellins. To study the role of endogenous GAs in leaf senescence, the GA content was analysed by combined gas chromatography-mass spectrometry. The 13-hydroxy GAs: GA_{19}, GA_{20}, GA_1, GA_8 and GA_{29} and the non-13-hydroxy GAs: GA_9 and GA_4 were identified in leaf extracts by comparing Kováts retention indices and full-scan mass spectra with those of reference GAs. In addition, GA_{15}, GA_{44}, GA_{24} and GA_{34} were tentatively identified by comparing selected ion monitoring results and KRIs with those of reference GAs. A number of GAs was detected in conjugated form as well. Concentrations of GAs in alstroemeria changed with the development of the leaves. The proportion of the biologically active GA_1 and GA_4 decreased with progressive senescence and the fraction of conjugated GAs increased.

3.2 Introduction

Chlorophyll degradation is the most striking physiological change occurring during leaf senescence and is influenced by various external and internal factors. With respect to growth regulators, the role of cytokinins in this process has been emphasized. However, in a number of species senescence is delayed more effectively by gibberellins. Whyte and Luckwill (1966) first described the delayed senescence of dark-incubated leaf discs of *Rumex* by GAs. Also *Taraxacum* (Fletcher and Osborne 1966), *Tropaeolum* (Beevers and

Guernsey 1967), *Lactuca* (Aharoni and Richmond 1978) and *Lilium* (Han 1995) showed a delayed senescence when GAs were applied. In alstroemeria, leaf senescence is delayed very effectively by application of GAs, whereas cytokinins exhibited less and auxins and polyamines no effect (Jordi *et al.* 1995).

It is widely accepted that GAs play a crucial role in the control of internode elongation in plants (*e.g.* reviewed by Graebe 1987). Other functions include stimulation of seed germination, control of flower development and fruit growth. In most species studied to date (maize, pea, rice, spinach) the major or only pathway in shoot tissues is the early 13-hydroxylation pathway yielding GA_1. However, these studies were limited to seedlings in which stem elongation is the major feature studied. Only a few reports, however, describe GA patterns in mature and older plant parts. Kurogochi *et al.* (1979) reported the presence of GA_1, GA_4 and GA_{19} in rice, and determined concentrations of GA_{19} the most abundant GA, throughout its life cycle. Endo *et al.* (1989) reported the presence of a number of 13-hydroxylated GAs in *Phaseolus vulgaris* shoots during their development. In tulip it was suggested that the major GA pathway is the non-13-hydroxylation pathway as GA_4, GA_9, GA_{12}, GA_{24} and GA_{34} were identified in different plant parts (Rebers *et al.* 1994).

Young leaves are thought to be major sites for gibberellin synthesis (Zeevaart and Gage 1993, Sponsel 1995), whereas senescing leaves contain a high concentration of conjugated GAs as determined by bioassay procedures (Aharoni and Richmond 1978, Zheng and Zhou 1995). Recently, Choi *et al.* (1995) reported high levels of GA_1, GA_{19} and GA_{53} in young leaves of rice compared to older leaves and suggested GA synthesis in the young leaves.

To our knowledge, only limited information is available discussing the role of endogenous GA in the regulation of leaf senescence using modern analytical techniques such as gas chromatography-mass spectrometry (GC-MS). Because leaf senescence in alstroemeria is retarded very effectively by applied GAs, endogenous GAs are most likely involved in the regulation of leaf senescence in this species. To obtain insight in this regulatory role, it is essential to clarify the nature of GAs endogenous in alstroemeria leaves and determine their concentrations during development. The present study revealed 11 GAs endogenous to alstroemeria leaves and reported the amounts of GAs found in three stages of leaf development. The results provide a basis for further studies on the role and mode of action of GAs in the process of leaf senescence.

3.3 Materials and methods

Chemicals

GA_1, GA_4, GA_8, GA_9, GA_{12}, GA_{15}, GA_{19}, GA_{20}, GA_{24}, GA_{34}, GA_{44}, [17-$^2H_2$]GA_1, [17-$^2H_2$]GA_4, [17-$^2H_2$]GA_8, [17-$^2H_2$]GA_9, [17-$^2H_2$]GA_{19}, [17-$^2H_2$]GA_{20}, were obtained from Dr L.N. Mander (Australian National University, Canberra, Australia), [2,3-$^3H_2$]GA_9 was from Dr. A. Crozier, Glasgow, UK, [1,2,3-$^3H_3$]GA_{20} was from Dr J. MacMillan, Bristol, UK and [1,2-$^3H_2$]GA_1 and [1,2-$^3H_2$]GA_4 from Amersham (Buckinghamshire, UK). The specific activity of [^{3}H]GAs was about 50.000 Ci mol^{-1}. Petroleum ether 40-60 was obtained from Lamers and Pleuger ('s Hertogenbosch, The Netherlands).

Plant material and experimental conditions

Alstroemeria hybrida cv. Westland plants were grown at a commercial nursery. The five uppermost leaves, positioned in a whorl directly below the flowers, were used for the experiments. Flower stalks were cut at approx. 60 cm from the top of the inflorescence. Three developmental stages were defined: (1) young leaves of 5-7 cm long, flower buds ± 0.5 cm; (2) full grown mature leaves of 10-12 cm long, flower still closed but petals of the first flower bud were already coloured red; (3) senescent yellow leaves, flowers open. To induce a homologous leaf senescence, stems were placed in demineralised water and kept in the dark for 8 days at 20°C and 70% relative humidity. Leaves were collected, weighed, plunged into liquid N_2 and stored at -80°C until extraction. A subsample of 100 mg was taken to analyse the chlorophyll (*a*+*b*) content as described in chapter 2.4.

Extraction and prepurification

The purification method was adapted from Rebers *et al.* (1994). All glass material was pre-silanised with dichloromethylsilane and rinsed thoroughly with demineralised water before use to reduce the loss of GAs caused by aspecific absorption. Frozen samples (about 50 -100 g FW) were ground to powder with pestle and mortar under liquid N_2. Ice-cold methanol (MeOH:tissue = 4:1 ($^w/_v$)) containing 0.01% ($^w/_v$) ascorbic acid was added, and the homogenate was stirred during 4 hours at 4°C. To quantify endogenous

amounts of GAs, [17-$^2H_2$]-gibberellins GA_1, GA_4, GA_8, GA_9, GA_{19} and GA_{20}, 100 ng each, were added at the start of the extraction period. After centrifugation (10 min, 4°C, 5000 rpm) the residue was re-extracted with 80% methanol overnight at 4°C. Methanol and part of the plant water were removed under reduced pressure. The aqueous residue was frozen at -20°C. After thawing, the volume of the residue was adjusted to 60 ml with H_2O and adjusted to pH 7.5-8.0 with 1 N KOH.
In the first experiments, [^{3}H]GAs (about 50.000 dpm each) were added to estimate recoveries during purification. The sample was partitioned against petroleum ether 40-60 (three times with an equal volume) and the aqueous phase passed down a polyvinylpyrrolidone column (about 2 g, pre-equilibrated with H_2O at pH 8.0), which was eluted with 10 ml of H_2O at pH 8.0. The eluate was adjusted to pH 2.5 with 6 N HCl and partitioned against ethyl acetate (four times). The combined organic phases were partitioned against 5% ($^w/_v$) sodium bicarbonate (three times). The aqueous phases were acidified to pH 2.5 with 6 N HCl and partitioned against ethyl acetate (four times) and subsequently against diethyl ether (once). The organic phases were combined and reduced to dryness. The sample was dissolved in 10 ml of H_2O and adjusted to pH 8.0, for QAE anion exchange chromatography.

Prepurification of conjugated gibberellins

The aqueous phase remaining after (the first) ethyl acetate partitioning may contain conjugated GAs. This phase was repartitioned with H_2O-saturated *n*-butanol (three times). For quantification, [17-$^2H_2$]-gibberellins GA_1, GA_4, GA_8, GA_9, GA_{19} and GA_{20}, 100 ng each, were added. The combined butanol phases were reduced to dryness with small amounts of H_2O added to remove final traces of butanol. The residue was dissolved in 10 ml of 0.5 M sodium acetate at pH 4.8. Cellulase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21) (Boehringer, Mannheim, Germany; about 3 U each) were added, and the sample was incubated for 24 h at 35°C. After hydrolysis, the pH was adjusted to 2.5 and the buffer phase was extracted with ethyl acetate (four times). The ethyl acetate phases were taken to dryness, and the residue was dissolved in 10 ml H_2O and adjusted to pH 8.0 for QAE anion exchange chromatography.

Anion exchange chromatography

The fractions resulting from the prepurification were each loaded on a column (10 cm x 1 cm, inner diameter) of QAE Sephadex A-25 (Pharmacia, Uppsala, Sweden), equilibrated with sodium formate (1% ($^{w}/_{v}$), at pH 8.0-8.5), and subsequently washed with H_2O (40 ml). GAs were eluted with 40 ml of 0.2M formic acid and loaded directly on a pre-equilibrated C_{18} Sep-Pak cartridge (Waters, Millipore Corporation, Milford, Massachusetts, USA). GAs were eluted with 10 ml of 80% methanol and evaporated to dryness.
GAs were further purified by reverse phase HPLC using a Chromspher 5 C_{18} column (Chrompack, Bergen op Zoom, The Netherlands; 250 x 10.0 mm). The column was eluted at a flow rate of 1 ml min^{-1} with 30% methanol for 15 min, followed by a linear gradient to 75% methanol over 35 min and subsequently to 90% methanol over 5 min (solvents contained 0.01% acetic acid). Samples were dissolved in 300 µl methanol and made up to 1000 µl with H_2O and injected into the column. Detection occurred with an UV absorbance monitor at 210 nm. For the qualitative analysis, 50 fractions of 1 ml were collected and taken to dryness under reduced pressure to remove all traces of acetic acid. For quantitative analysis, fractions of 18-21 min (A); 23-26 min (B); 26-29 min (C) and 40-48 min (D) were collected.
Extracts were methylated with excess ethereal diazomethane. The methylated extracts were taken to dryness under a N_2 stream and redissolved in 25 µl of a fresh mixture of BSTFA:TMCS:pyridine (20:1:79, $^{v}/_{v}$) and heated for 20 min at 70°C to produce the trimethylsilyl ethers of the methyl esters (MeTMSi) for GC-MS.

Capillary column GC-MS

Derivatized samples were analysed using a Hewlett Packard 5890 GC coupled to a HP 5970 mass selective detector (Hewlett Packard Company, Wilmington, Detroit, USA). Aliquots of 3 µl were injected splitless into a fused silica capillary column (CPSil 5CB; Chrompack, Bergen op Zoom, The Netherlands; 30 m x 0.25 mm x 0.4 µm) and separated by a temperature gradient: 0-2 min, 25°C min^{-1} to 250°C and 4°C min^{-1} to 280 min; 4 min hold. Helium was used as a carrier gas at 0.94 ml min^{-1}. The injector and interface temperature were 250°C and 290°C, respectively. GC-MS with selected ion monitoring (GC-SIM) was used to search HPLC fractions for some gibberellins not detected by full-scan MS. The system was set to monitor ions of *m/z* as follows: GA_{12}: 241, 269, 285, 300, 328, 360; GA_{15}: 239, 284, 312, 344; GA_{24}:

226, 286, 314, 342, 374; GA_{34}: 223, 288, 372, 416, 506; GA_{51}: 284, 328, 386, 418; GA_{53}: 207, 208, 251, 389, 448. Kováts Retention Indices (KRI) for standards and endogenous gibberellins were measured using co-injection of a mixture of *n*-alkanes (Gaskin *et al.* 1971).
Quantification of GAs was achieved using calibration curves constructed by mixing protonated and deuterated GAs in various amounts including corrections made for naturally occurring isotopes according to Hedden (1987) and Croker *et al.* (1994). The ion pairs monitored by GC-SIM were 506/508 (GA_1), 284/286 (GA_4), 594/596 (GA_8), 270/272 (GA_9), 434/436 (GA_{19}) and 418/420 (GA_{20}). GA_{29} was quantified against the [$^2H_2$]-GA_8 standard in the same injection, so the values should be taken as approximate although valid for comparison. Corrections for different abundances of GA_{29}- and [17-$^2H_2$]GA_8 standards were estimated by co-injection of both standards. GA_{24} and GA_{15} (against [17-$^2H_2$]GA_9 standard) and GA_{34} and GA_{44} (against [17-$^2H_2$]GA_4 standard) were estimated in the same way.

3.4 Results

Identification of gibberellins in mature leaf tissue

After fractionation of extracts by reverse phase HPLC, a total of six 13-hydroxylated GAs and five non-13-hydroxylated GAs were identified (Table 3.1). Based on a comparison of full scan mass spectra and KRIs with those obtained for standard GAs, HPLC fraction A was found to contain GA_8; fraction B GA_1; fraction C GA_4, GA_{19}, GA_{20} and GA_{44}; and fraction D GA_9. For GA_{29} in fraction A no standard was available and therefore we used reference spectra published by Gaskin and MacMillan (1991). GA_{34} in fraction C and GA_{15} and GA_{24} in fraction D were identified by comparing GC-SIM results and KRIs with standards. No evidence for the presence of GA_{12}, GA_{51} or GA_{53}, was found in any leaf extract as analysed by both full scan and SIM methods.
The aqueous phase left after the first ethyl acetate partitioning and successive enzymatic hydrolysis was found to contain GA_8 and GA_{29} (Table 3.3). In addition, traces of GA_1, GA_4, GA_9 and GA_{20} were found. When the aqueous phase was repartitioned against ethyl acetate instead of being hydrolysed, no (free) GAs could be detected, indicating that measuring free GAs as spill over instead of conjugated GAs is not likely.

Table 3.1 *GC retention times, Kováts retention indices (KRI) and characteristic ions for identification based on full scan monitoring of Me and MeTMSi derivatives of gibberellins from alstroemeria leaf tissue.*

Gibberellin	R_t (min)	KRI	Characteristic ions *m/z* and relative abundance (% base peak)
GA_1	13.7	2689	506 (M^+, 100), 491 (12), 448 (22), 376 (24)
GA_1 standard	13.7	2689	506 (M^+, 100), 491 (12), 448 (20), 376 (25)
GA_4	12.4	2522	418 (M^+, 27), 328 (43), 289 (54), 284 (100), 225 (63)
GA_4 standard	12.4	2522	418 (M^+, 29), 328 (35), 289 (48), 284 (100), 225 (65)
GA_8	15.0	2832	594 (M^+, 100), 579 (3), 535 (6), 448 (19), 379 (7)
GA_8 standard	15.1	2837	594 (M^+, 100), 579 (7), 535 (7), 448 (15), 379 (9)
GA_9	11.1	2319	330 (M^+,absent), 298 (100), 270 (59), 243 (51), 226 (53)
GA_9 standard	11.1	2319	330 (M^+, 9), 298 (100), 270 (61), 243 (46), 226 (51)
GA_{12}			not found, *i.e. less than 0.05 pmol (g FW)*$^{-1}$
GA_{12} standard	11.4	2359	360 (M^+, 2), 328 (30), 300 (100), 285 (19), 269 (7), 241 (25)
GA_{15}	13.2	2622	344 (M^+, 21), 312 (44), 284 (73), 239 (100)
GA_{15} standard	13.2	2624	344 (M^+, 25), 312 (27), 284 (71), 239 (100)
GA_{19}	13.1	2611	462 (M^+, 7), 434 (100), 402 (29), 374 (64), 345 (31)
GA_{19} standard	13.1	2610	462 (M^+, 7), 434 (100), 402 (31), 374 (60), 345 (22)
GA_{20}	12.3	2501	418 (M^+, 100), 403 (17), 390 (7), 375 (46), 359 (14)
GA_{20} standard	12.3	2501	418 (M^+, 100), 403 (15), 390 (3), 375 (52), 359 (15)
GA_{24}	12.0	2465	374 (M^+, 4), 342 (43), 314 (100), 286 (96), 226 (72)
GA_{24} standard	12.0	2465	374 (M^+, 4), 342 (31), 314 (100), 286 (96), 226 (97)
GA_{29}	13.7	2691	506 (M^+,100), 491 (18), 465 (absent), 447 (5), 375 (27), 303 (24)
GA_{29} standard*	13.7	2691	506 (M^+,100), 491 (14), 465 (2), 447 (7), 375 (17), 303 (20)
GA_{34}	13.6	2675	506 (M^+,100), 416 (8), 372 (10), 288 (19), 223 (31)
GA_{34} standard	13.6	2675	506 (M^+,100), 416 (8), 372 (10), 288 (21), 223 (39)
GA_{44} **	14.6	2788	432 (M^+, 61), 417 (7), 373 (9), 238 (47), 207 (100)
GA_{44} standard	14.6	2786	432 (M^+, 49), 417 (7), 373 (15), 238 (34), 207 (100)
GA_{51}			not found, *i.e. less than 0.05 pmol (g FW)*$^{-1}$
GA_{51} standard	12.5	2532	418 (M^+, 2), 386 (32), 328 (37), 284 (100)
GA_{53}			not found, *i.e. less than 0.05 pmol (g FW)*$^{-1}$
GA_{53} standard	12.4	2516	448 (M^+, 64), 389 (35), 251 (24), 208 (95), 207 (100)

*Note: M^+: molecular ion; *: obtained by interpolation of data of Gaskin & MacMillan (1991); **: detected in young leaves only. Identication of $GA_{15, 24, 34}$ was conducted by GC-SIM while other GAs were identified by full-scan GC-MS*

Table 3.2 *Seasonal variation in gibberellin concentrations (pmol (g FW)$^{-1}$) in mature leaves of alstroemeria.*

Time of harvest	Gibberellins, pmol (g FW)$^{-1}$					Number of analysis
	GA_{20}	GA_9	GA_1	GA_4	GA_8	
September 1994	3.64	1.20	0.66	0.72	6.58	3
January 1995	7.56	0.54	< 0.05	0.36	4.56	4
September 1995	3.63	2.05	0.74	0.79	7.58	2
October 1995	5.32	3.57	1.22	1.69	18.80	2

Variation in gibberellin content

Scintillation counting of samples after HPLC fractionation revealed that the recovery of individual GAs was approximately 60%. Conversion of [^{3}H]GA_1 and [^{3}H]GA_9 to their methyl esters yielded 98% efficiency as analysed by HPLC. To estimate the variation in the purification and analysis method used, we separately purified and analysed four samples originating from one batch of frozen leaves and determined concentrations of GA_8 and GA_9, representing a polar and an apolar gibberellin. For both GAs the variation was less than 8% of the mean value obtained (data not shown).
Concentrations of identified GAs varied from 0.04 pmol (g FW)$^{-1}$ for GA_{34} to 18.80 pmol (g FW)$^{-1}$ for GA_8. Mature leaves, harvested at different periods in time showed a considerable variation in GA concentrations (*e.g.* GA_9 varied from 0.54 to 3.57 pmol (g FW)$^{-1}$; Table 3.2). However, the amount of active GAs (GA_1 and GA_4) is significantly less compared to both precursor and inactivated GAs (Table 3.2).

Quantification of gibberellins in three leaf developmental stages

To relate leaf development to endogenous gibberellin content we determined GA concentrations in three stages of leaf development. Since dry matter content of the leaves did not change during development (data not shown), GA concentrations can be expressed as pmol per gram fresh weight. The time from stage 1 (young leaves) to stage 2 (mature leaves) was approximately 2 weeks. Total GA concentration increased strongly as the

leaves became older, mainly due to increases in (total) GA_8 and GA_{29} (Table 3.3). Concentrations of GA_8, GA_{15}, GA_{19}, GA_{20}, GA_{24} and GA_{29} increased during maturation. In contrast, concentrations of GA_1, GA_4, GA_9 GA_{34} and GA_{44} decreased.

Table 3.3 *Gibberellin concentration (pmol (g FW)$^{-1}$) in leaves of alstroemeria at three developmental stages. The data are means of 3 samples ± SE. Sampling was done in September.*

Leaf stage	Young	Mature	Senescent
Chlorophyll (mg (g FW)$^{-1}$)	0.77 ± 0.15	1.32 ± 0.09	0.24 ± 0.08
Free Gibberellin (pmol (g FW)$^{-1}$)			
non-13-OH GAs			
GA_{15}	0.36 ± 0.01	0.94 ± 0.05	< 0.05
GA_{24}	0.12 ± 0.01	0.49 ± 0.04	0.69 ± 0.16
GA_9	2.56 ± 0.11	1.20 ± 0.04	1.86 ± 0.45
GA_4	1.27 ± 0.03	0.72 ± 0.02	< 0.05
GA_{34}	0.66 ± 0.03	0.04 ± 0.02	2.27 ± 0.23
13-OH GAs			
GA_{44}	3.96 ± 0.29	< 0.05	< 0.05
GA_{19}	1.02 ± 0.03	1.27 ± 0.07	0.55 ± 0.29
GA_{20}	< 0.05	3.64 ± 0.19	4.82 ± 0.59
GA_1	0.83 ± 0.02	0.66 ± 0.05	0.20 ± 0.03
GA_8	1.15 ± 0.09	6.85 ± 1.47	15.23 ± 0.69
GA_{29}	< 0.05	2.96 ± 0.13	12.11 ± 1.12
Conjugated Gibberellin (pmol (g FW)$^{-1}$)			
GA_8	< 0.05	1.22 ± 0.36	6.57 ± 0.45
GA_{29}	< 0.05	0.15 ± 0.09	7.21 ± 0.88

In darkness, it took 8 days for the leaves to become completely senescent (stage 3). Chlorophyll content was less than 20% of the amount of mature leaves (Table 3.3). During senescence GA_1, GA_4, GA_{15} and GA_{19}

concentrations decreased, while GA_8, GA_{29} and GA_{34} concentrations increased.
After enzymatic hydrolysis of the aqueous fraction left after the first ethyl acetate partitioning, no GAs could be detected in extracts from young leaves. In similar extracts from mature leaves 1.22 pmol $(g\ FW)^{-1}$ GA_8 and 0.15 pmol $(g\ FW)^{-1}$ GA_{29} were detected making up 16% of total GA_8 and 5% of total GA_{29} analysed in these extracts. Also, traces of GA_1, GA_4, GA_9 and GA_{20} could be demonstrated in these extracts. Senescent leaves contain traces of GA_1, GA_9 and GA_{20} and 6.57 pmol $(g\ FW)^{-1}$ GA_8 and 7.21 pmol $(g\ FW)^{-1}$ GA_{29} (43% and 59%, respectively, of total GA_8 and GA_{29}) in the hydrolysed aqueous fraction.

3.5 Discussion

Surveys of GAs in mature tissues are rarely reported; most studies are restricted to GAs determined in seedlings or developing buds, all young and rapidly growing tissues. Rebers *et al.* (1994) analysed endogenous GAs in sprouts and bulbscales of tulip during storage and subsequental growth. Kurogochi *et al.* (1979) determined the gibberellin pattern in rice plants and found that GA_{19}, the most abundant GA, varied by a factor 15 throughout the growth season. However, growth and development could not be correlated with the GA_{19} pattern and the authors concluded that seasonal variation seemed a general feature in rice plants. In alstroemeria, large variations in GA concentrations were found in leaves of comparable developmental stage harvested at different moments in time suggesting that the history of the plants influenced GA concentrations. Although seasonal variation in gibberellin content was not the emphasis of this study, it is clear that statements correlating absolute GA concentrations to distinctive processes (in this case senescence) based on a single time point analysis must thus be interpreted with care.
The presence of (free) GA_1, GA_4, GA_8, GA_9, GA_{19}, GA_{20}, GA_{29} and GA_{44} in mature alstroemeria leaves was demonstrated by comparing full scan mass spectra and KRI with available standards. GA_{15}, GA_{24} and GA_{34} were detected by GC-SIM. The GAs identified in alstroemeria leaf extracts suggest that two major GA pathways, the early non-13-hydroxylation pathway to GA_4 and the early 13-hydroxylation pathway to GA_1, exist in alstroemeria. Both pathways are known to be operative in vegetative tissues of many plants (Sponsel 1995). Comparable results have been obtained in a related

plant species *Lilium elegans*, where GA_{12}, GA_{15}, GA_{24}, GA_9, GA_4, GA_{34} and GA_{51} were identified in addition to GA_{44}, GA_{19}, GA_{20}, GA_1 and 3-epi-GA_{34} (Takayama *et al.* 1993). No evidence was found for the presence of the early GA_{12} and GA_{53}. It is likely that these GAs are quickly metabolised and therefore present at concentrations below detection limits and require much larger scale extraction procedures. Neither GA_{51}, for which GA_9 is thought to be the precursor, nor the conjugated form of GA_{51} was found in any alstroemeria leaf extract.

We arranged the GAs detected in alstroemeria in three groups according to their supposed function in the metabolic pathway (Sponsel 1995). The ratio precursor : active : inactivated GAs could be calculated from Table 3.3 and changed from 0.67 : 0.17: 0.16 in young leaves to 0.21 : 0.005 : 0.78 in senescent leaves (Fig. 3.1). In young leaves of alstroemeria, the contribution of GAs assumed to be precursors was about 67% of the total GA pool indicating that these leaves have the possibility to rapidly synthesize the active GA_1 and GA_4. In rice seedlings it was also found that concentrations of precursor type GAs were higher in younger leaves when compared to older leaves (Choi *et al.* 1995).

Also in pea, cell-free extracts from young tissues have the highest activity for *ent*-kaurene synthesis (Coolbaugh 1985). It is generally accepted that young leaves are sites of GA biosynthesis (Zeevaart and Gage 1993, Sponsel 1995). In senescent leaves the concentrations of GA_9, GA_{20} and GA_{24} increased, although when related to the total amount of GAs, their proportion decreased. This indicates that even in senescent leaves GA metabolism occurs. For alstroemeria we consider both GA_1 and GA_4 as biologically active gibberellins as both GAs were effective in delaying leaf senescence (Jordi *et al.* 1995). Although the present experiments cannot exclude that GA_4 has to be converted to GA_1 before showing activity (Graebe 1987, Sponsel 1995), GA_4 was 2 to 3 orders of magnitude more effective in retarding chlorophyll in alstroemeria leaves (Jordi *et al.* 1995). A number of other studies also strongly suggest that GA_4 has intrinsic biological activity in *e.g. Arabidopsis* (Zeevaart and Talon 1992), *Cucumis* (Nakayama *et al.* 1991) and *Tulipa* (Rebers *et al.* 1994).

The total GA content of the leaf increased with maturation and subsequent senescence. This seems to be in conflict with the finding that GA concentrations decline rather than increase in senescent leaves (Fletcher *et al.* 1969, Chin and Beevers 1970). However, in those studies bioassay procedures were used and as a consequence only GAs active in the bioassay were determined, including some catabolites that show activity but excluding, for instance, inactive GA conjugates. In alstroemeria,

concentrations of the biologically active free gibberellins GA_1 and GA_4 declined after 8 days of darkness. This is in accordance with the study of Aharoni and Richmond (1978) which reported a decrease of GA_3-like substances in senescing lettuce leaves. During maturation and subsequent senescence of alstroemeria leaves the contribution of precursor type GAs decreased to 21% of the total amount. The contribution of free inactivated GAs increased to more than 78% in senescent leaves, which

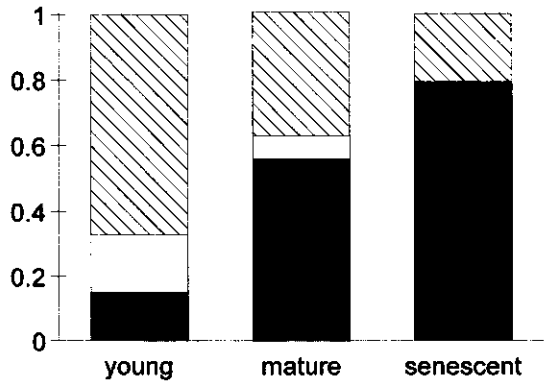

Figure 3.1 *Ratio's between different classes of gibberellins in leaves of alstroemeria in three stages of development. Classification is as follows: precursor type gibberellins (hatched), GA_{15} + GA_{24} + GA_9 + GA_{44} + GA_{19} + GA_{20}, active gibberellins (white), GA_1 + GA_4 and inactivated type gibberellins (gray), GA_8 + GA_{29} + GA_{34}. Data are calculated from Table 3.3.*

supports the view that these compounds are inactive forms of GAs. Previous studies using bioassay procedures have revealed the increase of GA-like substances in conjugated form during senescence of leaves of *Lactuca* (Aharoni and Richmond 1978) and *Rumex* (Zheng and Zhou 1995). Mature and senescent alstroemeria leaves were found to contain traces of a number of GAs in the hydrolysed aqueous fraction. The amounts of GA_8 and GA_{29} analysed in the aqueous fraction of mature and senescent leaves were substantial and suggest the occurrence of conjugated, glycosylated forms. However, in this study GA-conjugates could not be characterised individually, and therefore the total amounts of free and originally conjugated GA_8 and GA_{29} were considered as degradation products of the 13-hydroxylation pathway. The function of GA conjugates has been suggested by Sembdner *et al.* (1994) to be (temporarily) inactive, storage or transport forms. The large

increase of GA_8- and GA_{29}-conjugated GAs from trace amounts in young leaves to more than 25% of total GAs in senescent alstroemeria leaves suggest storage of inactive products and is apparently the result of conversion of free GAs to their conjugated forms. In senescent leaves, the conversion of precursors of GA_1 into GA_8-conjugate and GA_{29}-conjugate via GA_8 and GA_{29} seems to be higher than in mature leaves. Although GA_8 and GA_{29} increased two- and four-fold, respectively, during senescence the concentration of GA_{34} increased 57-fold as compared to mature leaves. Since GA_4 is also far more effective than GA_1 in delaying senescence of alstroemeria leaves (Jordi *et al.* 1995), the results suggest that non-13-hydroxylated GAs are involved in regulation of leaf senescence.

The present study established the identity of 11 GAs in alstroemeria leaves, which represent the occurrence of two major GA pathways in higher plants. The relative distribution of the different types of GAs varied throughout the development of the leaves. A shift of so-called precursor type GAs to inactivated GAs was found during maturation and subsequent senescence of the leaves. In previous work using exogenous application of gibberellins, it was suggested that endogenous GAs are important in the regulation of senescence of alstroemeria leaves (Van Doorn and Van Lieburg 1993, Jordi 1995). This study demonstrates that in alstroemeria leaves, the pattern of endogenous gibberellins correlates with leaf developmental stage. The possible causal relation between endogenous gibberellins and leaf senescence will be the basis for future studies.

Chapter 4 is based on the publication:

Kappers IF, Jordi W, Maas FM, Stoopen GM and Van der Plas LHW (1998) The effects of gibberellin and red light on senescence of alstroemeria leaves are independent. Physiologia Plantarum 103: 91-98.

4 Gibberellin and phytochrome control senescence independently

4.1 Abstract

In alstroemeria (*Alstroemeria hybrida*), leaf senescence is effectively retarded by the application of gibberellins and by low fluences of red light. In this study we examined the possible interaction of gibberellins and red light in the regulation of senescence. Determination of endogenous gibberellins revealed that leaf senescence is accompanied by significant changes in the concentrations of non-13-hydroxylated gibberellins, the onset of senescence coinciding with a dramatic drop in GA_4, whereas concentrations of 13-hydroxylated gibberellins are far less influenced. However, no direct effect of red light on a specific GA-metabolic step could be determined. When exogenously applied, non-13-hydroxylated GAs were more active than the 13-hydroxylated GAs. It appeared that the effect of red light is additive to that of active GAs. We hypothesise that GA_4 and phytochrome control senescence in alstroemeria mainly through separate mechanisms and have independent effects and that the observed differences in GA concentrations are a consequence of delayed leaf senescence rather than a cause for it.

4.2 Introduction

Leaf senescence in alstroemeria is strongly inhibited by gibberellins and red light (Van Doorn and Van Lieburg 1993, Jordi *et al.* 1995). In general, phytohormones, like cytokinins and in some systems gibberellins, delay the loss of chlorophyll whereas ethylene and abscisic acid enhance it (Thimann 1980). Previously, we studied the effects of various plant growth regulators

on the loss of chlorophyll and demonstrated that GA_4 and GA_7 were most effective in delaying leaf senescence in alstroemeria whereas cytokinins had less and auxins and polyamines had no effect (Jordi *et al.* 1995). A similar effect on chlorophyll retention was obtained when leaves were irradiated with a low fluence of red light that could be counteracted by far-red light (Van Doorn and Van Lieburg 1993). Next to a photomorphogenic effect involving phytochrome, the effect of light on senescence has been proposed to be photosynthetic (Goldthwaite 1988). In alstroemeria, both gibberellin and red light treatments resulted in a delayed loss of photosynthetic activity, chlorophyll and Rubisco, compared to permanently dark-treated leaves (Jordi *et al.* 1994, 1996). Both treatments also diminished the accumulation of a senescence-specific polypeptide (Jordi *et al.* 1996).

The process of leaf senescence in alstroemeria cut flowers thus appeared to be regulated both by hormones and phytochrome. The similarities between the effects of light and gibberellin might be explained if they share common signalling pathways. To date, extensive studies have been focused on the involvement of gibberellins in the red-inhibited / far-red-promoted response of stem and internode elongation (Martínez-García and García-Martínez 1992, López-Juez *et al.* 1995, Peng and Harberd 1997). In general, light can affect the plant growth regulator response pathway via several mechanisms. It can modify sensitivity (Firn 1986), *e.g.* light altered GA-sensitivity in elongating pea seedlings (Weller *et al.* 1994). Light can affect overall biosynthesis or rate of metabolism of a particular intermediate such as the light-induced decrease of GA_{20} metabolism in dark-grown pea (Sponsel 1986). It may be involved in the release of a free growth regulator from its conjugated form (Loveys and Wareing 1971) and, in theory, change accessibility and transport of the growth regulator.

Alstroemeria leaves offer a good model system with an easy detectable response. To study the role of gibberellins in leaf senescence, it is necessary to know which endogenous gibberellins occur. The presence of endogenous GA_1, GA_4, GA_8, GA_9, GA_{15}, GA_{19}, GA_{20}, GA_{24}, GA_{29}, GA_{34} and GA_{44} has been demonstrated in Chapter 3. As the leaves mature and senesce, the contribution of precursor-type gibberellins decreases while that of degradation-type gibberellins increases. In this study, we investigate the possibility of GA implication in the integration of light signals in the senescence of alstroemeria leaves.

4.3 Materials and methods

Chemicals

Origin and purity of GAs is described in Chapter 3.3. Stock solutions of GAs were prepared as 10^{-2} M in ethanol. The small amount of ethanol (maximal 1%) did not affect senescence in alstroemeria leaves (data not shown). Deflowered stems with whorl leaves were incubated in the absence or presence of different concentrations of various GAs (total volume 20 ml per stem), either in darkness or with red light irradiation.

Plant material and experimental conditions

Flower stalks of *Alstroemeria hybrida* cv. Westland plants were used and handled as mentioned in Chapter 2.4 (modelsystem with deflowered stems) The stems were placed in demineralised water in the absence or presence of a GA solution and kept in a climate room at 20 ± 1 °C and *ca* 65% relative humidity.

Light conditions

Irradiance levels and spectral photon distribution of green safe light, red light and far red light were measured as described in Chapter 2.4.

Chlorophyll analysis

Chlorophyll contents of the leaves were determined spectrophotometrically at 647 and 664.5 nm after extraction with dimethylformamide as described in Chapter 2.4. Non-destructive chlorophyll measurements were performed daily using a SPAD-502-meter (Minolta, Osaka, Japan) and experiments were ended when SPAD-measured chlorophyll level of the dark treatment had decreased below 20% of the initial amount (normally after 8 days).
Data are presented as percentage of the initial amount of chlorophyll in order to correct for the small differences between experiments.

Isolation and quantification of endogenous gibberellins

GAs were purified and determined by means of GC-MS as previously reported (Chapter 3.3). To quantify endogenous amounts of GAs, 50 ng of [17-$^2H_2$]-GAs GA_1, GA_4, GA_8, GA_9, GA_{19} and GA_{20} each were added at the beginning of the extraction.
The purification involved extraction in 100% and 80% methanol, followed by solvent partitioning yielding an acidic ethyl acetate fraction, QAE-25 anion exchange chromatography, C_{18} Sep-pak and reverse-phase HPLC. Four HPLC fractions were collected, methylated with ethereal diazomethane and dissolved in a fresh mixture of BSTFA:TCMS:pyridine (0.2:0.01:0.79 $^v/_{v/v}$) to produce the trimethylsilyl ethers of the methyl esters (MeTMSi) for GC-MS.
Quantification of GAs was achieved using selected ion monitoring (GC-SIM) as described in Chapter 3.3. The ion pairs monitored of GC-SIM were 506/508 (GA_1), 284/286 (GA_4), 594/596 (GA_8), 270/272 (GA_9), 434/436 (GA_{19}) and 418/420 (GA_{20}). GA_{29} was quantified against the [17-$^2H_2$]GA_8 standard in the same injection, so the values should be taken as approximate although valid for comparison. Corrections for different abundances of GA_{29}- and [17-$^2H_2$]GA_8 standards were estimated by co-injection of both standards. GA_{24} and GA_{15} (against [17-$^2H_2$]GA_9) and GA_{34} (against [17-$^2H_2$]GA_4) were estimated in the same way. Because the fresh weight of the leaves changed less than 2% throughout the course of the experiment (data not shown), gibberellin concentrations are expressed as pmol $(g\ FW)^{-1}$.

Experimental set-up and statistical analysis

Light and GA application experiments were performed with five replications per treatment and repeated at least twice. Experiments to determine endogenous concentrations were repeated at least once and included each three samples per time point. Logistic dose-response curves were calculated from the individual data points, using a non-linear regression method and are described by the formula $(R=R_{min}+(R_{max}-R_{min})/(1+([GA]/[GA]_{50})\text{^}p)$, where R is the response, R_{min} is the response in absence of exogenous GA, R_{max} is the maximum response, [GA] is the applied concentration, $[GA]_{50}$ is the concentration required for half-maximum response and p is the Hill coefficient representing the abruptness of the curve (Weyers *et al.* 1987). Data were statistically analysed using the Student *t*-test.

4.4 Results

Effects of different light treatments on chlorophyll content

Chlorophyll contents remained stable or decreased only slightly during the first 3 days in all treatments (Fig. 4.1). Leaves contained 1.67 ± 0.15 µmol chlorophyll (a+b) g^{-1} fresh weight at the onset of the experiments. In all experiments no significant changes in chlorophyll a/b ratio were determined (data not shown). The molar ratio chlorophyll a : chlorophyll b was 2.9. In darkness, chlorophyll content decreased rapidly from day 3 onwards and leaves were homogeneously yellow after 8 days of darkness. Experiments which combined various durations and fluences of red light demonstrated that the maximum effect on chlorophyll retention was already obtained with 5 µmol m^{-2} s^{-1} red light for 10 min day^{-1} (Chapter 2.5). Leaves irradiated with this amount of light showed a delay of the onset of leaf senescence as well as a decreased rate of chlorophyll loss during the following 4 days (Fig. 4.1). This behaviour is comparable to that of leaves kept at natural day and night conditions which resulted in a retention of 68.8 ± 1.9% of the inital chlorophyll content after 8 days. A response to red irradiation was observed when the treatment was started within the first 4 days of darkness. A red light treatment after 4 days of darkness could not delay senescence. Leaves given this treatment had only 30 ± 6 % of their chlorophyll left after 8 days (data not shown). A single 10 min red light pulse, 70 to 80 h after the leaves were placed in darkness, delayed the onset of chlorophyll loss for *ca* 1 day without affecting the rate of senescence (Fig. 4.1). Far-red light slightly accelerated the onset of chlorophyll loss. Daily irradiation of leaves for 10 min with 5 µmol m^{-2} s^{-1} red light followed by 10 min 5.4 µmol m^{-2} s^{-1} far-red light did not prevent chlorophyll loss and resulted in similar kinetics as the dark treatment. The chlorophyll level after 8 daily red / far-red pulses was 21 ± 2 % of the initial level. Furthermore, we demonstrated that photosynthesis was negligible at this red light intensity (5 µmol m^{-2} s^{-1}) as the measured light-compensation point was determined at 3.7 µmol m^{-2} s^{-1} and the leaves were irradiated for only 10 min daily.

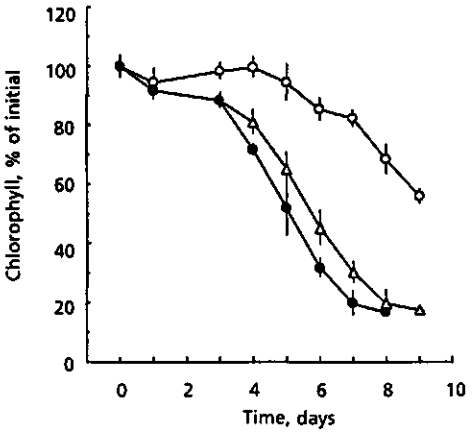

Figure 4.1 *Chlorophyll (a+b) content (% of initial amount) of alstroemeria leaves under different light conditions: ●, continuous darkness; ○, 10 min daily 5 µmol m^{-2} s^{-1} red light; △, 72 hrs darkness, 10 min 5 µmol m^{-2} s^{-1} red light followed by darkness. Data are means ± SE of 3 experiments, each consisting of 5 replications.*

Gibberellin concentrations during senescence in dark- and light-treated leaves.

We analysed endogenous gibberellin concentrations in alstroemeria leaves placed either in permanent darkness or subjected to a daily 10 min 5 µmol m^{-2} s^{-1} red light irradiation during 8 days. Initial concentrations differed substantially between experiments. However, the relative changes in gibberellin concentrations during senescence were comparable and therefore only one representative set of experimental data is presented. During the first 4 days, the period directly preceding the rapid loss of chlorophyll, GA_4 concentration in dark-treated leaves decreased to less than 0.1% of the initial concentration. In red light treated leaves, the GA_4 concentration remained at approximately 40-50 % of that of the initial (Fig. 4.2).

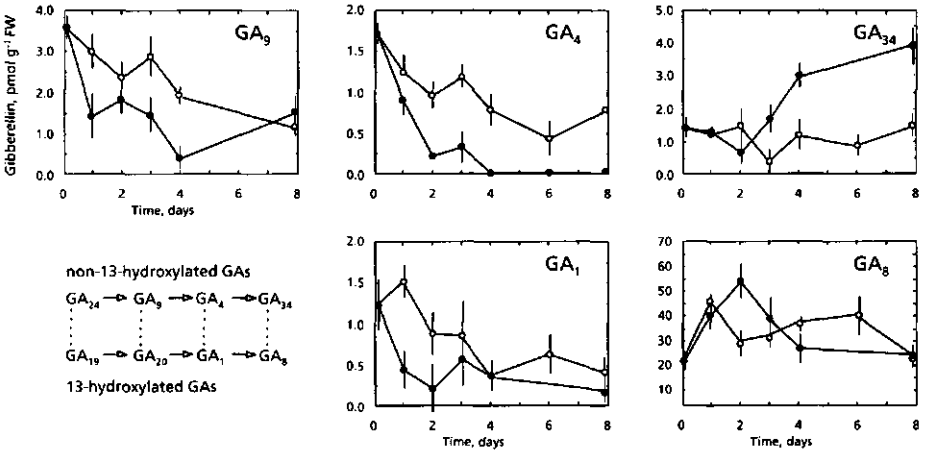

Figure 4.2 *Gibberellin concentrations (pmol g^{-1} FW) in leaves of alstroemeria placed for 8 days in darkness with (○) or without (●) a 10 min daily red light irradiation of 5 µmol m^{-2} s^{-1}. Inset shows metabolic grid of gibberellins endogenous in alstroemeria. Arrows indicate known conversions and dotted lines corresponding GAs in both pathways.*

In addition, the proportional decrease in GA_9 concentration was slightly less in light-treated leaves. In contrast to GA_4, GA_1 was still present at day 4 in dark-treated leaves. The concentration of GA_{34} showed a clear increase from day 2 onwards in darkness but not in the red light-treated leaves. The concentration of GA_{24} was highly variable during the senescence period (data not shown). Both in leaves placed in either darkness or under daily red light irradiation GA_8, GA_{19}, GA_{20} and GA_{29} did not change significantly ($p<0.05$) during the 8 days of the experimental period and remained at the initial concentrations that were 21, 4, 5 and 9 pmol (g fresh weight)$^{-1}$ respectively. For reasons of clarity only GA_8 is shown in Fig. 4.2. A 3 h irradiation with 5 µmol m^{-2} s^{-1} red light following a 24 h dark period did not change the levels of gibberellins, neither during the irradiation period nor during the subsequent 30 or 60 min dark period following the red light treatment. The levels of GA_1, GA_4, GA_8, GA_9, and GA_{34} found at these different times deviated less than 10 % from those in fully mature leaves and were, respectively, 0.66 ± 0.03, 0.72 ± 0.02, 6.9 ± 1.5, 1.20 ± 0.04, and 0.04 ± 0.02 pmol (g fresh weight)$^{-1}$.

Responses to exogenously applied gibberellins in combination with red light irradiation

To test whether light and GAs act independently, leaves were treated with various gibberellins by incubating the deflowered stems in a range of gibberellin concentrations and placing them in permanent darkness or subjecting them to a daily 10 min, 5 µmol m^{-2} s^{-1} red light irradiation. After 8 days the amount of chlorophyll in untreated leaves (no added GAs, permanent darkness) decreased to *ca* 20 - 30% of the initial amount. GA_1, GA_4, GA_9 and GA_{20} exhibited a clear concentration-dependent effect on the amount of chlorophyll present in leaves after 8 days of darkness (Fig. 4.3). No effect on delaying chlorophyll loss was obtained with GA_8 and GA_{24}. Dose-response curves were fitted for the GAs that delayed chlorophyll loss (Fig. 4.3). The threshold concentration of the active GAs required to delay chlorophyll breakdown in darkness was lowest for GA_4 and highest for GA_{20}. GA concentrations required for half-maximum response varied between > 500 nM for GA_{20} and about 19 nM for GA_4. Within different experiments a substantial variation (24%) in the half-maximum responses $[GA]_{50}$ was found. However, the ranking order of efficacy ($GA_4 > GA_9 > GA_1 > GA_{20} >> GA_{24} = GA_8$ = no effect) did not differ among the experiments.
Treatment of leaves with the stipulated GAs in combination with a daily red irradiation, showed an additive effect on the retention of chlorophyll in case of GA_1, GA_4, GA_9 and GA_{20} (Fig. 4.3). The combination of GA_8 or GA_{24} with red light did not increase the chlorophyll content compared to red light irradiation alone. The abruptness (p-value) of the calculated curves did not change significantly for GA_1 and GA_{20} when GA application in either darkness or combined with a red light irradiation were compared. For GA_9 and GA_4 small but significant ($p<0.1$) changes in abruptness were determined for leaves treated with gibberellins compared to leaves treated with both gibberellins and red light. The calculated $[GA]_{50}$ did not differ significantly for all GAs in darkness with or without red light irradiation.

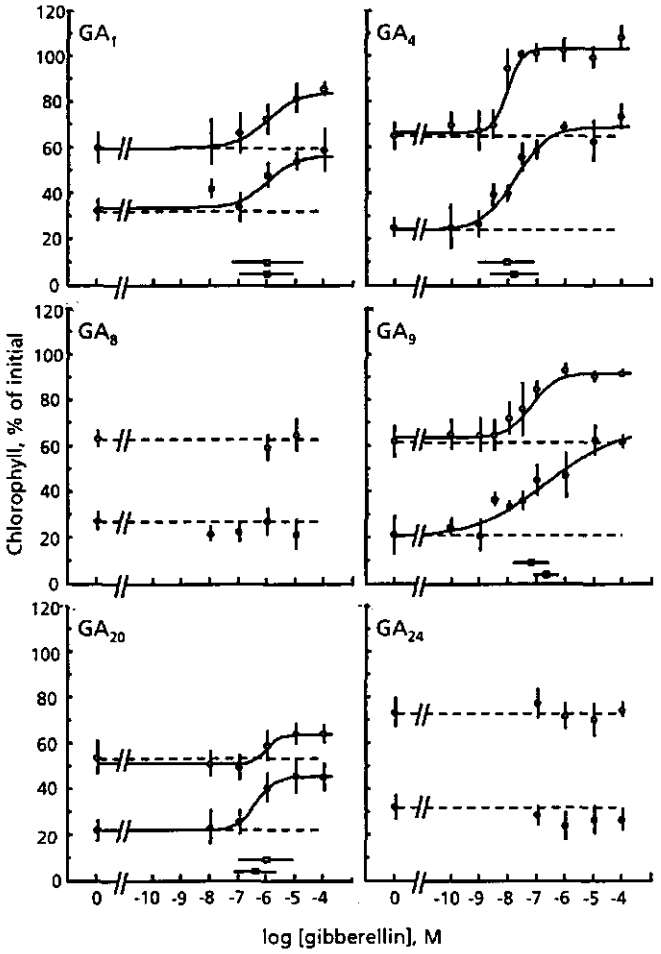

Figure 4.3 *Chlorophyll (a+b) content (% of initial amount) of alstroemeria leaves influenced by the application of different concentrations of GA_1, GA_4, GA_8, GA_9, GA_{20} or GA_{24} and placed in darkness (●); or subjected to 10 min day^{-1} 5 µmol m^{-2} s^{-1} red light (O). Data are means of 5 replications ± SE and are representative for a number of experiments. When appropriate, the data were fitted as logistic dose response curves and the calculated $[GA]_{50}$ ± SE are depicted by ■ (darkness) and □ (red light). Dashed lines represent chlorophyll content after 8 days of darkness without GAs (lower line) or daily red light treatment without GAs (upper line).*

4.5 Discussion

Both gibberellins and low amounts of red light delay leaf senescence in alstroemeria. We studied whether these two factors could possibly interact in their regulation of leaf senescence. Although to date, the interactive effects of light and gibberellins have been thoroughly studied in stem elongation (*e.g.* Sponsel 1986, López-Juez *et al.* 1995) and seed germination (*e.g.* Derkx *et al.* 1994), their effects on senescence are far less noted. The observation that in alstroemeria senescence is very effectively retarded by low concentrations of gibberellins and low fluences of red light, makes this species well suited to study the interaction of both factors in the regulation of senescence.

Non-13-hydroxylated gibberellins are correlated with senescence

To test whether red light affects endogenous gibberellin concentrations, we determined the latter under different light conditions during an 8-day period. Non-13-hydroxylated GA concentrations were more strongly affected by red light irradiation than the 13-hydroxylated GAs. In more detail: concentrations of GA_4 and to a lesser extent GA_{34} were correlated with chlorophyll loss. After 3 days of permanent darkness no GA_4 could be detected in the leaves while the amount of GA_{34} increased steadily during 8 days of permanent darkness. The disappearance of GA_4 was well correlated with the onset of chlorophyll loss. Whether this suggested threshold concentration for GA_4 is causal for senescence remains to be studied. The concentrations of GA_4 and GA_{34} in red light-irradiated leaves remained more or less stable during the experimental period. No consistent differences in the concentrations of GA_1, GA_8, GA_{19}, GA_{20}, GA_{24} and GA_{29} could be detected between dark-treated and red light treated leaves despite a substantial decrease in chlorophyll content in the former (Fig. 4.1). From these results we hypothesise that the GAs of the non-13-hydroxylated pathway are predominantly involved in senescence whereas those of the 13-hydroxylated pathway are at least not primarily involved. Further argumentation for this hypothesis is the observation that application of the non-13-hydroxylated GAs GA_4 and GA_9 to leaf tips was substantially more effective in delaying leaf senescence than that of the 13-hydroxylated GAs GA_1 and GA_{20} (Fig. 4.3).
From Fig. 4.2 the question arises whether the decrease in GA_4 concentration is causal for light-regulated senescence or occurs as a consequence of

senescence. Reid *et al.* (1968) found a transient increase in total GA_3-like activity within 30 minutes after application of a red light pulse to young barley leaves. Inhibitors of gibberellin biosynthesis diminished the increase of gibberellins after red light irradiation. Other authors have reported altered GA concentrations after red irradiation (Campbell and Bonner 1986) or a higher concentration in light-grown plants compared with dark-grown plants (Gawronska *et al.* 1995). The absence of such a distinct rapid light-induced transient change in GA concentration in alstroemeria leaves does not entirely rule out the involvement of GA metabolism in response to light. For example, light might direct the compartmentalisation of GAs within the cell and thus alter the availability of (any of the) GAs at their sites of action without a change in the overall GA concentration. Although we observed a smaller decrease in GA_4 concentration in red-irradiated leaves than in dark-treated leaves, this seems unlikely to have caused the delay in senescence, since, extrapolating from the data in Fig. 4.3, GA_4 concentrations in red-irradiated leaves would have to be at least a hunderd to a thousand-fold higher than in dark-treated leaves to be able to explain the delayed senescence. This further argues against delayed senescence by increased GA_4 biosynthesis. However, it should be emphasised that GA_4 is obviously an integral part of the early process of senescence of alstroemeria leaves as this compound inhibits chlorophyll and protein degradation and the increase of a senescence-specific polypeptide with a strong sequence homology with isopropylmalate dehydrogenases (Jordi *et al.* 1996).

Red light is additive to active gibberellins

The endogenous GA concentrations obtained under different light conditions prompted us to hypothesise that GAs and red light have mainly independent effects. To test this hypothesis, dose response curves were determined for a number of exogenously applied GAs. The effects of the applied 13-hydroxylated GAs GA_1, GA_8 and GA_{20} were less or even nil compared to the GAs of the non-13-hydroxylated pathway GA_4, GA_9 and GA_{24} (Fig. 4.3). Of the GAs tested, GA_4 was the most effective as was reported earlier (Jordi *et al.* 1995). The calculated dose for the half-maximum response was 19 nM, which is about 2 orders of magnitude lower than the calculated $[GA]_{50}$ for GA_1. Graded series of activities exist in GA-biosynthetic pathways, the lowest being for the intermediates early in the pathway (Phinney and Spray 1982). No effect of GA_{19} (data not shown) and GA_{24} on delay of chlorophyll breakdown was demonstrated, neither in dark

incubated leaves with or without a daily red light irradiation. Although the presence of endogenous GA_{19} and GA_{24} was demonstrated in alstroemeria leaves, application of these GAs to leaf tips had no effect on chlorophyll retention. Control experiments in which leaves were incubated with [17-$^2H_2$]-GA_{19} and subsequently analysed by GC-MS, showed that [17-$^2H_2$]-GA_{19} was taken up by the leaf (data not shown). Apparently, the leaf in this state is incapable of converting a C20-GA ($GA_{19,24}$) to a C19-GA ($GA_{9,20}$). Alternatively, the plasma membrane transport rate of these hydrophylic GAs is too low to observe activity. In all species studied to date GA_9 and GA_{20} have to be converted to GA_4 and GA_1 via 3β-hydroxylation by cytosolic enzymes to become active (Smith *et al.* 1990). Therefore, the activity of GA_9 and GA_{20} most probably depends on the degree of metabolism to GA_4 or GA_1. GA_9 can also be converted to GA_1 via GA_{20} (Graebe 1987), or to GA_3 via the sequence $GA_9 \rightarrow GA_{20} \rightarrow GA_5 \rightarrow GA_3$ (Smith *et al.* 1991). However in our experiments, GA_{20} was less active than GA_9 when exogenously applied. As for the second possibility, neither GA_5 nor GA_3 could be demonstrated as endogenous GAs in alstroemeria leaves (Chapter 3).

Assuming a 10 min light pulse with a low intensity (5 μmol m^{-2} s^{-1}) will not change the uptake of individual exogenously applied GAs, the total response of leaves incubated in GA_1, GA_4, GA_9 or GA_{20} increased when leaves were irradiated daily (Fig. 4.3). Responses to GA_8, GA_{19} and GA_{24} did not (significantly) change. The increase in the abruptness of the curve at the half-maximum response dose ($[GA]_{50}$) for both GA_9 and GA_4 suggested that red light enhanced the responsiveness to these gibberellins. However, it is dangerous to give a biological interpretation to these values, based solely on dose response curves (Weyers *et al.* 1987). In contrast, changes in $[GA]_{50}$ may be regarded as true shifts in sensitivity. From this parameter it becomes clear that red light irradiation of the leaves did not lead to a significant shift of GA sensitivity. Indeed, the significant additional effect of red light to that of GA_1, GA_4 or GA_9, strongly indicates that red light has an independent effect on senescence and will not be mediated through the gibberellin pathway. It has been proposed that phytochrome can influence 3β-hydroxylation of GA_{20} (Martínez-García and García-Martínez 1992). However, based on the observed data as shown in Fig. 4.3, the combined treatment of GA_9 and red light gave higher chlorophyll levels than GA_4 in darkness and therefore it is clear that in alstroemeria, changes in the 3β-hydroxylation of GA_9 can not explain the observed additive effect of red light. Therefore, our results do not support the suggestion made by Van Doorn and Van Lieburg (1993) who suggested that red light delays leaf senescence in alstroemeria by stimulating gibberellin synthesis. Our experiments suggest that the higher

concentrations of GA_1 and GA_4 in red light compared to dark-treated alstroemeria's results from an inhibition of the metabolic conversion of GA_1 and GA_4 into their respective metabolic inactivation products GA_8 and GA_{34}, since the concentrations of the latter gibberellins increased more slowly in the leaves given a daily red light irradiation as compared to continuous darkness. Thus, the observed changes in gibberellins are more likely the consequence rather that this cause of the delayed leaf senescence.

In conclusion, we have demonstrated that non-13-hydroxylated GAs are predominantly involved in the early process of leaf senescence in alstroemeria. The high activity of applied GA_4 and the presence of considerable amounts of GA_4 in leaves of alstroemeria indicates that GA_4 has intrinsic biological activity with respect to leaf senescence. Based on analogy to other systems, is the effect of GA_9 presumably due to conversion to GA_4 and red light could possibly enhance the responsiveness to these gibberellins. The endogenous concentrations of GA_4 decreased as a result of placing leaves in permanent darkness, whereas an amount of red light sufficient for the delay of senescence also maintained the concentration of GA_4 at higher concentrations. Furthermore, if the effect of red light on GA_4-levels is a direct one, no GA metabolic step seems to be a likely candidate for phytochrome control. We have strong indications that phytochrome and gibberellins have basically independent actions and result in additive effects on leaf senescence in alstroemeria.

Chapter 5 is based on the publication:

Kappers IF, Tsesmetzis N, Jordi W, Maas FM, Van der Plas LHW (1998) GA_4 does not require conversion into GA_1 to delay senescence of *Alstroemeria hybrida* leaves. Journal of Plant Growth Regulation: 17: 89-93.

5 GA_4 does not require conversion into GA_1 to delay senescence

5.1 Abstract

The biological activity and metabolism of applied GA_1 and GA_4 were studied in leaves of alstroemeria (*Alstroemeria hybrida*). It appeared that GA_4 was two orders of magnitude more active in delaying leaf senescence than GA_1. GA_3-13-OMe, a GA-analogue that has no free OH-group at the the 13-C position, also retarded chlorophyll loss although less efficiently. Tritiated and deuterated GA_1, GA_4 and GA_9 were applied to leaves, and their metabolites were analysed. According to HPLC and GCMS analyses, GA_9 was converted into GA_4 and GA_{34}, and GA_4 was converted into GA_{34} and more polar components. No evidence was found for the conversion of both GA_9 and GA_4 into GA_1, even at the relatively high concentrations that were taken up by the leaf. The results strongly suggest that GA_4 is recognised directly by a receptor involved in regulation of leaf senescence in alstroemeria.

5.2 Introduction

Gibberellins play an important role in the control of growth and development of plants. It has been hypothesised that different types of gibberellins are involved in different processes; vegetative and generative processes might be regulated by specific GAs (Sponsel 1995).
There is considerable evidence that GA_1 has biological activity *per se* especially in regulating seed germination and shoot and internode elongation (Graebe 1987, Sponsel 1995). Next to GA_1, a number of studies also indicate GA_4 as biologically active in regulation of stem elongation.

Metabolism of GA_4 has been studied in various systems, and in seedlings of *Phaseolus* (Turnbull and Crozier 1989), *Zea*, *Oryza* and *Arabidopsis* (Kobayashi *et al.* 1991), GA_4 can be metabolised to GA_1. Thus, the biological activity of applied GA_4 may be due to its conversion to GA_1. However, GA_4 has been suggested to be active *per se* for stimulation of stem elongation in *Cucumis sativus* (Nakayama *et al.* 1991), *Arabidopsis thaliana* (Talon *et al.* 1990), and *Tulipa gesneriana* (Rebers *et al.* 1995) based on a higher endogenous GA_4 concentration as compared to GA_1. In addition, GA_{34} is a common metabolite of GA_4 in vegetative tissues of *Phaseolus coccineus* (Turnbull and Crozier 1989). These references suggest the existence of the non 13-hydroxylation pathway yielding GA_4 as intrinsically active gibberellin. Limited information is available as to whether GA_1 is also the most active gibberellin in regulation of other plant developmental processes such as senescence. Alstroemeria leaves offer a good model system to study senescence closely related to the *in situ* situation. It has the advantage of being highly sensitive to GAs, and the time course of the dark-induced senescence of detached leaf tips is very comparable to that of attached leaves (Jordi *et al.* 1993). In previous studies with alstroemeria, endogenous GA concentrations have been related to leaf senescence (Chapter 3). It has been shown that several gibberellins of both the 13-hydroxylation and of the non-13-hydroxylation pathways were present in these leaves. In fresh leaves, GA_4 and GA_1 were present in comparable concentrations (approximately 0.7 pmol (g FW)$^{-1}$). GAs of the non-13-hydroxylation pathway changed in concentration due to a senescencing inducing treatment (darkness) while 13-hydroxylated GAs did not. GA_4 especially, was influenced: after 3 days of dark treatment no GA_4 could be detected anymore. Under low red light conditions, which delayed leaf senescence, the concentration of GA_4 remained at a much higher level (Chapter 4). Apparently, the decrease in GA_4 is a very early response of the senescence process. Exogenously applied GA_4 was found to be active at a concentration that was several orders of magnitude lower than that of GA_1 (Jordi *et al.* 1995, Chapter 4). This observed difference might be explained in terms of uptake of gibberellins by the tissue or in terms of metabolism of active GAs. Additionally, applied GA_4 could function as a precursor (slow release form) for GA_1 while exogenously applied GA_1 is inactivated rapidly.

The focal point of this study with alstroemeria leaves is to compare biological activity and metabolism of exogenously added GA_1 and GA_4 to elucidate whether the involved putative receptor has the ability to recognise GA_4. Dose response experiments were performed with GA_1, GA_4, and GA_3-13-OMe, a GA_3-analogue that is unlikely to be converted back into GA_3, which

is hydroxylated at the 13-C position like GA_1. In metabolic studies with [$^2H_2$]- and [$^3H_3$]- GA_1, GA_4, and GA_9, GA metabolites were determined with HPLC and GC-MS. Our results suggest that in delaying senescence in alstroemeria leaves GA_4 is biologically active *per se* and is probably recognised by the receptor involved.

5.3 Materials and methods

Chemicals

Origin and purity of GAs are described in Chapter 3.3. GA_3-13-OMe was a gift from Dr MH Beale (Bristol, UK). GC-MS control experiments demonstrated that GA_3-13-OMe contains ≤ 0.01% of other GAs such as GA_1 and GA_4.

Plant Material and Dose Response Experiments

The five uppermost leaves of *Alstroemeria hybrida* cv. Cinderella, were used for leaf tip experiments as described in Chapter 2.4. Logistic dose-response curves were calculated from the individual data points, using a non-linear regression method described by the formula ($R=R_{min}+(R_{max}-R_{min})/(1+([GA]/[GA]_{50})^p$), where R is the response, R_{min} is the response in absence of exogenous GA, R_{max} is the maximum response, [GA] is the applied concentration, $[GA]_{50}$ is the concentration required for half-maximum response and p is de Hill coefficient representing the abruptness of the curve (Weyers *et al.* 1987).

Chlorophyll Analysis

Chlorophyll (*a*+*b*) content of the leaves was calculated using the molar extinction coefficient in dimethylformamide as described in Chapter 2.4.

Uptake and Metabolism of Deuterated and Tritiated GAs

Tritiated GAs were first purified by HPLC before feeding. Each leaf was placed in 3 ml of a 10^{-7} M GA solution enriched with 50,000 dpm tritiated labelled GA. In experiments where combinations of deuterated and tritiated GAs were fed, GAs were diluted in 0.1 M citrate phosphate buffer, pH 5.0. Leaf tips were incubated in darkness at 20°C. After incubation, leaves were frozen in liquid N_2 and stored at -80°C until extraction. Experiments were performed in duplicate with four replications in each.

Extraction and Purification of GAs

Leaves were homogenised in ice cold 100% MeOH, 5 ml g FW^{-1}, containing 0.1% ascorbic acid. When leaves were fed both deuterated and tritiated GAs, 3 leaves were combined making 1 sample. After extraction for 2 h at 4°C and continuous shaking, the extract was centrifuged (5 min at 5000 x*g*) and the pellet was washed twice with 80% MeOH. The pooled MeOH extracts were evaporated to the water phase under reduced pressure at 35°C. The aqueous phase (± 2 ml) was adjusted to pH 2.5 with 1 N HCl and partitioned against ethyl acetate (3 times 2 ml). The combined ethyl acetate fractions were evaporated to dryness, dissolved in 2 ml of water and adjusted to pH 8.0 with 1 N NaOH. The extract was then further purified through a QAE Sephadex A-25 (Pharmacia, Uppsala, Sweden) column (4 cm x 0.25 cm i.d.) equilibrated with sodium formate (1% ($^{w}/_{v}$)) and fed directly through a pre-equilibrated C_{18} column (Waters, Millipore Corporation, Milford, Massachusetts, USA). GAs were eluted with 80% MeOH and evaporated to dryness under reduced pressure prior to HPLC and GCMS analysis.

HPLC and GCMS

The HPLC system and operating conditions are described in Chapter 3.3. The column was eluted at a flow rate of 1 ml min^{-1} with 30 % methanol for 15 min, followed by a linear gradient to 75% methanol over 35 min, and subsequently to 90% methanol over 5 min (solvents contained 0.01 % acetic acid).
To analyse metabolic conversion products, successive 0.5 ml fractions were collected, and the radioactivity in an aliquot of each fraction was assayed by liquid scintillation counting. In experiments with both tritiated and

deuterated labelling, fractions containing radioactivity were analysed by GC-SIM as described in Chapter 3 and 4. For the detection of $[^2H_2]$GAs, their characteristic ions were monitored together with the characteristic ion of the endogenous GA. The labelled metabolites were identified based on co-chromatography with the endogenous GAs, which had been identified previously using Kováts retention indices and full-scan mass spectra compared with those of authentic standards (Chapter 3.3) except for GA_8- and GA_{34}-catabolites which were tentatively identified by comparison with data from Gaskin and MacMillan (1991).

5.4 Results and discussion

Gibberellins Delay Senescence

There was no significant loss of chlorophyll during the first 5 days in all treatments (data not shown). Thereafter, chlorophyll decreased rapidly from day 6 onward in leaves that were placed in darkness and leaves were uniformly yellow after 10 days. Application of a number of GAs delayed the loss of chlorophyll (Jordi *et al.* 1995). We determined the effect of the various treatments after 10 days of incubation, when dark-treated leaves without GA retained only 20% of the initial amount of chlorophyll.
Figure 5.1 shows the effects of various concentrations of GA_1, GA_4 and GA_3-13-OMe on chlorophyll loss in leaf tips of alstroemeria placed in darkness for 10 days. Both GA_1 and GA_4 showed a concentration-dependent effect on the retention of chlorophyll with a maximum effect of about 80% of the initial amount of chlorophyll left (Fig. 5.1). However, the efficacy of GA_4 is two orders of magnitude higher than GA_1 as can be seen from the calculated concentrations needed for the half-maximum effect ($[GA]_{50}$) (2.1 and 220 nM, respectively). These data indicate that the receptor involved will recognise exogenously applied GA_4. However, a number of arguments can be proposed which argue against the simplicity of this conclusion. Therefore, we further investigated the relation between GA_1 and GA_4.

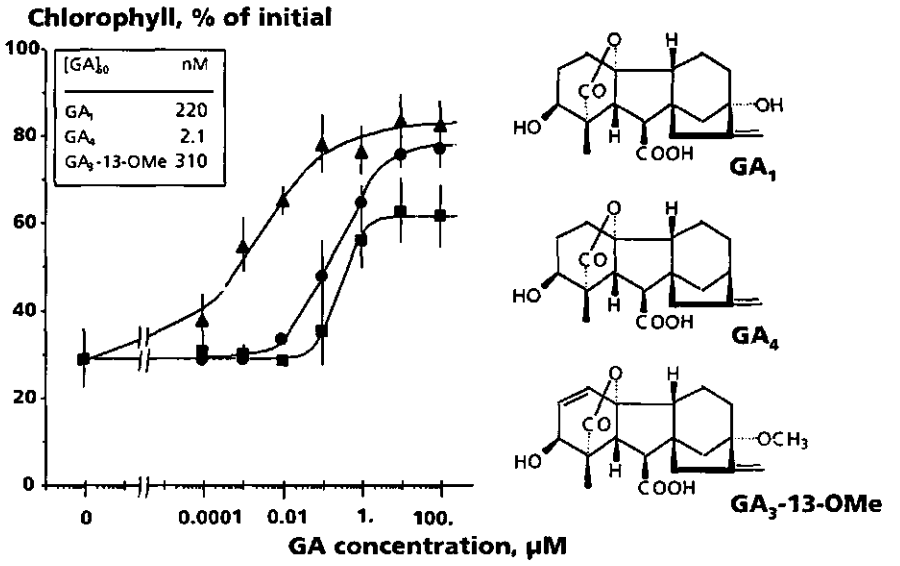

Figure 5.1 *Effect of various concentrations of GA_1 (●), GA_4 (▲) and GA_3-13-OMe (■) on chlorophyll retention in alstroemeria leaf tips placed in darkness for 10 days. Data are means of two experiments with 5 replications each. Vertical bars represent the standard error. The inset shows the dosis needed for the half maximum response of the tested gibberellins ($[GA]_{50}$).*

Table 5.1 *Uptake of $[^3H_2]$-GA_1 and $[^3H_2]$-GA_4 by alstroemeria leaf tips during 48 hours in darkness*

Incubation In dark (h)	$[^3H_2]$-GA_1 (dpm)	% of applied label	$[^3H_2]$-GA_4 (dpm)	% of applied label
5	123	0.2	178	0.4
10	355	0.7	366	0.7
24	560	1.1	467	0.9
48	802	1.6	970	1.9

Uptake of [^{3}H] GA_1 and [^{3}H] GA_4

Firstly, differences in the uptake of GA_1 and GA_4 might explain the differences found in efficacy. Leaves were incubated with a 10^{-7} M GA solution enriched with tritium labelled GA. Leaves were shown to have a comparable uptake during the experimental period of 48 hours (Table 5.1). Thus, differences in GA-uptake can not explain the differences in efficacy of GA_1 and GA_4.

Necessity for 13-Hydroxylation ?

It is thought that active GAs have two characteristics in common: a 10-C-19-C lactone bridge below the plane of the A-ring and a hydroxyl group at the 3-C position (Pearce *et al.* 1994). According to Stoddart (1986) hydroxylation of the 13-C position enhances efficacy further. The presence of hydroxyl groups cannot simply explain activity in alstroemeria leaves because the less hydroxylated GA_4 was much more active than GA_1. To find out whether conversion of GA_4 into GA_1 and hence a 13-OH group is a prerequisite for activity we used a GA analogue. This modified GA has an O-CH_3 group on the 13-C position and is unlikely to be converted back to its basis GA (Dr MH Beale, personal communication). From previous work it is known that an order of efficacy exist in gibberellin acitivity from $GA_4 = GA_7 > GA_9 > GA_1 > GA_3$ (Jordi *et al.* 1995). We used GA_3-13-OMe to test whether a 13-OH group is neccesary for biological activity. From the results in Fig. 5.1 it is clear that GA_3-13-OMe showed a concentration-dependent effect on delay of chlorophyll loss. No GA_1, GA_3, or GA_4 was detected in an aliquot of 100 ng of GA_3-13-OMe analysed by GC-MS. Although the calculated concentration needed for the half-maximum response is 310 nM for GA_3-13-OMe compared to 2.1 nM for GA_4, it is clear that GA_3-13-OMe delays leaf senescence. The observed difference in maximum activity could be partly due to the difference found between maximum activity of GA_4 and GA_3. The $[GA]_{50}$ of GA_3 is comparable to that of GA_1 (unpublished results, IF Kappers). We conclude that hydroxylation on the C-13 position seems not essential for delaying chlorophyll loss.

Metabolism of GA_1, GA_4 and GA_9

Our results show that uptake of GAs can not explain the differences found in efficacy. Furthermore, 13-hydroxylation of GAs is not a prerequisite for biological activity in delay of leaf senescence. We have further studied the metabolism of deuterated GA_4 and GA_1 to elucidate the possible conversion pathways *in vivo* of these GAs.

In alstroemeria leaves, endogenous amounts of GA_1 and GA_4 in mature (fresh) leaves are almost identical; 0.66 ± 0.05 pmol (g FW)$^{-1}$ and 0.72 ± 0.02 pmol (g FW)$^{-1}$ respectively (Chapter 3). To get insight in their metabolism, we followed the fate of deuterated and tritiated labelled compounds. Although to date it is nearly impossible to estimate exact turnover rates, we can compare relative turnover rates of both GAs. To ensure a high uptake of deuterated GAs into the leaf, they were dissolved in 0.1 M citrate phosphate buffer at pH 5.0. Previous experiments showed that uptake was maximal at this pH (data not shown). The amount of deuterated GA taken up by the leaf was calculated from the tritiated GAs. Assuming no discrimination in the uptake of deuterated and tritiated GA the uptake of deuterated GA was estimated to be 65 pmol GA_1, 59 pmol GA_4 and 182 pmol GA_9 (g FW)$^{-1}$, resulting in an increase of the endogenous concentration of GA_1, GA_4 and GA_9 with a factor 98, 82 and 152, respectively. When leaves were incubated with $^2H_2$-GA_4 the ratio *m/z* 286/284 found after 48 hrs of labelling was 35 times the ratio found in non-labelled leaves which confirmed an increased endogenous concentration of GA_4 (Table 5.2). The discrepancy between the total uptake of 82 times the endogenous concentration and the enrichment of the ratio between deuterated and endogenous GA (35 times) is due to the metabolism of the applied GA. The increased ratio's of 508/506 and 373/371 found at the retention times of GA_{34} and GA_{34}-catabolite suggested the presence of deuterated GA_{34} and possibly GA_{34}-catabolite, demonstrating the conversion of GA_4 into GA_{34}. This step involves a 2β-hydroxylation, known to inactivate GAs (Graebe 1987). GA_{34} was found in alstroemeria leaves at a low concentration of 0.04 pmol (g FW)$^{-1}$ (Chapter 3). After incubation with GA_4, endogenous GA_1 was detected, but the *m/z* 508/506 ratio observed corresponded with the natural abundance of the isotope. This indicated that even at a very high endogenous GA_4 concentration no significant extra GA_1 was formed (*i.e.* less than 3%). To exclude as much as possible experimental errors in demonstrating GA_4 metabolism, we also incubated leaves with labelled GA_9 (Table 5.2). It can be seen that the ratio's of GA_9, GA_4 and GA_{34} differed from the endogenous values. Also in this case no increase in endogenous GA_1 could be found. Incubation of leaves with

Table 5.2 *GC-SIM data on metabolites from $[^2H_2]GA_1$, $[^2H_2]GA_4$ and $[^2H_2]GA_9$ incubation of alstroemeria leaves during 48 hours. Data are means of four independent experiments ± SD. Enrichment is calculated by ([m/z+2] / [m/z] of sample) / ([m/z+2] / [m/z] of standard).*

GA fed To the leaves	Deuterated GAs KRI	Ga_n	Relative peak area ratio [m/z+2] / [m/z]**			Enrichment
GA_1 standard	2689		0.150		(508/506)	
GA_4 standard	2522		0.053		(286/284)	
GA_8 standard	2837		0.254		(596/594)	
GA_9 standard	2319		0.034		(300/298)	
GA_{34} standard	2675		0.169		(508/506)	
GA_8-catabolite*	2723		0.182		(536/534)	
GA_{34}-catabolite*	2569		0.294		(373/371)	
$[^2H_2]GA_1$ fed	2689	GA_1	2.257	± 0.12	(508/506)	15.05
	2522	GA_4	0.059	± 0.003	(286/284)	1.11
	2837	GA_8	1.056	± 1.89	(596/594)	4.22
	2319	GA_9	0.039	± 0.004	(300/298)	1.15
	2675	GA_{34}	0.153	± 0.009	(508/506)	0.91
	2723	GA_8 –cat	0.319	± 0.18	(536/534)	1.75
	2569	GA_{34}-cat	0.291	± 0.06	(373/371)	0.99
$[^2H_2]GA_4$ fed	2689	GA_1	0.138	± 0.01	(508/506)	0.92
	2522	GA_4	1.864	± 0.09	(286/284)	35.17
	2837	GA_8	0.272	± 0.04	(596/594)	1.07
	2319	GA_9	0.040	± 0.002	(300/298)	1.18
	2675	GA_{34}	17.601	± 1.27	(508/506)	104.15
	2723	GA_8 –cat	0.178	± 0.04	(536/534)	0.98
	2569	GA_{34}-cat	0.641	± 0.12	(373/371)	2.18
$[^2H_2]GA_9$ fed	2689	GA_1	0.162	± 0.005	(508/506)	1.08
	2522	GA_4	1.532	± 0.08	(286/284)	28.91
	2837	GA_8	0.292	± 0.02	(596/594)	1.15
	2319	GA_9	3.029	± 0.13	(300/298)	89.08
	2675	GA_{34}	16.431	± 0.88	(508/506)	97.22
	2723	GA_8 –cat	0.180	± 0.05	(536/534)	0.99
	2569	GA_{34}-cat	0.451	± 0.04	(373/371)	1.53

Note: * obtained by interpolation of data of Gaskin & MacMillan (1991)

** ions representing [m/z+2] and [m/z] for the different GAs are given in parentheses.

deuterated GA_1 lead to increased ratio's for GA_1 and GA_8 and possibly GA_8-catabolite (Table 5.2).

Although the estimated concentration of the biologically active hormones was the same for GA_1 and GA_4, the turnover rate, that is, the number of molecules per time unit that is formed and subsequently turned over, seemed to be different. From the calculated enrichment (Table 5.2) it can be concluded that the non-13-hydroxylated GA_{34} and GA_{34}-metabolite were formed relatively more than their corresponding 13-hydroxylated counterparts, GA_8 and GA_8-catabolite. Although the uptake of both GA_1 and GA_4 is comparable, the enrichment of the leaf with GA_1 is lower after 48 hours. Within the limits of this experiment, this indicates a higher turnover rate for GA_1 than for GA_4. However, it is obvious that even at high concentrations of GA_4 in the leaf no conversion into GA_1 occurs. Combined with the greater biological efficacy of applied GA_4 towards delay of senescence this makes it very likely that the non-13-hydroxylation pathway yielding GA_4 is a part of early processes regulating senescence in alstroemeria leaves. As no conversion into GA_1 is needed, GA_4 will be directly recognised by the receptor involved in the regulation of leaf senescence.

6 *Meta*-topolins are involved in red light signalling

6.1 Abstract

In alstroemeria (*Alstroemeria hybrida*), leaf senescence is effectively retarded by the exogenous application of cytokinins and by low photon fluences of red light. In this study we examined the interaction of both isoprenoid and aromatic cytokinins and red light in the regulation of senescence. Leaf senescence was accompanied by a sharp drop in endogenous zeatin riboside during the first day while aromatic cytokinin concentrations did not significantly alter. Irradiation of the leaves with red light induced a transient increase in endogenous *meta*-topolin and *meta*-topolin riboside concentration which could be partly counteracted by a subsequent far red irradiation. Red light irradiation had no significant effect on isoprenoid cytokinins in the leaves. When exogenously applied, the concentrations of *meta*-topolin (riboside) and zeatin (riboside) needed for the half-maximum effect were in the same range. However, application of *meta*-topolin (riboside) resulted in complete chlorophyll retention whereas Z(R) did not. We suggest that the phytochrome-induced delay of leaf senescence is at least partly mediated by a transient and rapid increase in *meta*-topolin.

6.2 Introduction

Cytokinins stimulate chloroplast development, promote bud break and enhance the resistance of plant to various forms of stress (Kamínek 1992). In addition, there is abundant evidence in many plant species that cytokinins play a role in the regulation of leaf senescence (Smart 1994).

Generally, three groups of cytokinins are reported to be present in plants, *i.e.* Z-, DHZ-, and iP-type cytokinins. Senescing leaves generally contain lower concentrations of free cytokinins, while O- and N-glucosides increase strongly in older leaves (Badenoch-Jones *et al.* 1987). In a number of plants aromatic cytokinins are reported (Ernst *et al.* 1983, Nandi *et al.* 1989, Strnad *et al.* 1992a).

In alstroemeria, leaf senescence can be effectively delayed by application of cytokinins and gibberellins (Jordi *et al.* 1995). A comparable effect on chlorophyll retention was obtained when leaves were irradiated with a low fluence of red light which could be counteracted by far-red light (Chapter 4). The process of leaf senescence in alstroemeria appears thus to be regulated both by growth regulators and phytochrome. The similarities between the effects of light and growth regulators might be explained if they share common signalling pathways. In a preceding paper we demonstrated that the effects of gibberellins and red light on leaf senescence are independent (Chapter 4).

In the present study we examine whether isoprenoid or aromatic cytokinins could function as mediator for the transduction of the red light signal in the regulation of leaf senescence. Interaction of light and cytokinin responses has been described earlier for processes such as chloroplast development and gene expression (Longo *et al.* 1990, Reski *et al.* 1991). In contrast, Su and Howell (1995) reported independent and additive effects of cytokinin and light on hypocotyl elongation. Hewett and Wareing (1973) reported an effect of light on the increase of cytokinins in poplar. In this study, we monitored the levels of aromatic and isoprenoid cytokinins after red light pulses before any visible decrease of chlorophyll appeared. Furthermore, the effect of exogenously applied cytokinins on chlorophyll retention was studied. The results are consistent with topolins functioning as intermediates in the phytochrome response towards leaf senescence.

6.3 Materials and Methods

Chemicals

BAP (purity > 99%) was obtained from Duchefa (Haarlem, The Netherlands), DHZ, iPR, Z and ZR (purity > 97%) from Apex Organics (Devon, UK) and *m*T, *m*TR and *o*T (purity > 97%) from Olchemin (Olomouc, Czech Republic). Pre-

immunoaffinity chromatography (pre-IAC) and IAC columns were prepared as described in Strnad *et al.* (1997). Specific properties of the antibodies used in the ELISA methods were also described elsewhere (Strnad *et al.* 1992a,b, Strnad 1996).

Light conditions

Light conditions are described in detail in Chapter 4.3. The photon fluence rate of the red light was 5 μmol m^{-2} s^{-1} and of the far red light 5.4 μmol m^{-2} s^{-1} at the level of the leaves.

Plant material and growth conditions

Alstroemeria hybrida cv. Westland plants were used and handled as described in Chapter 2.4.

Extraction and purification of cytokinins

Cytokinins were purified according to the method of Strnad *et al.* (1997) with modifications. Leaves (1-2 g FW) were ground to powder under liquid nitrogen, extracted in ice-cold 70% EtOH (20 ml $(g\ FW)^{-1}$) containing sodium diethyldithiocarbamate as anti-oxidant (400 μg $(g\ FW)^{-1}$) for 2 h and centrifugated at 15,000 rpm for 20 min at 0°C. The pellet was re-extracted for another 2 h. The combined supernatants were poured through a C_{18} column (activated with 80% MeOH) and thereafter evaporated until ethanol free. After freezing and thawing, the sample was dissolved in 20 ml 40 mM NH_4COOCH_3 and adjusted to pH 6.5 with 5 M NH_4OH. A C_{18} column (activated with subsequently 100% MeOH, water and 40 mM NH_4COOCH_3) was coupled to a DEAE column (2 ml $(g\ FW)^{-1}$), equilibrated with 40 mM NH_4COOCH_3. After loading of the sample, the column was washed with 40 mM NH_4COOCH_3 and the free bases, ribosides, and 3-, 7-, 9-, and O-glucosides were eluted and collected on the C_{18}-column. The C_{18}-column was removed and a new C_{18}-column (activated with MeOH, water and 6% formic acid) was coupled to the DEAE column. Nucleotides were eluted from DEAE and collected on the second C_{18}-column by applying 6% formic acid. Both C_{18}-columns were eluted with 80% MeOH to collect cytokinins. Samples were evaporated to dryness, taken up in 50 μl 70% EtOH and 450 μl phosphate

buffer (50 mM NaH_2PO_4, 15 mM NaCl, pH 7.2) and applied to a pre-IAC column and subsequently to an IAC column according to Strnad *et al.* (1997). Cytokinins were eluted with ice-cold MeOH and evaporated to dryness. O-glucosides were not bound by IAC. Therefore, the eluent was collected and dried prior to enzymatic cleavage of the O-glucoside by β-glucosidase (EC 3.2.1.21) to yield free cytokinins (30 min, 0.1 M NH_4COOCH_3, pH 5, 37°C). Subsequently, the sample was applied to the IAC column as described before.

HPLC

The HPLC system consisted of a Spectra Physics SP8800 solvent delivery system coupled to a SPD-M6A photodiode array UV-Vis detector and SPD-MXA data station (Shimadzu). The injection was performed by a Rheodyne 7010 injection loop (100 µl). The gradient system used was solvent A 10% MeOH (pH 3.35 adjusted with triethylamine) and solvent B 80% MeOH (pH 3.35). Initial conditions were 95% A and 5% B, then a linear gradient to 60% A and 40% B at 20 min, a linear gradient to 50% A and 50% B at 30 min, and 100% MeOH for 5 min (column wash). A Microsorb C18 column was used (4.6 x 100 mm, 3 µm) at a flow of 0.6 ml min^{-1}.
Samples were dissolved in 50 µl 70% EtOH and made up to 250 µl with water. An aliquot of 100 µl was injected and subsequent fractions of 0.5 ml were collected and dried. Aliquots of 50 µl from appropriate fractions were tested for cytokinin activity with a direct ELISA method as described by Strnad *et al.* (1996). Antibodies against BAPR, DHZR, iPR, *m*TR, oTR and ZR were used. Specific properties of the antibodies were described previously (Strnad *et al.* 1992a, b, Strnad 1996). The method was validated by GC-MS (Strnad *et al.* 1997).

Chlorophyll analysis

Chlorophyll (*a*+*b*) content of the leaves was determined spectrophotometrically after extraction in dimethylformamide as described in Chapter 2.4. Data are presented as percentage of the initial amount of chlorophyll in order to correct for small differences between experiments.

Dose response experiments

Leaf tips (fresh weight approximately 500 mg, leaf area approximately 7 cm^2) were incubated in various concentrations of cytokinins (total volume 3 ml) in darkness. After 8 days the chlorophyll concentration was determined. Logistic dose-response curves were calculated, using a non-linear regression method described by $(R=R_{min}+(R_{max}-R_{min})/(1+([CK]/[CK]_{50})^p)$, where R is the response, R_{min} is the response in absence of exogenous cytokinin, R_{max} is the maximum response, [CK] is the applied cytokinin concentration, $[CK]_{50}$ is the concentration required for half-maximum response and p is the Hill coefficient representing the abruptness of the curve (Weyers *et al.* 1987).

6.4 Results

Endogenous cytokinins

Identification of endogenous cytokinins in alstroemeria leaves

Mature green leaves of alstroemeria contained a variety of cytokinins (Table 6.1). Next to the isoprenoid cytokinins, aromatic cytokinins were detected. All classes of cytokinins known to be endogenous in plants were identified with Z-type cytokinins being predominantly present (33% of total cytokinin content). The major cytokinin was ZR: 4.1 pmol $(g\ FW)^{-1}$. Of the total cytokinin content, more than 30% was aromatic (BAP, *m*T or oT type) of which BAPR had the highest concentration of 1.4 pmol $(g\ FW)^{-1}$ (Table 6.1). About 10% of the analysed cytokinins were of the *m*T-type, of which the free base was present in the highest concentration.

Zeatin and meta-topolin concentration during senescing period

Chlorophyll breakdown was effectively delayed by a daily red irradiation (10 min day^{-1} 5 µmol $m^{-2}\ s^{-1}$) of the leaves (data not shown). This low red light irradiation resulted in a retention of approximately 45 to 60% of the initial chlorophyll content after 8 days while non irradiated leaves had only 20% of

Table 6.1 *Cytokinins identified in mature green leaves of alstroemeria (pmol (g FW)$^{-1}$). Data are means of 4 replications ± SD.*

Cytokinin	Concentration (pmol (g FW)$^{-1}$)	Cytokinin	Concentration (pmol (g FW)$^{-1}$)
IPRMP	0.52 ± 0.21	*o*T	0.20 ± 0.09
IP	0.30 ± 0.09	*o*TR	0.41 ± 0.01
IPR	0.50 ± 0.30	*o*T9G	0.18 ± 0.07
IP9G	0.26 ± 0.08	*o*TOG	0.08 ± 0
		*o*TROG	0.12 ± 0.01
ZRMP	1.05 ± 1.07		
Z	0.42 ± 0.00	BAP	1.01 ± 0.45
ZR	4.06 ± 0.54	BAPR	1.42 ± 0.82
Z9G	0.52 ± 0.03	BAP9G	1.04 ± 0.55
ZOG	0.39 ± 0.39		
ZROG	0.16 ± 0.04	*m*TRMP	0.38 ± 0.02
		*m*T	0.60 ± 0.10
DHZ	1.54 ± 0.18	*m*TR	0.39 ± 0.11
DHZR	2.27 ± 1.55	*m*T9G	0.21 ± 0.03
DHZ9G	0.28 ± 0.00	*m*TOG	0.20 ± 0.11
DHZOG	0.40 ± 0.35	*m*TROG	0.25 ± 0.16
DHZROG	0.84 ± 0.15		

BAP-type	17.4%
DHZ-type	26.6%
IP-type	7.9%
*M*T-type	10.2%
*O*T-type	4.9%
Z-type	33.0%

the chlorophyll left. A rapid decrease in ZR concentration to 10% of the initial content (4.1 pmol (g FW)$^{-1}$) was found after 24 hours of darkness. In this period ZROG concentration increased from 0.16 to 2.8 pmol (g FW)$^{-1}$. No significant changes in concentrations of other cytokinins during the first 24 hours were detected (data not shown). During the following 7 days cytokinin concentrations decreased slowly but were still detectable 8 days after the start of the experiment.

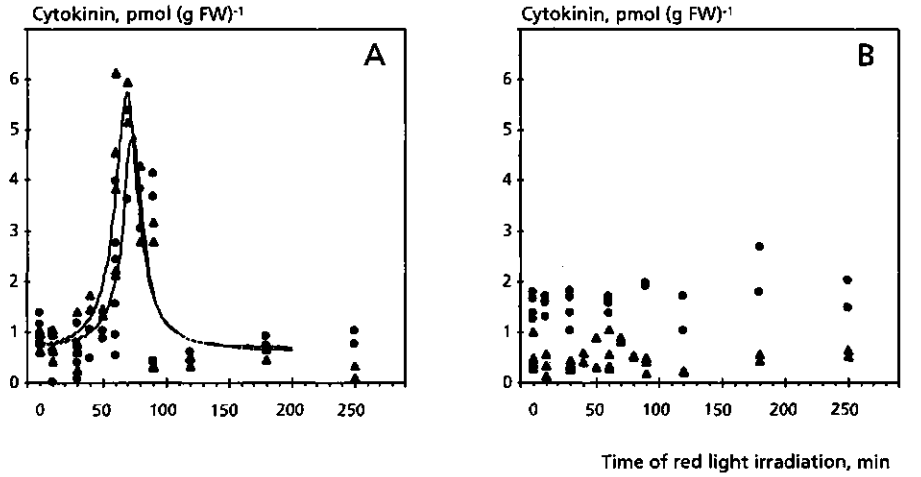

Figure 6.1 *Cytokinin concentrations (pmol (g FW)$^{-1}$) in alstroemeria leaves placed in darkness for 24 hours and subsequently irradiated with 5 µmol m^{-2} s^{-1} red light. A: mT (Δ) and mTR (●), B: Z (Δ) and DHZ (●). Non-linear regression curves were calculated for mT and mTR data points: mT: 0.61+5.18/(1+((T-68.9)/-11.1)^2), r^2=0.86 and mTR: 0.68+4.27/(1+((T-73.8)/9.6)^2), r^2=0.74.*

Figure 6.1 compiles three independent experiments in which leaves were placed in darkness for 24 hours and subsequently irradiated with 5 µmol m^{-2} s^{-1} red light. In all these experiments after 60 - 70 minutes of red light irradiation a rapid and transient increase in *m*T and *m*TR concentration was observed (Fig. 6.1A). For all other cytokinins endogenous in alstroemeria leaves (see Table 6.1) no significant changes due to the irradiation could be detected. As an example the concentrations of Z and DHZ are shown (Fig. 6.1B). A daily red light irradiation (10 min day^{-1}) of the leaves caused a transient increase in *m*T(R) but during 8 days did not alter the overall cytokinin concentrations (including *m*T(R)) when the leaves were analysed immediately after each of the red light pulses (data not shown).
The concentration of *m*T in the leaves increased 9-fold in one hour when leaves were irradiated for only 10 min after 24 hours of darkness (Table 6.2). When the red light pulse was followed immediately by 10 min of far red light which counteracted the delay in leaf senescence, the increase in *m*T concentration was initially the same (after 50 min) but significantly different at 60 and 70 minutes (Table 6.2). For *m*TR, a similar pattern was observed. No significant changes were found in other type of cytokinins due to this light treatment (data not shown).

Table 6.2 *mT and mTR concentrations in (pmol (g FW)$^{-1}$) ± SD in alstroemeria leaves placed in darkness for 24 hours (t=0) and subsequently irradiated with 5 µmol m^{-2} s^{-1} red light for 10 min or 10 min red light immediately followed by 5.4 µmol m^{-2} s^{-1} far red light for 10 min. Control leaves remained in the dark during the experiment.*

	mT (pmol (g FW)$^{-1}$)		mTR (pmol (g FW)$^{-1}$)	
Leaves placed in darkness for 24 hrs	0.88 ± 0.13		0.95 ± 0.28	
for 25 hrs			1.12 ± 0.52	
Leaves placed in darkness and subsequently irradiated with	R 10 min	R / FR 10+10 min	R 10 min	R / FR 10+10 min
50 min after start of light treatment	3.85 ± 0.71	4.28 ± 1.56	4.83 ± 0.87	6.10 ± 1.70
60 min after start of light treatment	7.84 ± 1.48	2.91 ± 0.52	6.19 ± 1.36	3.11 ± 1.29
70 min after start of light treatment	6.95 ± 1.27	3.06 ± 0.62	4.80 ± 1.65	3.18 ± 0.13

Exogenous cytokinins

The effects of various concentrations of 8 cytokinins on chlorophyll retention in excised leaf tips of alstroemeria was tested. Chlorophyll decrease started after approximately 4 days in untreated leaves (darkness, no addition of cytokinins). After 8 days the amount of chlorophyll was approximately 20% of the initial amount. Table 6.3 shows the cytokinin concentrations needed for the half maximum effect on leaf senescence. A range of activity exists for the different cytokinins in the order of *m*TR > ZR ≈ *m*T > Z > DHZ > BAP ≈ iPR >> *o*T = no effect. Figure 6.2 shows the dosis response curves for the most active cytokinins in delaying leaf senescence, *m*T(R) and Z(R). Both *m*T and *m*TR showed a clear concentration dependent effect on the chlorophyll content after 8 days and all chlorophyll in the leaves was retained by high concentrations of *m*T or *m*TR. Also Z and ZR showed a concentration dependent effect on chlorophyll retention, ZR being slightly more effective (Fig. 6.2B). However, for Z and ZR no saturation of chlorophyll retention was obtained in this range of cytokinin concentrations (Fig. 6.2B). The effect of *m*T(R) in terms of amplitude (R_{max}-R_{min}) was significantly greater than the effect of Z(R) (Fig. 6.2).

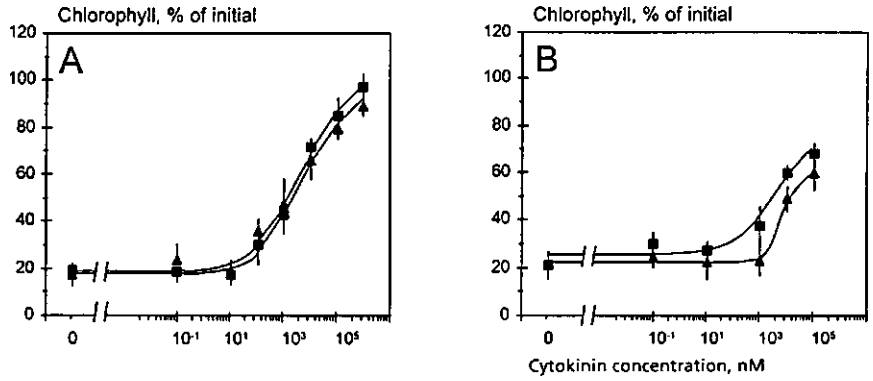

Figure 6.2 *Chlorophyll (a+b) content (% of initial amount) of alstroemeria leaves affected by various concentrations of mT(▲), mTR (■) (A) and Z (▲), ZR (■) (B) after placement for 8 days in darkness. Data are means of 5 replications ± SD. Initial chlorophyll concentration was 1.89 mg (g FW)$^{-1}$.*

Table 6.3 *Cytokinin concentration (μM) needed for the half maximum effect ($[CK]_{50}$) on inhibition of leaf senescence of alstroemeria leaf tips placed for 8 days in darkness.*

Cytokinin	$[CK]_{50}$, μM
BAP	> 2
DHZ	1.56 ± 0.09
iPR	> 2
mT	0.54 ± 0.06
mTR	0.36 ± 0.03
oT	no significant effect
Z	0.67 ± 0.08
ZR	0.50 ± 0.05

Discussion

Both low photon fluences of red light and a number of cytokinins delay leaf senescence in alstroemeria. We studied whether these two factors interact in their regulation of leaf senescence.

A range of cytokinins was detected in mature, fully expanded alstroemeria leaves: the free bases, their ribosides, nucleotides and O-glucosides (Table 6.1). The ribosides, ZR, DHZR, and BAPR were dominant cytokinins in the leaf. Overviews of all cytokinins in plant tissues are rarely reported. Most studies have been limited to the determination of isoprenoid-derived cytokinins (Wagner and Beck 1992, Dieleman *et al.* 1997). In these studies, Z and DHZ type cytokinins were predominant. In addition, in some plants aromatic cytokinins are reported (Ernst *et al.* 1983, Nandi *et al.* 1989, Strnad *et al.*, 1992a, 1997). In alstroemeria, aromatic cytokinins amounted to approximately one third of the total cytokinin content.

Isoprenoid cytokinin biosynthesis is hypothesised to start with the synthesis of iP-type cytokinins which are subsequently converted to Z-type and DHZ-type cytokinins (Letham and Palni 1983). De *novo* synthesis of cytokinins in leaves was reported for pea (Chen *et al.* 1985), *Perilla* (Grayling and Hanke 1992) and rose (Dieleman *et al.* 1997). In mature leaves of alstroemeria, approximately 8% of the total cytokinin pool was of the iP-type. The incorporation of [^{14}C]-adenine into cytokinins decreased with maturation and senescence in tobacco leaves (Singh *et al.* 1992b) suggesting that a difference in cytokinin biosynthesis in young versus mature leaves may play a role in the control of leaf senescence.

Aromatic cytokinins posess a benzylic side chain and are hypothesised to originate from the metabolism of phenolics (Strnad 1997). Although the biosynthetic pathways of isoprenoid and aromatic cytokinins are not yet firmly established, the present knowledge suggest individual pathways with separate control mechanisms for both groups of cytokinins (Kamínek *et al.* 1987).

The decrease in ZR concentration during the first day after harvest occurs before changes in chlorophyll or protein concentration. This decrease might be caused by removal of the stems from the root system, where cytokinins are proposed to be synthesised. ZR is suggested to be the major translocation form of cytokinins (Wagner and Beck 1992, Dieleman *et al.* 1997). In alstroemeria leaves, an increase in ZROG was found in the same time period, suggesting a conversion of ZR to ZROG. However, the decrease in ZR is not strictly coupled to the induction of the leaf senescence process, since a red light irradiation which very effectively delayed leaf senescence, did not

significantly alter the concentrations of any of the isoprenoid cytokinins in alstroemeria (Fig. 6.1B).

In strong contrast with isoprenoid cytokinins, an increase in *m*T and *m*TR concentration was found 60 to 70 minutes after the onset of the red light, followed by a rapid decrease when the leaves remained irradiated (Fig. 6.1A). Leaves irradiated for shorter periods showed a more gradual decrease in *m*T(R) (data not shown), suggesting a light-regulated induction of both synthesis and breakdown. In poplar leaves an increase of *m*T(R) was found 2 hours after sunrise under field conditions (Strnad, unpublished results). Based on bio-assays, Hewett and Wareing (1973) reported an increase in cytokinin-like activity in poplar leaves as a result of short periods of irradiation and phytochrome involvement was suggested.

A far red light irradiation directly following red light irradiation completely counteracted the effect of red light on chlorophyll retention (Kappers *et al.* 1998). This far red light treatment only partly counteracted the increase in *m*T(R) due to a red irradiation (Table 6.2). Most likely, the increase in *m*T(R) concentration is due to phytochrome activation directly after the onset of the light. The subsequent transduction of signals leading to the increase in *m*T(R) after 10 min of red light can thus only partly be deactivated by the inactivation of phytochrome due to far red light. The time scale from phytochrome activation to the start of the increase in *m*T(R) is about 40 minutes (Table 6.2), indicating that *m*T(R) can be considered as a 'second order signal'. The increase in *m*T(R) could be due to a rapid *de novo* synthesis starting from AMP. Conversely, as suggested by Kamínek *et al.* (1987), BAP(R) or *o*T(R) can be converted into *m*T(R) by a hydroxylation at the benzyl ring. However, we could not detect a clear decrease in either BAP-type or *o*T-type cytokinins. Also no increase in *m*TRMP, hypothesised to be the precursor for *m*T(R), was detected. Metabolic studies should provide more information about the origin of *m*T(R).

To relate changes in endogenous *m*T(R) concentration due to red light with delay of senescence, the biological activity of a number of aromatic and isoprenoid cytokinins was tested. No biological effect was found when oT was applied, which confirmed that a hydroxylation at the *o*-position inactivates the aromatic cytokinin (Kamínek *et al.* 1987). Chlorophyll was about equally well retained by both the aromatic cytokinin *m*T(R) and the isoprenoid cytokinin Z(R) when $[CK]_{50}$-values were compared (Table 6.3). However, *m*T(R) was capable to retain all chlorophyll present in the leaves (100%-level) after 8 days whereas Z(R) could not effectuate a complete chlorophyll retention (Fig. 6.2). The effect of *m*T(R) on chlorophyll retention could not be saturated by high concentrations (Fig. 6.2). Therefore,

additional evidence that *m*T(R) and red light act via similar pathways could not be confirmed by application studies.

Our results show that a number of cytokinins delay chlorophyll loss in alstroemeria leaves. Although isoprenoid cytokinins and in particular Z(R) delay chlorophyll breakdown, the failure to observe an increase in isoprenoid cytokinin concentration in the leaves due to red light irradiation, suggest that these cytokinins are not directly involved in mediating the phytochrome signal in leaf senescence in alstroemeria. In contrast, *m*T-type cytokinins showed an approximately five fold increase after phytochrome activation. Furthermore, exogenously applied *m*T-type cytokinins were most effective in retaining chlorophyll in the leaves. These data strongly suggest that *meta*-topolins mediate the red light induced delay of leaf senescence.

7 Differential effects of cytokinins, gibberellins and phytochrome on chlorophyll metabolism and functioning

7.1 Abstract

In alstroemeria (*Alstroemeria hybrida*), leaf senescence is effectively retarded by a number of cytokinins, gibberellins and by low photon fluences of red light. Although the visual effect is similar, *i.e.* a net retention of chlorophyll, the mode of action of the regulators can be different. This study focused on the effects of *meta*-topolin, GA_4 and red light on parameters of chlorophyll metabolism and functioning of the chloroplast during the course of senescence. It was found that both red light and *meta*-topolin had positive effects on chlorophyll biosynthetic reactions as well on the rate of photosynthesis and expression of genes encoding for chlorophyll binding proteins (*cab*). GA_4 did not affect any of these parameters. Neither *meta*-topolin, nor GA_4 or red light did differentially affect Mg-dechelatase activity, as an example of a chlorophyll catabolic reaction.

7.2 Introduction

In mature leaves there is a balance between anabolic and catabolic chlorophyll biosynthetic reactions. During senescence of the leaves there is a net decrease in the amount of chlorophyll molecules due to a lower activity of chlorophyll-synthesising enzymes and / or a higher activity of chlorophyll-catabolising enzymes.

In alstroemeria, chlorophyll loss is effectively delayed by three factors: GA_4 (Chapter 4), *meta*-topolin riboside (*m*TR), an aromatic cytokinin (Chapter 6) and

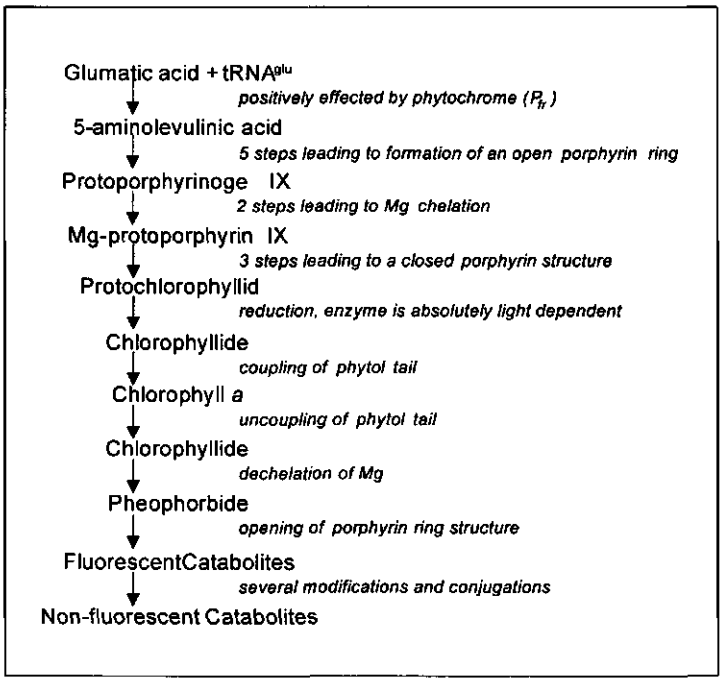

Figure 7.1 *Schematic representation of the biosynthetic reactions leading to chlorophyll and the subsequent catabolic reactions of chlorophyll degradation.*

low photon fluences of red light (Chapter 2 and 4). Although the visible results of red light, GA and CK are similar, *i.e.* a net retention of chlorophyll and thus green leaves, it is possible that these factors influence chlorophyll metabolism and chlorophyll functioning in photosynthesis in a different way. In Figure 7.1 the biosynthetic reactions leading to chlorophyll and the subsequent catabolism of chlorophyll are summarised. Light, via activation of phytochrome, stimulates the formation of 5-aminolevulinic acid, an early precursor of chlorophyll (Castelfranco and Beale 1983). The porphyrin ring of chlorophyll originates from 8 molecules of 5-aminolevulinic acid. The conversion of protochlorophyllide into chlorophyllide in the biosynthetic pathway of chlorophyll is known to be absolutely light dependent (Von

Wettstein *et al.* 1995). Furthermore, light is known to increase the transcription of *cab* genes, which encode for the apoprotein of the light-harvesting chlorophyll a/b binding protein (LHCP) (Yamamoto *et al.* 1991).
For cytokinins it has been established that transcription and stability of the *cab* mRNA are enhanced by benzyl adenine (BA) (Flores and Tobin 1986, Longo *et al.* 1990). In etiolated seedlings CKs minimised the lag-time needed for chlorophyll synthesis after an inducing light pulse (Castelfranco and Beale 1983).
Although gibberellins are reported to delay senescence in a number of species, to our knowledge no information is available about specific steps in chlorophyll metabolism or chloroplast functioning which are regulated by gibberellins. Previously, GAs were shown to have only a minor effect on the decline of photosynthetic activity in senescing alstroemeria leaves (Jordi *et al.* 1994).
To get insight into the mode of action of CKs, GAs and red light we studied a number of steps in chlorophyll metabolism and chloroplast functioning during the course of senescence. Levels of precursors of chlorophyll, Pchlide and Chlide, and the activity of Mg-dechelatase, a chlorophyll-degrading enzyme were determined. In addition, the expression of *cab* mRNA and the photosynthetic activity of the leaves were followed during senescence to study the effects of CKs, GAs and red light on chlorophyll functioning in photosynthesis.
In strong contrast to *m*TR incubation and red light irradiation, GA_4, although very effective in chlorophyll retention, did affect neither chlorophyll synthesis, nor *cab* expression nor maintained photosynthetic capacity.

7.3 Material and Methods

Plant material and experimental conditions

Leaves of *Alstroemeria hybrida* cv. Cinderella plants were used and handled as described in Chapter 2.4 (deflowered stem system). The stems were placed in demineralised water in the absence or presence of a growth regulator solution, transferred from daylight to darkness and kept in a climate room at 20 ± 1°C and ca 65% relative humidity.

Light conditions

Light sources were described previously in Chapter 2.4. The photon fluence rate of the red light source was 5.0 µmol m^{-2} s^{-1} and of the far red light source 5.4 µmol m^{-2} s^{-1} at the level of the leaves.

Growth regulators

Origin and purity of GA_4 and *m*TR are described in Chapter 3.3 and 6.3. Stock solutions were prepared as 10^{-2} M in ethanol. The small amount of ethanol (maximal 1%(v/v)) did not affect senescence in alstroemeria leaves (data not shown).

Chlorophyll

Chlorophyll *a* + *b* contents of the leaves were determined spectrophotometrically at 647 and 664.5 nm after extraction with dimethylformamide as described in Chapter 2.4.

Protochlorophyllide and chlorophyllide

All handlings were performed under dim green safe light. Leaves were extracted in 80% acetone + 0.025%(v/v) ammonia. After precipitation of the debris, the extract was transferred to another tube and partitioned against an equal volume of hexane for two times. Chlorophyll, fully esterified with an apolar phytol-tail, is partitioned in the hexane fraction, whereas carboxylic non phytol-tail containing precursors of chlorophyll remain in the aqueous acetone phase. Pchlide and Chlide were quantified spectrofluorometrically according to Hukmani and Tripathy (1992). Pchlide and Chlide, when excited at 440 nm, fluoresce at 638 nm (E_{440} F_{638}) and 675 nm (E_{440} F_{675}), respectively. To estimate the net synthesis of Pchlide, leaves were exposed to white light (450 µmol m^{-2} s^{-1}) for 10 min. Control experiments demonstrated that a 5 min light treatment was sufficient to exhaust the phototransformable Pchlide content of the leaves. The Pchlide accumulated in the leaves was phototransformed into Chlide after this light treatment. Subsequently, the leaves were placed in darkness for 10 h. After this period Pchlide content was estimated and net synthesis was calculated

by subtracting the initial nonphototransformable Pchlide content from the final total Pchlide pool. The net synthesis of Chlide was estimated by measuring the Pchlide content after depletion by a saturating light pulse (450 µmol m^{-2} s^{-1} for 10 min). During the course of the experiment and independent of treatments, approximately 90 - 95% of the Pchlide was converted to Chlide in the light (data not shown).

Preparation of chloroplast membranes

Chloroplast membranes were prepared from leaves incubated under different experimental conditions. Leaves were blended for 3 s in a medium containing 0.4 M sorbitol, 25 mM tricine KOH buffer, pH 8.0, 2 mM Na-EDTA, 1 mM $MgCl_2$, 0.1% (w/v) BSA, 5 mM polyethylene glycol 4,000 and 10 mM cysteamine-HCl at 0°C. After filtration through a 25 µm nylon gauze, thylakoids were concentrated by centrifugation (3 min 6,000 g). The pellet was resuspended in 0.4 M sorbitol, 25 mM Tricine KOH buffer, pH 8.0. Aliquots of the suspension containing the thylakoid equivalent of 0.5 g fresh leaf tissue were then pipetted into 1.5 ml eppendorf tubes. After centrifugation (4 min, 6,000 g) the supernatants were removed and the thylakoid pellets frozen in liquid N_2 until required for measurement. Prior to incubations, the pellets were thawed and resuspended in 500 µl 50 mM Tris/Tricine buffer, pH 9.0 containing 1% (v/v) Triton X-100). For complete solubilisation of thylakoids the preparations were kept on ice for 30 min and agitated at intervals.

Mg-dechelatase activity

The assay is based on the assay described by Vicentini *et al.* (1995). In this assay, chlorophyllin instead of Chlide is used as a substrate for Mg-dechelatase. Chlorophyllin is a Mg-chlorin derivative from chlorophyll and enzymatically converted into its pheoderivative. It has the advantage of a more sensitive shift in absorbance at conversion, is easily prepared and stable for some weeks when stored at -20 °C.
The assay mixture (final volume 1.0 ml) contained 50 mM Tris/Tricine buffer, pH 9.0, 20 µl of chlorophyllin stock solution to yield a final concentration of 64 nM (A_{644} = 1.4), 0.1% Triton X-100 and 5-50 µl chloroplast membrane preparation. Changes of absorbance at 687 nm were recorded between the fifth and tenth minute of incubation. All determinations were made at 20°C.

Activities were calculated as ΔA_{687} min^{-1}. Determinations are the means of 10 parallel incubations with a mean standard deviation of 8.8%.

Photosynthesis

The photosynthetic capacity of the leaves was measured using a leaf chamber analyser. The ingoing air had a constant composition of 33 Pa CO_2. The average conditions within the leaf chamber were 19.8 °C and the vapour pressure deficit was 0.57 kPa. The photosynthetically active radiation was 800 µmol m^{-2} s^{-1}, previously determined to be a saturating photon fluence rate for fresh mature leaves (data not shown). Rates of photosynthesis were calculated from the flow rates and the measured concentrations of CO_2 and vapour in the ingoing and outgoing air stream as described by Von Caemmerer and Farquhar (1981).

Cab mRNA expression

Leaves of three stems were pooled, frozen in liquid nitrogen and ground to a fine powder. Total RNA was isolated from 100 mg frozen tissue by using the purescript RNA isolation kit (GENTRA systems Inc., Minneapolis, MI, USA) followed by DNase treatment, phenol / chloroform purification and ethanol precipitation according to standard protocols. The precipitated RNA was washed twice with 70% ethanol and dissolved in water. Equal volumes of the RNA samples were separated on 1.2% agarose gels, blotted onto nylon membranes (Hybond N, Amersham, UK) and hybridised at 65°C according to standard protocols.
Probes (Cab Lhca3.St.1 gene from chrysanthemum, obtained from Maarten Jongsma, CPRO-DLO, The Netherlands) were labelled by random priming (Ready To Go, DNA labelling Beads (-dCTP), Pharmacia Biotech). Filters were briefly washed in 2x SSPE / 0.1% SDS at room temperature, followed by 30 min washes in 1x SSPE/0.1% SDS and 0.1x SSPE/0.1% SDS at 35°C. Blots were exposed to phosphor image plates and subsequently analysed with a imager apparatus (Fuji Bas 2000 system).
Control experiments demonstrated that 10 independent RNA isolations had a standard deviation of 13.4%. *Cab* mRNA expression patterns showed highest expression between 10.00 hr and 16.00 hr when leaves were placed under normal day light conditions (data not shown). In tomato, maximum *cab*

mRNA expression was found around noon also (Piechulla and Gruissem, 1987). Samples were taken at 13.00 hr, 2 hours after the light pulse.

7.4 Results

The individual experimental parameters were determined separately at least 3 times for each experimental parameter. The data presented in this paper represent plants from the same harvest period. This enabled us to compare the different measurements that were performed. RNA isolations were taken from a pooled sample of 3 individual leaves.

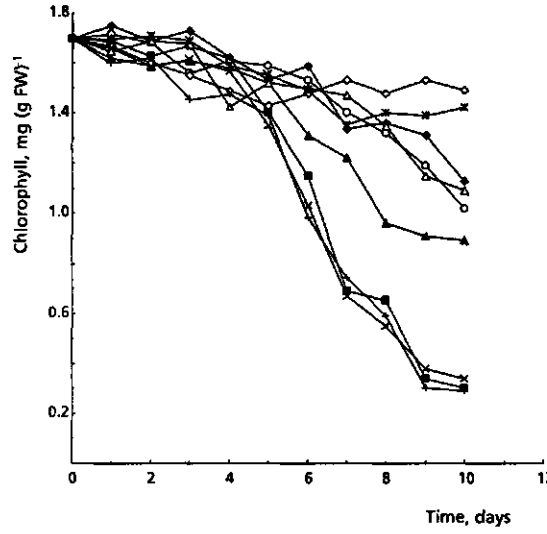

Figure 7.2 *Chlorophyll (a+b) concentration (mg (g FW)$^{-1}$) of alstroemeria leaves under different conditions: ■, continuous darkness; +, 10 min day^{-1} 5.4 µmol m^{-2} s^{-1} far-red light; X, 10 min day^{-1} 5 µmol m^{-2} s^{-1} red light immediately followed by 10 min day^{-1} 5.4 µmol m^{-2} s^{-1} far red light; O, 10 min day^{-1} 5 µmol m^{-2} s^{-1} red light; ◆, continuous darkness and incubated in 10^{-6} M GA$_4$; ▲, continuous darkness and incubated in 10^{-5} M mTR; ◊, 10 min day^{-1} 5 µmol m^{-2} s^{-1} red light and incubated in 10^{-6} M GA$_4$; Δ, 10 min day^{-1} 5 µmol m^{-2} s^{-1} red light and incubated in 10^{-5} M mTR; ✱, continuous darkness and incubated in 10^{-6} M GA$_4$ and 10^{-5} M mTR. Data are means of 5 replications. Standard deviation is < 5% of the values.*

Chlorophyll concentrations did not decrease significantly during the first 4 days in all treatments (Fig. 7.2). In darkness, the concentration of chlorophyll decreased from day 4 onwards and leaves were homogeneously yellow after 10 days, remaining about 20% of the initial concentration of chlorophyll. Far-red irradiation did not prevent chlorophyll loss and resulted in similar kinetics of chlorophyll breakdown as in leaves placed in continuous darkness. Leaves irradiated with 5 µmol m^{-2} s^{-1} red light for 10 min per day showed a delay of net chlorophyll breakdown; this treatment resulted in a retention of about 60% of the initial chlorophyll content after 10 days. A red light irradiation immediately followed by a far-red irradiation did not delay chlorophyll breakdown. Incubation of leaves in either 10^{-6} M GA_4 or 10^{-5} M *m*TR resulted in retention of 67% and 52% of the initial chlorophyll, respectively. Combination of GA_4 or *m*TR incubation with a daily red light irradiation resulted in 88% and 64%, respectively, of the initial chlorophyll, whereas application of both GA_4 and *m*TR retained 84% of the chlorophyll after 10 days. The molar ratio chlorophyll *a* : chlorophyll *b* was 2.8 and did not change during the course of the experiment in all treatments (data not shown).

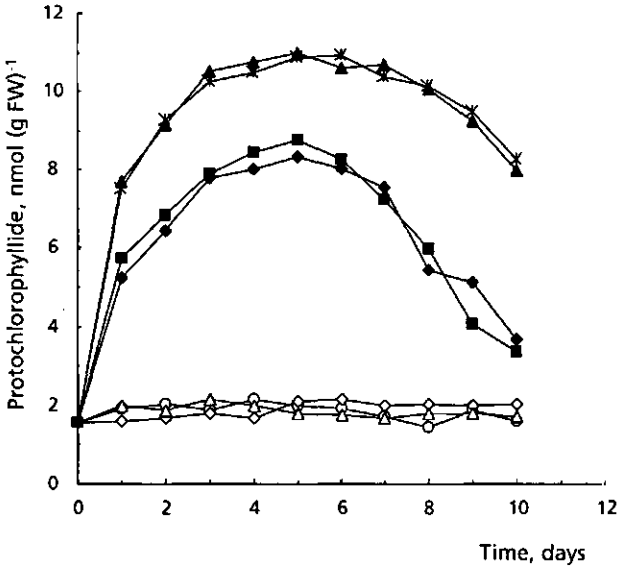

Figure 7.3 *Protochlorophyllide concentration (nmol (g FW)$^{-1}$) of alstroemeria leaves under different conditions: for explanations of symbols see Fig. 7.2. Data are means of 4 replications. Standard deviation is < 10% of the values.*

Biosynthesis / Metabolism

To study effects of red light, *m*TR and GA_4 on chlorophyll biosynthesis and catabolism, Pchlide concentrations and Mg-dechelatase activity were determined. Pchlide accumulated up to 5 days of darkness and then slowly declined (Fig. 7.3). Leaves irradiated daily with red light did not show a significant increase in total Pchlide during the course of the experiment. Pchlide accumulation during the first 5 days was enhanced by *m*TR but not by GA_4. When leaves were incubated in either *m*TR or GA_4 and also irradiated daily with red light, the accumulation of Pchlide was absent. A combined incubation in *m*TR and GA_4 resulted in a similar pattern as *m*TR incubation alone. In contrast to Pchlide, the chlorophyll precursors protoporphyrin IX or Mg-protoporphyrin IX did not accumulate in dark-incubated leaves (data not shown).

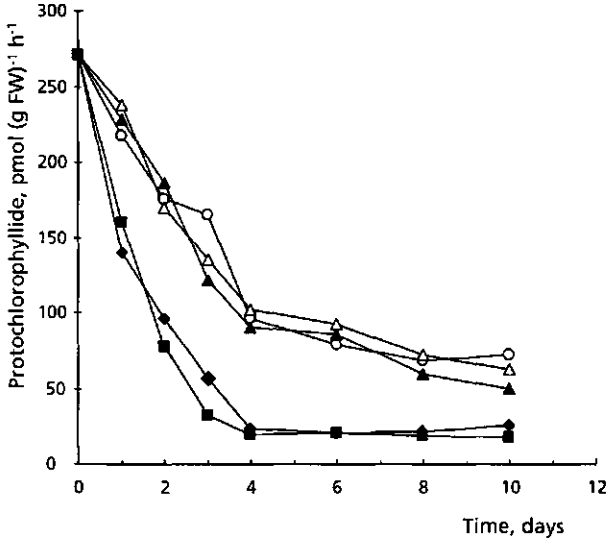

Figure 7.4 *Net synthesis of protochlorophyllide (pmol (g FW)$^{-1}$ h^{-1}) of alstroemeria leaves under different conditions: for explanations of symbols see Fig. 7.2. Data are means of 4 replications. Standard deviation is < 10% of the values.*

The absence of Pchlide accumulation in irradiated leaves could be due to either a decline in the biosynthetic reactions or due to a conversion of the formed Pchlide into Chlide. Therefore, the rate of Pchlide synthesis was

determined. The rate of net synthesis of Pchlide decreased to 60% of the initial value after 1 day of darkness (Fig. 7.4). After 4 days of darkness no Pchlide synthesis was detected anymore. Both red light and *m*TR treatments maintained an approximately two-fold higher rate of Pchlide synthesis. Neither GA_4 nor far-red light increased Pchlide synthesis, the kinetics being similar to that of the dark-treated leaves. Also the subsequent red/far-red irradiation had no effect on the Pchlide synthesis. Combined incubation in *m*TR and red light irradiation was as effective as obtained by *m*TR or red light alone whereas GA_4-incubation did not result in an additive effect to that of red irradiation (data not shown). These results are consistent with a *m*TR-induced stimulation of Pchlide synthesis in darkness.

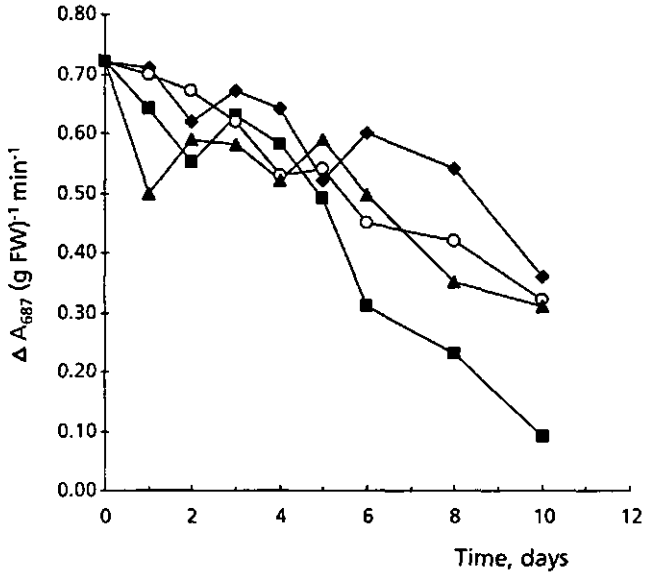

Figure 7.5 *Mg-dechelatase activity (arbitrary units) of alstroemeria leaves under different conditions: for explanations of symbols see Fig. 7.2. Data are means of 4 replications. Standard deviation is < 10% of the values.*

The assay system to measure Mg-dechelatase activity described by Vicentini *et al.* (1995) proved to be suitable for alstroemeria leaves. Addition of solubilised thylakoids to a buffer solution containing chlorophyllin as substrate resulted in a decrease of the absorption maximum of chlorophyllin at 644 nm and a progressive increase of a new peak at 687 nm. Time courses of increasing absorption at 687 nm were linear for over 20 min and the rates

of absorption change were proportional to the thylakoid-enzyme dose (data not shown).

Mg-dechelatase activity was highest in thylakoids prepared from mature fresh leaves. When leaves were placed in permanent darkness, Mg-dechelatase activity declined gradually from day 0 onwards (Fig. 7.5). The decrease in Mg-dechelatase activity was less when leaves were incubated in either GA_4 or *m*TR or irradiated with red light. A net decrease in chlorophyll content would be expected to be correlated with an increase in Mg-dechelatase activity. However, a linear relation was found between *in vitro* activity and chlorophyll concentration (r^2= 0.893) and thus, the specific activity per unit chlorophyll remained rather stable during senescence. Irradiation of the leaves with red light or incubation in either GA_4 or mTR did not result in a change of the specific activity of Mg-dechelatase.

Chlorophyll functioning in photosynthesis

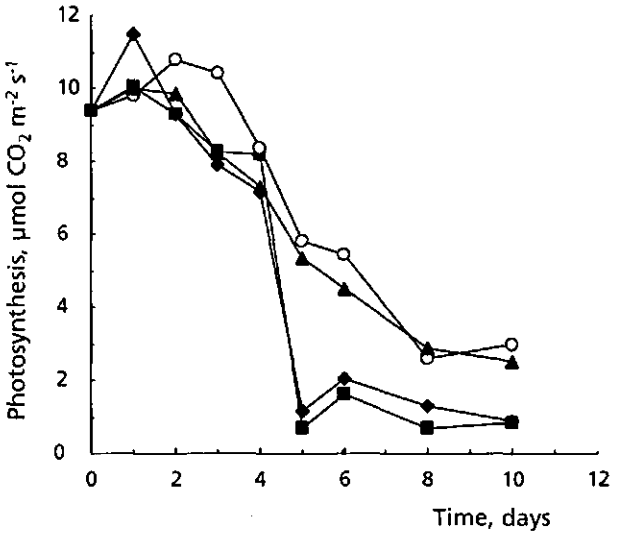

Figure 7.6 *Photosynthesis of alstroemeria leaves under different conditions: for explanations of symbols see Fig. 7.2. Data are means of 4 replications. Standard deviation is < 10% of the values.*

Net photosynthesis was approximately 9.5 µmol CO_2 m^{-2} s^{-1} in mature fresh alstroemeria leaves and decreased steadily from day 0 onwards (Fig. 7.6).

After 5 days of incubation the maximum capacity of photosynthesis had decreased dramatically in dark- and GA_4 treated leaves compared to the initial rate of photosynthesis. Both daily irradiations with small photon fluences of red light or incubation in *m*TR kept the maximum photosynthetic activity higher than in leaves placed in permanent darkness or treated with GA_4 from day 4 onwards. The specific photosynthesis per unit of chlorophyll showed a similar pattern: comparable activities of the dark- and gibberellin-treated leaves resulting in hardly any photosynthetic activity from day 5 onwards whereas chlorophyll in red light- and *m*TR-treated leaves still showed some photosynthetic activity after 10 days of treatment.

Expression of cab mRNA

Figure 7.7 shows the abundance of *cab* mRNA, encoding for light harvesting chlorophyll proteins (LHCP) in dark and red light treated leaves. The aliquots represent equal amounts of leaf fresh weight and are therefore not corrected for a possible decline in total RNA content during senescence.

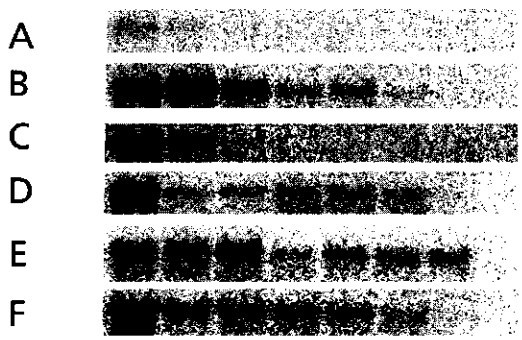

Figure 7.7 *Northern blot analysis of* cab *mRNA in alstroemeria leaves placed in darkness (A), irradiated daily with 5 µmol m^{-2} s^{-1} red light for 10 min (B), incubated in 10^{-6} M GA_4 in darkness (C), incubated in 10^{-5} M* mTR *in darkness (D), incubated in 10^{-6} M GA_4 and irradiated daily with 5 µmol m^{-2} s^{-1} red light for 10 min (E), incubated in 10^{-5} M* mTR *and irradiated daily with 5 µmol m^{-2} s^{-1} red light for 10 min (F).*

In dark-incubated leaves a drastic decrease in *cab* mRNA is observed from day 1 onwards and after 4 days no *cab* mRNA could be detected. Leaves irradiated with red light for 10 min per day and analysed 2 hours after this irradiation retained a higher expression of *cab* mRNA during the whole experimental period compared to the dark treatment. Incubation of the leaves in GA_4 had no effect on *cab* mRNA expression compared to dark-incubated leaves, nor was there an additional effect when GA_4 incubation was combined with red irradiation: *cab* mRNA levels were similar to those in red irradiated leaves. In contrast, incubation of the leaves in mTR in darkness retained more *cab* mRNA compared to dark treated leaves.

7.5 Discussion

Chlorophyll synthesis and breakdown

The biosynthetic pathway of chlorophyll consists of 13 steps of which the first and the next-to-last, the conversion of Pchlide to Chlide, are light dependent (Fig. 7.1). Finally, chlorophyll is formed by esterification with phytol.
Estimated from the synthesis of Pchlide in fresh leaves *in vitro*, 5.8 µg chlorophyll per g fresh weight is synthesised per day. This means that about 0.34% of the total chlorophyll pool in these leaves is replenished daily. To date, limited information is available about the amount of chlorophyll synthesis in mature leaves. Leaves are reported to contain 5 to 20 µg Pchlide per g fresh weight (Böddi *et al.* 1994). In mature alstroemeria leaves chlorophyll synthesis may be low, but the capacity to synthesise chlorophyll is still present.
Determination of the effect of phytochrome activation by red light on Pchlide accumulation is complicated by the phototransformation of the formed Pchlide to Chlide. Therefore, we determined both total accumulation and the capacity to synthesise Pchlide by incubation of leaves during a 10 hour period of darkness.
During senescence Pchlide increased until day 5. Since the leaves were incubated in darkness, the decline in Pchlide from day 5 onwards could not be due to its phototransformation to Chlide but must be caused by a net degradation. The capacity to synthesise Pchlide was strongly reduced during the first day in darkness and was neglectable after 4 days of incubation.

Incubation of leaves in *m*TR increased Pchlide accumulation, indicating that *m*TR stimulated Pchlide biosynthesis. However, the net degradation of Pchlide during the later stages of senescence could not be abolished by *m*TR, as the shapes of the curves were similar (Fig. 7.3). Similar observations were made by Hukmani and Tripathy (1994) in barley leaves treated with kinetin. Red light prevented accumulation of Pchlide, also when *m*TR was present, suggesting that a photon fluence of 5 μmol m^{-2} s^{-1} during 10 min is sufficient to convert Pchlide into Chlide. According to Griffiths *et al.* (1985) a trace amount of Pchlide reductase is sufficient to carry out the conversion to chlorophyll in the presence of light. Irradiation with red light and incubation in *m*TR both resulted in a higher net synthesis of Pchlide (Fig. 7.4). Thus, although no Pchlide accumulation was found when leaves were irradiated, red light did stimulate the synthesis of Pchlide. Previously, phytochrome involvement in the regulation of Pchlide reductase mRNA was reported by Apel (1981). Although GA_4 is very effective in delaying net chlorophyll decrease (Fig. 7.2), it did not cause accumulation or synthesis of Pchlide as the curves for GA_4 coincide with the dark-treated leaves (Fig. 7.3 and 7.4). Previously, we found that the effects of red light and gibberellin on chlorophyll retention were additive and independent (Chapter 4), while aromatic cytokinins and red light did not exhibit additive effects. Even more: aromatic cytokinins could function as intermediates for the transduction of the red light signal (Chapter 6). Apparently, red light and *m*TR both act on chlorophyll synthesis and the effect of GA_4-induced retention of chlorophyll is not caused by maintaining chlorophyll synthesis.

Using a variety of different methods Mg-dechelatase activity was demonstrated in presenescent as well as in senescent leaves in various species by Langmeier *et al.* (1993) and Vicentini *et al.* (1995). In mature, fresh leaves of alstroemeria Mg-dechelatase was found present but neither red light, GA_4 nor *m*TR were shown to have an effect on the enzyme activity per unit of chlorophyll. In alstroemeria, this step is probably not a point of regulation by phytochrome or hormones.

Chlorophyll functioning in photosynthesis

For photosynthetic activity it is essential that chlorophyll is complexed with chlorophyll binding proteins. To determine the performance of chlorophyll during the senescence period, photosynthetic activity and *cab* mRNA encoding for the light harvesting chlorophyll *a/b* complex apoprotein were

measured. At day 5 approximately 80% of the initial amount chlorophyll was still present in these leaves but apparently with extremely low capacity for photosynthetic activity (Fig. 7.6). Leaves incubated in GA_4 did not show a maintenance of photosynthetic activity, nor expression of *cab* mRNA, although the GA_4-treatment resulted in the highest level of chlorophyll in the leaves during the period of 10 days. Previously, Jordi *et al.*, (1994) found only marginal effects of GA_3 on photosynthetic activity and fluorescence parameters in alstroemeria leaves treated with GA_3 compared to dark-incubated leaves. We propose that a high concentration of GA in the leaves prevent a normal chlorophyll breakdown. Thus, although the leaf in fact is in a senescent phase, no breakdown of chlorophyll occurred. As disassembly of the pigment-protein complex holds the risk of photooxidative damage of free chlorophyll molecules, chlorophyll breakdown may be regarded as a process of detoxification (Matile *et al.* 1996). In GA_4-treated leaves the LHCP-chlorophyll complex is no longer intact, therefore the chlorophyll still present in the leaves is not functional anymore.

In contrast, leaves irradiated with red light or incubated in *m*TR retained a slightly but significant higher photosynthetic activity. Both *m*TR and phytochrome activation resulted in higher levels of *cab* mRNA in leaves. Flores and Tobin (1986) observed that Pfr and BA substantially increased transcription of *cab* genes independently, since the effects of the two stimuli were additive. BA appeared to affect *cab* mRNA expression post-transcriptionally, probably by increasing stability of the mRNA. In watermelon, it has been reported that BA enhanced transcription of *cab* mRNA (Longo *et al.*, 1990). Conversion of Pr to Pfr resulted in transiently higher aromatic cytokinin concentrations (Chapter 6). Our results demonstrate that other aromatic cytokinins than BA modulate *cab* mRNA abundance. Previously, we showed that *meta*-topolin cytokinins could function as intermediates for the transduction of the phytochrome signal (Chapter 6). Consistent with a role of *m*TR as an intermediate in phytochrome activation, this study shows that both phytochrome and *m*TR (partly) prevent loss of photosynthetic capacity, stimulate Pchlide biosynthesis and enhance *cab* mRNA expression.

8 General discussion

8.1 Introduction

The work presented in this thesis aims to elucidate the role of the phytohormones gibberellins and cytokinins in the phytochrome-mediated delay of leaf senescence. In this chapter, an attempt is made to integrate the results of the foregoing chapters to ascertain to what extent this aim is achieved.

The large number of similarities found between light and hormonal effects on plant growth and development raises the question whether light and hormones act independently or whether the latter play an integral role as second messengers in any sequence of events initiated by active photoreceptors. Alternatively, hormones may serve as integrators of distinct signalling pathways by 'cross-talk', thereby influencing the capacity for transmission of light-related signals. The senescence characteristics of alstroemeria leaves are affected by low photon fluences of red light as well as by GAs and CKs. To find out whether these hormones could function as intermediates for the transduction of the phytochrome signal, it is important to know which GAs and CKs are endogenous in leaves of alstroemeria and thus can be involved in senescence. Secondly, it must be cleared whether endogenous concentrations of gibberellins and cytokinins are affected by irradiation. Another possibility is that the sensitivity of the tissue for gibberellins or cytokinins is altered by irradiation. Figure 8.1 presents schematically the relations proposed between irradiation, gibberellins, cytokinins and the concentration of chlorophyll present in the leaf. In the following paragraphs the different aspects of this model will be discussed.

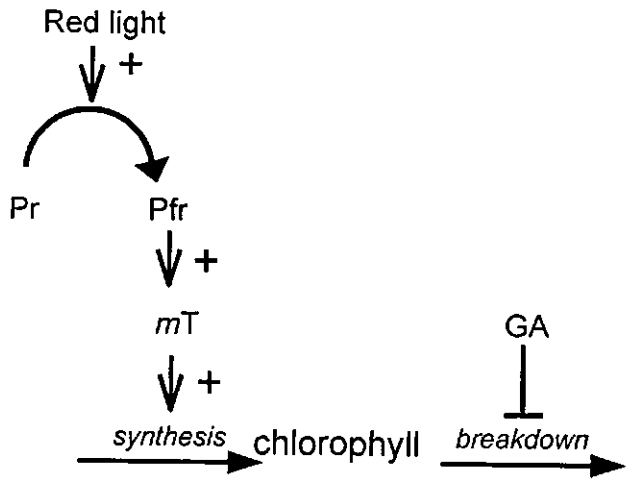

Figure 8.1 *Model for interaction of phytochrome, GA, and mTR on chlorophyll levels in alstroemeria leaves. Arrows represent sequential processes, + represent promotive effects,—| represent inhibitory action.*

8.2 Light involvement in senescence

Leaf senescence is the sequence of biochemical and physiological events comprising the final stage of development, from the mature, fully expanded state until death (Smart 1994). The changes taking place during senescence proceed in a genetically programmed sequence, with close co-ordination at the cell and tissue levels. The most obvious sign of leaf senescence is the change of colour due to preferential degradation of chlorophylls compared to carotenoids, and due to the synthesis of new compounds such as anthocyanins and phenolics (Matile 1992). This is accompanied by a disassembly of functional chloroplasts and hence the loss of the photosynthetic capacity.

Senescence is often induced by external parameters such as light, nutrition, and temperature. The influence of light can be via the photoperiod, irradiance level or the spectral distribution. Brief irradiations with red light suppressed degradation of chlorophyll in rice leaves and this effect was nullified by far-red light (Okada *et al.* 1992). In this thesis we demonstrated that in alstroemeria leaves senescence was effectively delayed by irradiations

with low photon fluence red light of about 5 μmol m^{-2} s^{-1} for 10 min daily (Chapter 2). The effect was completely abolished when far-red irradiation immediately followed the red light, indicative of a classical low fluence phytochrome–mediated response (LFR). This type of response is induced by a single pulse of red light, and shows reversibility by subsequent far-red irradiation. The photon fluence dose, defined as (photon fluence rate) x (time of irradiation), required for saturation of the response by red light is about 900 μmol m^{-2} (Chapter 2), and lies in the range from 1 to 1000 μmol m^{-2} s^{-1}, typical for LFR responses (Smith and Whitelam 1990). A single red light pulse delays the onset of senescence approximately for one day, and repeating the irradiation pulse daily delays the onset of senescence for a longer period, although every following day with less effect.

8.3 Transduction of the phytochrome signal

Three classes of photoreceptor systems are known in plants: phytochromes, blue light/UV-A receptors and UV-B receptors. The phytochrome system is sensitive to the relative irradiance of photosynthetically active red light to non-photosynthetically far-red light. This red / far-red ratio provides the plant with useful information about changes in light quality and the suitability of the light for photosynthesis. The red / far-red light absorbing phytochromes are the best-characterised photoreceptors in plants and are encoded by a multigene family. In *Arabidopsis* the PHY gene family has been described and consists of five members (Clack *et al.* 1994). In tomato, at least seven different PHY genes are present (Pratt 1995). It is assumed that present day angiosperm PHYs arose as a consequence of gene duplications and sequence divergence from a progenitor phytochrome (Pratt 1995). From work with phytochrome mutants it is suggested that there is a redundancy between the different photoreceptors (Kerckhoffs 1996). *PhyA* and *phyB* are supposed to function through different transduction chains before converging to result in the same terminal response. *PhyB* is the principal phytochrome responsible for the classical red / far-red reversible responses and for the responses of light-grown plants to the low R/FR ratio.
The cascade of events following the amplification of the perceived signal is still largely a black box (Bowler and Chua 1994, Kerckhoffs 1996). Mutants with deficiencies in phytochrome signal transduction are very helpful in elucidation of the pathway leading from light perception to photomorphogenesis. Using single-cell micro-injection techniques, chloroplast

development, anthocyanin accumulation and expression of a photoregulated CAB-β-glucuronidase (GUS) reporter gene were monitored in hypocotyl cells of the tomato *aurea* mutant after injection of purified oat phyA in combination with inhibitors, activators and putative signalling compounds (Neuhaus *et al.* 1993, Bowler *et al.* 1994). This has led to a model, in which PHYA leads to activation of G-proteins and this subsequently activates three pathways: a cGMP pathway, leading to anthocyanin accumulation; a Ca^{2+} dependent pathway acting via calmodulin, resulting in the biogenesis of chloroplasts and a combined Ca^{2+}-calmodulin/cGMP pathway, leading to fully mature chloroplasts (Neuhaus *et al.* 1993, Bowler *et al.* 1994). It seems likely that signalling compounds like Ca^{2+}, cGMP and G-proteins will be involved in numerous pathways that are triggered by phytochrome activation. To date, most studies are limited to early developmental processes such as the hypocotyl system. Whether processes later in the development of the plant are also regulated in a similar way remains largely to be elucidated. Although the alstroemeria model system proved very useful in elucidating light-hormone interactions in senescence, more basal knowledge about amplification and transduction reactions requires a model system in which mutants are relatively easy to obtain. Very recently, regeneration and transformation of alstroemeria has been described by Van Schaik (1998).

8.4 Methods to determine endogenous gibberellins and cytokinins

To determine the effects of environmental stimuli on plant hormones, the changes in their endogenous concentrations must be determined. To date, methods of determination are based on quantification in extracts of plant organs. This implies that an overall concentration of a tissue is determined and not the concentration in a specific cell organelle or near the receptor. In general, the change in concentration must, therefore, be quite large to lead to detectable differences. From the data presented in Chapter 4 it seems likely that red light does not induce changes in GA concentrations directly. However, it can not be excluded that either small changes in concentration occurred or that a shift of GA molecules from one cell compartment to another took place. Recently, new analytical techniques were designed which combined gas chromatography with a double mass spectrometry (GC-MS-MS) (Moritz and Olsen 1995). This technique enables accurate measurements of GAs in small tissue samples (approximately 10 mg fresh weight). With this technique, more precise statements can be made about

local shifts in GA concentrations along, *e.g.* a stem gradient or within a leaf. However, to be able to determine effects of (external) stimuli on plant hormone compartmentalisation within a cell, other, highly sensitive techniques using, *e.g.* immuno labelling should be further developed for plant growth regulators.

8.5 Seasonal effects

In general, not much information is available about GA and CK concentrations throughout the development of the plant and seasonal variation therein. The characteristics of leaf senescence were very similar throughout the year. In contrast, the data presented in Chapter 3 show that the absolute GA concentrations in leaves of comparable developmental stages differ substantially. Variation in endogenous concentration was also demonstrated for CKs in alstroemeria leaves of comparable developmental stage (unpublished results). This implies that no conclusions should be made based on single measurements of absolute phytohormone concentrations in time. Secondly, variation in the absolute phytohormone concentration throughout the year suggests strongly that it is not the absolute concentration of a phytohormone that determines whether or not a process is triggered. It is tempting to assume that the threshold value for the target process also varies during the season. The observation that the ratios of precursor, active and inactivated GAs remained more or less the same, independent of the season (Chapter 3), corresponds with such a variable threshold concentration. Furthermore, substantial variation was found within different experiments in the concentrations of applied GA and CK needed to obtain half-maximum responses $[GA]_{50}$ or $[CK]_{50}$. The rate of senescence of alstroemeria leaves is therefore probably independent of the absolute endogenous GA and CK concentration in the leaves.

8.6 Endogenous gibberellins and cytokinins

The GAs endogenous to alstroemeria were characterised and 11 GAs and 2 GA-conjugates were identified (Chapter 3). Based on conversions demonstrated in other plant species (Sponsel 1995) and feeding experiments with deuterated GAs (Chapter 5), it is suggested that two metabolic GA

routes operate in alstroemeria leaves: the 13-hydroxylated pathway and the non-hydroxylated GA pathway yielding GA_1 and GA_4, respectively, as physiologically active GAs.
Next to isoprenoid CKs, aromatic CKs were detected in alstroemeria leaves (Chapter 6). Isoprenoid CK biosynthesis is thought to start with synthesis of iP type CKs, which are subsequently converted to Z and DHZ type CKs. All forms of these CKs were found present in alstroemeria leaves. The presence of iP type CKs in alstroemeria leaves may indicate CK synthesis in these leaves. As, in general, levels of CK free bases and ribosides decline in senescing leaves (Badenoch-Jones *et al.* 1987, Singh *et al.* 1992a), CK synthesis in alstroemeria leaves may have a role in suppressing their leaf senescence, as was suggested previously for tobacco (Singh *et al.* 1992b).
To date, information about the biosynthetic origin and pathway of aromatic CKs is very limited. Based on the structure of isoprenoid and aromatic CKs it is very likely that the pathways diverge at an early point in the biosynthesis. Strnad (1997) stated in his review about aromatic CKs that these must be widespread in plant. However, to date, only dicots were reported to contain aromatic CKs, because to our knowledge, no attention was given to the possible occurrence of aromatic CKs in monocot species. The presence of aromatic CKs in the monocot alstroemeria indicates that, next to isoprenoid CKs, aromatic CKs are indeed endogenous to both mono- and dicots.

8.7 Interaction between phytochrome and gibberellins and cytokinins

The levels of the free GAs and CKs were measured during senescence under conditions of continuous darkness and daily red irradiation (Chapter 4 and 6). To correlate an increase or decrease in hormone level during senescence with the phytochrome induced delay of chlorophyll breakdown, a change should be limited to either dark-incubated or red light irradiated leaves. The results showed that the levels of some GAs indeed changed during the experimental period. However, differences found in GA concentration between dark incubated and red irradiated leaves probably reflect the difference between a physiologically old (senescing) leaf and a younger one, rather than a phytochrome mediated change in GA level. This is supported by the absence of direct effects of red light on specific steps of GA-biosynthesis (Chapter 4). Therefore, phytochrome-controlled regulation of senescence seems not to require direct effects on GA biosynthesis. In

contrast, GA biosynthesis has been indicated to be under phytochrome control in pea (Campell and Bonner 1986), cowpea (Martínez-García and García-Martínez 1992), *Arabidopsis* (Xu *et al.* 1995) and the Sorghum ma_3^R mutant (Foster and Morgan 1995).
About 60 to 70 minutes after exposure to red light, alstroemeria leaves showed a transient increase in two aromatic CKs (*m*T and *m*TR), while the other CKs did not change in concentration (Chapter 6). This increase could be partially prevented if red light was followed by a far-red treatment. Phytochrome activation leads to a transient increase in *m*T and mTR (Fig. 8.1). The time scale from phytochrome activation to the start of the increase in *m*T(R) is about 40 min. It can be assumed that in these 40 min primary signal transduction reactions took place, like the activation of genes and G-proteins and, as a secondary response resulting in the increase in *m*TR. Several studies indicated that transient increases in CKs in seeds are due to interconversion of CKs from storage forms to biologically active forms rather than to *de novo* synthesis (Thomas *et al.* 1997).

A second possibility for the involvement of plant hormones in the transduction of the phytochrome signal is a change in sensitivity of the tissue for these compounds. Although the total response to exogenously applied GA_4 increased when leaves were irradiated with red light, the parameters of the dose response curves did not change significantly (Chapter 4) and it was concluded that the sensitivity of the leaves to GAs was not altered. Based on these results it is concluded that GAs will not function as intermediates in the phytochrome-induced delay of senescence in alstroemeria leaves.
The best described physiological processes involving regulation by light and GAs are the induction of seed germination and the control of cell elongation. Several studies have examined the relationships between light and GAs in these processes, and provided somewhat contrasting arguments for an involvement of GAs in the light-signalling pathway. In some plants, the data suggest that light may affect the concentrations of GAs, perhaps by regulating their synthesis, while in other plants, it seems clear that phytochrome acts by decreasing the responsiveness to GAs. The *phyB*-deficient ma_3^R mutant of sorghum (Foster *et al.*, 1994) and the *Brassica ein* mutant (Rood *et al.* 1990) have elevated GA levels, suggesting that phyB controls GA biosynthesis in these species. However, the ein mutant has elevated GA levels only under some physiological conditions and the ma_3^R mutant was shown to be phase-shifted in a diurnal rhythm regulating GA accumulation (Foster and Morgan 1995). These results suggest that the effects of light on GA metabolism are far from simple. The opposite effects

on GA synthesis has been observed in transgenic tobacco overexpressing *PHYA* (Jordan *et al.* 1995). These plants have a dwarfed phenotype and lower levels of a number of GAs, suggesting that phytochrome can inhibit GA biosynthesis. In *Arabidopsis*, the level of GA20-oxidase mRNA increased in long-day photoperiods (Xu *et al.* 1995).
In other species, phytochrome affect the responsiveness to GAs. The *phyB* mutants of cucumber (*lh* mutant) and pea (*lv* mutant) have GAs levels comparable to the wild type, but show enhanced elongation responses to exogenous GAs (Weller *et al.* 1994, López-Juez *et al.* 1995). In fuchsia, differences in stem length caused by differential day and night temperature and variation in the blue light : PAR ratio were not accompanied by changes in GA sensitivity (Maas and Van Hattum, 1997). No significant difference in endogenous GA concentration was found between wild type *Arabidopsis* and the *phyB* mutant (Reed *et al.* 1996). These results indicate that the phyB mutation causes an increase in responsiveness to GAs in these species, while concentrations of endogenous GAs were not affected. However, one unifying principle for the interaction of phytochrome and GAs is not yet emerged and will need more precise measurements of GA levels and the identification of a GA receptor (Chory and Li 1997).

The maximum effect of the aromatic *m*T(R) in terms of amplitude (R_{max}-R_{min}) was significantly higher than that of the isoprenoid Z(R). In contrast, the needed for the half-maximum effect for retention of chlorophyll were about similar. Red light irradiation of these leaves incubated in Z(R) or *m*T(R) did not alter the $[CK]_{50}$ values of the dose response curves (data not shown). Thus, red light probably also did not affect the sensitivity of the leaf towards either Z or *m*T. Similarities between phytochrome and cytokinin effects have been described for dormancy release of light sensitive seeds, cotyledon expansion, leaf development and chloroplast differentiation. Although these similarities in effects led to the suggestion that red light and CKs might operate via similar biological mechanisms, other observations indicated that CKs and the light signal act independently. For example, CK and light-responses were found to be independent and additive in the inhibition of hypocotyl elongation in *Arabidopsis* (Su and Howell 1995). In alstroemeria, is it likely that the aromatic cytokinin *m*T(R) functions as an intermediate in the transduction of light signals.

8.8 Differential effects on chlorophyll metabolism

The results obtained in this study reveal that threshold concentrations of the phytohormones GA_4 and *m*T(R) could be control specific steps in the regulation of senescence in alstroemeria leaves. In addition, phytochrome activation, can be an important regulation parameter. Although the visible effects of these three parameters are similar, namely a net retention of chlorophyll, it is possible that chlorophyll metabolism and chlorophyll functioning in photosynthesis are differentially affected by GAs, CKs and phytochrome.
The regulation of chlorophyll biosynthesis and breakdown is a very complex process and occurs at the level of transcription and translation of chloroplast and nuclear genes regulated by phytochrome-mediated and hormonal responses. Several compounds in the chlorophyll biosynthetic pathway, Mg-protophyrin IX, protochlorophyllide, and chlorophyllide have feedback effects on the early precursors steps, such as the formation of aminolevulinic acid. Mg chelation is also an important site of metabolic regulation as it is the first step in the Mg-porphyrin pathway and dependent on ATP. Other regulatory mechanisms from protochlorophyllide onwards will be probably very complex because the formed chlorophyll molecules must be inserted at specific sites in the membrane, in association with proteins and lipids. To date, these processes are beginning to be unravelled but very little is known about their regulation.

In mature alstroemeria leaves, the biosynthetic reactions leading to the foprmation of chlorophyll are still present but they decline strongly when leaves are placed in darkness. The capacity to synthesise protochlorophyllide, remained longer present when leaves received a daily red light pulse or were incubated in *m*TR (Chapter 7). Therefore, both *m*TR and phytochrome stimulate this pathway. We hypothesise that phytochrome effects on chlorophyll synthesis are mediated by CKs. In alstroemeria, red light caused a transient increase in *m*T(R) concentration in the leaves (Chapter 6), suggesting that the latter can act as intermediate transduction of the phytochrome signal (Fig. 8.1). This hypothesis is supported by the observation that both mT(R) and red light were found to increase the transcription of *cab* mRNA encoding for the light harvesting chlorophyll a/b binding proteins of photosystem II (Chapter 7). In addition, both maintained the photosynthetic capacity of alstroemeria leaves for about 6 days while the rate of photosynthesis declined to almost zero in leaves placed in darkness or treated with GA_4. In other plant species, BA increased the accumulation of

proteins encoded by light regulated genes involved in photosynthesis, such as *cab* and *rbcs* (encoding for the small subunit of RuBisCo) (Tessendier de la Serve *et al.* 1985, Ohya and Suzuki 1991). Chory *et al.* (1994) demonstrated that low concentrations of CK caused de-etiolation of dark-grown *Arabidopsis* wild-type seedlings in a way similar to that observed in the *det1* and *det2* mutants. This implies that an increase in CK is sufficient to override a light requirement for leaf and chloroplast development. Flores and Tobin (1986) proposed that phytochrome and BA independently alter the pool size of a common intermediate, which then more directly regulates gene expression.

The breakdown of chlorophyll into phytol, Mg^{2+} and cleavage products of the porphyrin moiety occurs in three steps, catalysed by chlorophyllase, Mg-dechelatase and pheophorbide *a* oxygenase. Chlorophyllase and Mg-dechelatase activity was demonstrated in pre-senescent as well as in senescent leaves (Matile 1996). In mature leaves of alstroemeria Mg-dechelatase was found present but neither red light, GA_4 nor mTR were shown to have an effect on the enzyme activity. The third enzymatic step in chlorophyll catabolism, pheophorbide *a* oxygenase, was studied by Hörtensteiner *et al.* (1995), and they reported that the enzyme was only detectable in senescent leaves. This step, therefore, may represent a key enzyme in chlorophyll breakdown and thus subject for regulation. However, control of the pathway at such a late step seems strange and it is likely that a senescence-specific regulation point is located earlier in the breakdown pathway (Matile 1996). Recently, Matile *et al.* (1997) reported for barley and oilseed rape leaves that chlorophyllase is located in the inner envelope membrane of the chloroplast. This indicates that breakdown of chlorophyll is likely to require an additional machanism that mediates between the chlorophyll, present in the thylakoids and the catabolic enzymes, that are at least partly located in the inner envelope membrane.

Although a GA_4 treatment resulted in the highest retention of chlorophyll in the leaves during the senescence period of 10 days, it did not maintain chlorophyll synthesis or performance. A high concentration of GA_4 in the leaves prevents a normal chlorophyll breakdown. Thus, although the leaf in fact is in a senescent phase, no breakdown of chlorophyll occurs. Free chlorophyll molecules holds the risk of photooxidative damage and chlorophyll breakdown may therefore be regarded as a process of detoxification of the pigment-protein complex (Matile 1996). GA_4 is not capable to keep the LHCP-chlorophyll complex functional, and the

chlorophyll still present in the leaves is not functional anymore. The effect of GA_4 on delay of senescence is probably restricted to chlorophyll breakdown processes as no effect of GA_4 on chlorophyll biosynthetic processes and photosynthetic performance was found. Furthermore, the results of this study show clearly that GA_4 does not act as an intermediate in transduction of the phytochrome signal in regulation of senescence (Fig. 8.1).

8.9 Implications for further research and genetic engineering

The complex interplay of light with hormone metabolism and response pathways is an area for which there are still more questions than clear answers.

The alstroemeria leaf system has been proven to be very suitable for the type of studies described in this thesis. It appeared that both GAs (indirectly, long term) and CKs (directly, minutes) levels respond to phytochrome activation. Thus, genes encoding for the enzymes involved in the metabolism or perception of these phytohormones will have specific expression patterns during senescence. Detection of these genes and determination of their expression levels during senescence will give opportunities to enlarge the knowledge about the process of senescence and the possibilities to manipulate it. However, it is very important to estimate the consequences of genetic engineering in advance. For alstroemeria it seems less logic to manipulate genes involved in GA metabolism, as there seems to be no effect on the performance of the leaves, while genes involved in aromatic CK metabolism seems more promising in this view. However, for cosmetic effects (green leaves, no photosynthesis required) the use of GA-related genes is also possible.

To date, studies to obtain fundamental insights in phytochrome signal transduction focussed on a few species, such as *Arabidopsis*, tomato and tobacco. In these plants, stem elongation or other processes that occur in young developing plants act as model system. To extrapolate the obtained results to plant development in general, it should be tested whether similar transduction pathways also operate in later developmental processes. The other way around, it would be interesting to study whether the observed effects of phytochrome activation on mT(R) concentrations in leaves, also occur in other plant developmental systems and thus, if mT(R) can be considered as a more general intermediate for phytochrome-mediated responses.

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Summary

Light controls almost every step in the plant life cycle. Although the structure and function of a number of photoreceptors are well characterised, the sequence of events occurring after light perception are largely unknown. Phytohormones have been proposed to be involved in these transductions because they have many overlapping aspects with light in regulation of plant development. One of the developmental processes influenced by light as well as phytohormones is senescence. In this thesis the putative role of the phytohormones cytokinins and gibberellins as mediators for the light signal on leaf senescence was studied.
Therefore, cytokinin and gibberellin levels were analysed during the period of senescence under different light conditions, and the effect and metabolism of applied plant growth regulators on chlorophyll metabolism and performance was studied.

Leaves of two alstroemeria cultivars were used as a model system. The senescence characteristics of these leaves were determined under various conditions (Chapter 2). The effects of irradiation with red light were studied and it was found that low photon fluence rates were sufficient to ensure maximal delay of chlorophyll and protein breakdown. The effect of red light could be completely counteracted by a subsequent far red irradiation and it was concluded that the photoreceptor involved was phytochrome.
An inventory was made of gibberellins, in young, mature and senescent leaves (Chapter 3). By combined gas chromatography-mass spectrometry (GC-MS) and GC-selected ion monitoring (SIM), GA_1, GA_4, GA_8, GA_9, GA_{15}, GA_{19}, GA_{20}, GA_{24}, GA_{29}, GA_{34} and GA_{44} were detected. Among these gibberellins, GA_1 and GA_4, were considered as active gibberellins, the others being precursors (GA_9, GA_{15}, GA_{19}, GA_{20}, GA_{24} and GA_{44}) or inactivation products (GA_8, GA_{29} and GA_{34}). During senescence the relative concentration of precursors and active gibberellins decreased whereas the relative concentration of inactivated gibberellins strongly increased. Irradiation of the leaves with red light

resulted in delayed senescence and a higher GA_4 concentration in senescing leaves compared to dark-incubated leaves, while that of other gibberellins did not show differences that could be correlated with the senescence pattern (Chapter 4).

The biological activity of a number of GAs and CKs were was tested using a model system of detached leaves (Chapter 4 and 6). The ranking order in efficacy of the concentration needed for the half maximum response was for GAs: $GA_4 > GA_9 > GA_1 > GA_{20} >> GA_{24} = GA_8$ = no effect and for CKs:, *m*TR > ZR ≈ mT > Z > DHZ > BAP ≈ iPR >> *o*T = no effect. However, the efficacy of GA_4 was over two orders of magnitude higher than of the most efficient cytokinin, *m*TR.
The observation that both GA_1 and GA_4 have biological activity in delaying senescence of alstroemeria leaves and both gibberellins are endogenous in these leaves led to the question whether GA_4 has biological activity itself or functions as a precursor for GA_1. Incubation of leaves in deuterated and tritiated GA_1, GA_4, and GA_9 and subsequent analysis of the labelled gibberellins formed during the incubation period, demonstrated that both gibberellins were metabolised but no GA_1 was formed after incubation in either GA_4 or GA_9 (Chapter 5), demonstrating that GA_4 itself has biological activity in alstroemeria.

The endogenous cytokinins analysed in alstroemeria leaves belonged to two major classes, namely isoprenoid derived and aromatic cytokinins (Chapter 6). Alstroemeria leaves were found to contain cytokinins of all subgroups: dihydrozeatin-, isopentenyl-, and zeatin type cytokinins (isoprenoid derived cytokinins) and benzyladenine-, *meta*-topolin-, and *ortho*-topolin type cytokinins (aromatic cytokinins).
Irradiation of leaves with red light resulted in a transient increase in *meta*-topolin and *meta*-topolin riboside, the active forms of this group of cytokinins. Approximately one hour after the onset of the light, *m*T and *m*TR increased 5 to 6 fold the initial concentration. No light related changes in concentration were found for other cytokinins in these leaves. From the results, it is suggested that aromatic cytokinins are primarily involved in regulation of leaf senescence and can function as a mediator for the transduction of the phytochrome signal.

Although the visible effect of red light, gibberellins and cytokinins are similar, *i.e.* a net retention of chlorophyll and thus green leaves, their mode of action is different. In Chapter 7, an attempt is made to distinguish

between the effects of the different regulators. It was found that both red light and *m*TR had a positive effect on chlorophyll biosynthetic reactions as well as on the rate of photosynthesis and expression of genes encoding for chlorophyll binding proteins. GA_4 did not affect these parameters. The removal of Mg from the chlorophyll molecule, an important step in chlorophyll catabolism, was not affected by either red light, *m*TR or GA_4.
In conclusion, two major findings were the results of this study. Firstly, *meta*-topolin might be considered as a transmitter of the phytochrome signal towards delay of leaf senescence whereas the concentration of gibberellins did not show a causal relation with the red light treatment. Secondly, although GA_4 very effectively kept the leaves green, the remaining chlorophyll had no functional capacity anymore. Thus, to improve plant quality by genetic modification, the use of *m*TR-related genes seems preferable.

Samenvatting

Vrijwel elke stap in de levenscyclus van een plant wordt beïnvloed door licht. Alhoewel van een aantal lichtreceptoren structuur en functie zijn beschreven, is van de gebeurtenissen die optreden nadat het lichtsignaal is ontvangen nog weinig bekend. Plantenhormonen hebben vaak vergelijkbare effecten op de groei en ontwikkeling van planten als licht en worden daardoor beschouwd als mogelijke intermediairen, dat zijn stoffen betrokken bij de overdracht van het lichtsignaal tussen lichtreceptor en ontwikkelingsproces. Eén van de ontwikkelingsprocessen in de plant die kan worden beïnvloed door licht is bladveroudering. In dit proefschrift wordt de mogelijke rol van de planten-hormonen gibberellinen en cytokininen in de regulatie van bladveroudering door licht onderzocht.
Hiertoe werden in de eerste plaats de concentraties aan gibberellinen en cytokininen bepaald in alstroemeria bladeren die gedurende de verouderings-periode aan verschillende lichtcondities waren blootgesteld. Daarnaast werden gibberellinen en cytokininen toegediend aan de bladeren en werd bestudeerd wat er vervolgens in de bladeren met deze hormonen gebeurde en wat de effecten waren op het chlorofyl metabolisme en het functioneren van het chlorofyl.

Voor het onderzoek werden de bladeren van twee alstroemeria cultivars gebruikt. De verouderingskarakteristieken van deze bladeren werden bepaald bij verschillende condities (Hoofdstuk 2). Het bleek dat een korte dagelijkse belichting van de bladeren met een lage intensiteit rood licht een maximale vertraging van de afbraak van chlorofyl en eiwit bewerkstelligde. Dit effect werd echter volledig teniet gedaan indien direct na de rood belichting een verrood belichting werd gegeven. Deze activatie door rood licht en inactivatie door verrood licht is kenmerkend voor door de fotoreceptor fytochroom gereguleerde ontwikkelingsprocessen.
Met behulp van de gecombineerde technieken gas chromatografie en massa spectrometrie (GC-MS) werden de van nature in alstroemeria bladeren

voorkomende gibberellinen geïdentificeerd (Hoofdstuk 3). Van deze gibberellinen worden GA_1 en GA_4 beschouwd als biologisch actieve gibberellinen, terwijl de overigen als precursors (GA_9, GA_{15}, GA_{19}, GA_{20}, GA_{24} en GA_{44}) of inactieve gibberellinen (GA_8, GA_{29} en GA_{34}) fungeren. De concentraties van de verschillende gibberellinen verschilden in jonge, volgroeide en verouderde bladeren: de relatieve concentraties aan precursor en actieve gibberellinen namen af gedurende de veroudering, terwijl die van geïnactiveerde gibberellinen sterk toenamen. Rood licht vertraagde de bladveroudering (chlorofylafbraak) in sterke mate en leidde tot een hogere GA_4-concentratie in de bladeren dan in bladeren die permanent in het donker waren geplaatst. Tussen het verloop in concentratie van andere gibberellinen en het verloop van de bladveroudering werd geen verband aangetoond (Hoofdstuk 4).

Met behulp van een modelsysteem van afgesneden bladeren werd van een aantal gibberellinen en cytokininen de biologische activiteit bepaald (Hoofdstuk 4 en 6). De mate van effectiviteit, gedefinïeerd als de concentratie nodig voor bereiken van het half-maximale effect, was voor GAs: $GA_4 > GA_9 > GA_1 > GA_{20} >> GA_{24} = GA_8$ = geen effect, en voor CKs: *m*TR > ZR ≈ *m*T > Z > DHZ > BAP ≈ iPR >> *o*T = geen effect. Om een vergelijkbaar effect te krijgen was de benodigde concentratie GA_4 echter meer dan twee grootte-ordes lager dan de concentratie van het effectiefste cytokinine, *m*TR. De waarneming dat zowel GA_1 als GA_4 voorkomen in alstroemeria bladeren en de bladveroudering vertragen, leidde tot de vraag of deze GAs beide biologisch actief zijn of dat GA_4 eerst omgezet moet worden in GA_1. Hiertoe werden in experimenten gedeutereerde en getritieerde GA_1, GA_4 en GA_9 toegediend aan bladeren en na verschillende tijdsperioden de gevormde producten geanalyseerd. Zowel GA_1 als GA_4 werden in de bladeren gemetaboliseerd maar na toediening van GA_9 of GA_4 werd geen GA_1 gevormd. Deze resulaten maken het zeer aannemelijk dat GA_4 eigen biologische activiteit bezit ten aanzien van het vertragen van bladveroudering in alstroemeria (Hoofdstuk 5).

De cytokininen die in alstroemeria voorkomen behoren tot twee verschillende typen: de isoprenoid afgeleide cytokininen en de aromatische cytokininen (Hoofdstuk 6). Cytokininen uit alle tot nu toe bekende subgroepen werden aangetoond in alstroemeria bladeren: dihydrozeatine, isopentenyl en zeatine (isoprenoid afgeleide cytokininen) en benzyladenine, *meta*-topoline en *ortho*-topoline (aromatische cytokininen).

Blootstellen van alstroemeria bladeren aan rood licht resulteerde in een tijdelijke toename van *m*T en *m*TR, de veronderstelde biologisch actieve vormen van deze aromatische cytokininen. Ongeveer een uur na aanvang van de belichting was de concentratie van deze cytokininen in de bladeren toegenomen tot *circa* 5-6 keer de initiële concentratie. Van de concentraties van de overige, in alstroemeria voorkomende, cytokininen kon geen verband worden gelegd met de rood belichting. Uit de verkregen resultaten werd geconcludeerd dat aromatische cytokininen primair betrokken zijn bij regulatie van bladveroudering en dat deze fungeren als intermediair in de transductie van het lichtsignaal.

Alhoewel het toedienen van rood licht, gibberellinen en cytokininen leidde tot hetzelfde effect, namelijk het vertragen van de bladveroudering, kan de wijze waarop zij dit bewerkstelligen verschillen. Het effect van deze bladveroudering vertragende factoren op een aantal verschillende processen in de chlorofyl-synthese, -afbraak en het functioneren van chlorofyl werd gemeten teneinde vast te stellen of alle behandelingen op eenzelfde of verschillende wijze beinvloeden (Hoofdstuk 7). Zowel rood licht als *m*TR hadden positieve effecten op chlorofylsynthese processen, fotosynthese en de expressie van genen betrokken bij chlorofyl binding eiwitten. GA_4 had geen effect op deze processen. Rood licht, noch *m*TR of GA_4 waren van invloed op een belangrijke stap in de afbraak van chlorofyl; het verwijderen van magnesium uit het chlorofyl molecuul.
Op basis van de in dit proefschrift verkregen resultaten wordt geconcludeerd dat *m*T(R) fungeert als een intermediair in de overdracht van het via fytochroom waargenomen lichtsignaal naar de processen betrokken bij de regulatie van de bladveroudering. Daarnaast wordt geconcludeerd dat GA_4 zeer effectief bladeren groen houdt maar dat het aanwezige chlorofyl niet functioneel meer is.
Tenslotte worden een aantal implicaties van dit onderzoek voor toekomstig onderzoek besproken. De belangrijkste hiervan is dat onze resultaten laten zien dat het meer zinvol lijkt om *meta*-topoline gerelateerde genen te gebruiken voor genetische manipulatie om plantkwaliteit te beïnvloeden dan GA gerelateerde genen.

Account

Parts of this thesis have been or will be published elsewhere:

Chapter 3
Kappers IF, Jordi W, Maas FM and Van der Plas LHW, 1997
Gibberellins in leaves of *Alstroemeria hybrida*: identification and quantification in relation to leaf age. Journal of Plant Growth Regulation 16: 219-225.

Chapter 4
Kappers IF, Jordi W, Maas FM, Stoopen GM and Van der Plas LHW, 1998.
The effects of gibberellin and red light on senescence of alstroemeria leaves are independent. Physiologia Plantarum: 103: 91-98.

Chapter 5
Kappers IF, Tsesmetzis N, Jordi W, Maas FM and Van der Plas LHW, 1998.
GA_4 does not require conversion into GA_1 to delay senescence of *Alstroemeria hybrida* leaves. Journal of Plant Growth Regulation: 17: 89-93.

Chapter 6
Kappers IF, Strnad M, Jordi W, Maas FM and Van der Plas LHW, 1998.
Meta-topolin cytokinins are involved in red light signalling. Submitted.

Chapter 7
Kappers IF, Stoopen GM, Jordi W, Maas FM and Van der Plas LHW, 1998.
Differential effects of cytokinin, gibberellin and phytochrome on chlorophyll metabolism and functioning. Submitted.

Kappers IF, Stoopen GM, Jordi W and Maas FM, 1996.
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Kappers IF, Stoopen GM, Jordi W and Maas FM, 1997.
Leaf senescence of *Alstroemeria hybrida*, Regulation by gibberellins, cytokinins and red light. Acta Botanica Neerlandica 46: 426.

Curriculum vitae

Iris Kappers werd geboren op 30 april 1966 in Ede. Na het doorlopen van de middelbare school begon zij in september 1984 met de studie tot botanisch analist aan de Hogere Landbouwschool in Wageningen. Het diploma werd in juni 1998 behaald. In september van dat jaar vervolgde zij haar opleiding met de studie Plantenveredeling aan de Landbouwuniversiteit in Wageningen. Het doctoraalexamen met als specialisatie Plantenfysiologie, werd in januari 1992 behaald. In datzelfde jaar begon zij als gastmedewerker bij de afdeling Gewasfysiologie van het Centrum voor Agrobiologisch Onderzoek in Wageningen (CABO-DLO, later Instituut voor Agrobiologisch en Bodem-vruchtbaarheidsonderzoek, AB-DLO). Het onderzoek betrof het project 'Regulatie van de etherische olie opbrengst bij karwij met behulp van *in-vitro* technieken'. Vervolgens werd zij in december 1993 aangesteld als onderzoeker in opleiding (OiO) voor de toenmalige Stichting Levenswetenschappen (SLW, nu Aard- en Levenswetenschappen, gefinancierd door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) en de Directie Wetenschap en Kennisoverdracht van het ministerie voor Landbouw, Natuurbeheer en Visserij, op de afdeling Plantenfysiologie van het AB-DLO binnen de Onderzoekschool Experimental Plant Sciences (EPS) van de Landbouwuniversiteit Wageningen. De aanstelling betrof het project 'Regulatie van bladveroudering van alstroemeria door fytochroom en gibberellinen'. Het onderzoek heeft geleid tot dit proefschrift. Tijdens het promotieonderzoek werd een korte werkperiode doorgebracht als gastmedewerker bij de Faculty of Natural Sciences van de Palacký University in Olomouc, Tsjechië. Zij heeft een beurs gekregen van DLO om vanaf oktober 1998 onderzoek te starten in samenwerking met de groep in Olomouc.

Nawoord

Wetenschap is leuk, of beter gezegd het doen van onderzoek is fantastisch. Ik denk dat een hoop collega's dat met me eens zijn maar om nu uit te leggen wat er dan zo fantastisch aan is, is nog niet zo makkelijk. Na deze promotieperiode bedenk ik dat er de afgelopen jaren vooral veel is gecommuniceerd: Gepraat en gediscusieerd over hoe het nu precies geregeld is in die planten. Heel vaak heb ik gedacht en gezegd dat ik er graag binnen in zou willen kruipen om met eigen ogen te kunnen zien hoe een lichtsignaal wordt opgevangen of een gibberellinemolecuul wordt gemaakt, maar we moeten het doen met praten en denken over mógelijke mechanismen, bedenken van de meest geëigende proeven en dan met de resultaten in de hand weer verder praten over waarom en hoe het allemaal toch weer net iets anders zou kunnen zijn. Wetenschap is communiceren; communiceren doe je met je hart; het doen van onderzoek is fantastisch.
Veel is er gepraat: in het lab tijdens experimenten, op de gang, tijdens congressen en de borrels en de laatste maanden tussen het schrijven van het proefschrift door. Het proefschrift is de weergave van zo'n vier jaar praten, denken en experimenteren en daar hebben een aantal mensen hun steen aan bijgedragen. Dit nawoord is dan ook voornamelijk voor hen bedoeld.
In de eerste plaats het begeleidende team. Met veel plezier zal ik terugdenken aan de besprekingen met ons vieren en al die tussendoortjes bij zovele gelegenheden.
Prof. Dr van der Plas was mijn promotor en begeleider vanuit de vakgroep Plantenfysiologie. Linus, dank je wel voor de ruimte die ik heb gekregen om die onderzoekslijn te kiezen waarvan ik dacht dat het goed was. Je interesse, begrip en hulpvaardigheid waren steun en vreugde.
Dr Jordi en Dr Maas waren de dagelijkse begeleiders die elk op hun eigen wijze betrokken waren bij zowel het onderzoek als de uitvoerende oio. Wilco, jouw enorme werklust en groot enthousiasme voor onderzoek laten grote indruk bij mij achter. Nieuwe ideeën kwamen vaak binnenrollen op soms onverwachte momenten. Ik vraag me nog steeds af of die eindeloze

liters thee daar van invloed op zijn geweest. Frank heeft mij in het begin van het project grondig wegwijs gemaakt met de zaken die bij lichtonderzoek van belang zijn. Na twee jaar ben je zelf ook met gibberellinenbepalingen begonnen en werden we kamergenoten hetgeen de discussies over gibberellinen en daarnaast ook andere aardse zaken zeer ten goede kwam. Zou ik al jouw dubbele bodems hebben doorgrond, denk je?
Linus, Wilco, Frank, heb dank dat ik altijd bij jullie terecht kon. Jullie stimulerende en richtinggevende invloed is waarschijnlijk nog groter geweest dan ik me realiseer.
Zonder de gedegen hulpvaardige handen van Geert Stoopen had dit proefschrift er heel anders uitgezien. Eindeloos veel alstroemeria's hebben we samen geknipt en vervolgens gemalen, geëxtraheerd en ontelbare keren uitgeschud. Heel veel gepraat is er ook door jou, ik heb genoten van vele schaats- en andere verhalen en daarnaast ook nog heel wat opgestoken van jouw labvaardigheden.
Harro Bouwmeester, via jouw karwijonderzoek ben ik binnen de muren van het toen nog CABO, binnengekomen. Jouw brede interesse en belangstelling waren zeer stimulerend en ik vond het erg plezierig om met je te werken. Vlak nadat ik als oio begon aan de andere kant van het gebouw, kwam ook jij op die afdeling werken en konden we verder blijven praten over terpenen want zijn gibberellinen tenslotte ook geen terpenen?
Geert en Harro, natuurlijk zijn júllie mijn paranimfen.
Een afdeling met betrokken collega's is onmisbaar voor een goede sfeer en het goed verlopen van het onderzoek. Vooral Evert Davelaar en Jacques Davies zijn in de beginperiode zeer behulpzaam geweest. Met z'n tweeën hebben ze mij zorgvuldig alles geleerd over het opzuiveren van gibberellinen uit planten-materiaal en dat vervolgens analyseren met behulp van de GC-MS. In een later stadium is Francel Verstappen leermeester geweest in het bepalen van cytokininen. Bedankt voor het aanleren van een gedegen basis voor het 'hormoonbepalen'.
De studenten Wilfred Janssen en Marco Strik hebben heel wat alstroemeria-bladeren in hun handen gehad. Ook al zijn veel van jullie experimenten niet of nauwelijks meer in dit proefschrift terug te vinden, toch is jullie bijdrage waardevol geweest voor de loop van het onderzoek. Nicolas Tsesmetzis came all the way from Greece to Wageningen to collect numerous HPLC-fractions. Your work contributed substantially to Chapter 5. Thank you Nicolas. I guess, when you read these lines your comment will be: 'so what'.
Anja Dieleman, wie kaatst kan de bal terug verwachten. Ruim twee jaar hebben we een kamer gedeeld en daar heel wat hormoondiscussies gevoerd. Van jouw duidelijke manier van presentatie in woord en daad heb ik veel

geleerd. Samen met de andere aio's van de afdeling Plantenfysiologie hebben we getracht om ook buiten universitaire vakgroepen het begrip aio een zinvolle invulling te geven.
In September 1997, I was able to visit the Institute of Plant Growth Regulators of the Palacký university of Olomouc in the Czech Republic. What started as a small excursion into the world of aromatic cytokinins turned out to be one of the major conclusions of this thesis. I would like to thank Miroslav Strnad, Hana Martínková, Thomas Werner, Karel Dolezal and Jan Hanuš for their help in finding my way in and around the lab and the city of Olomouc. I'm looking forward to come back to Olomouc in October this year.
Zonder alstroemeria's geen onderzoek, en dankzij de families Janssen uit Huissen en van Daalen uit Brakel was de aanvoer van alstroemeria's vrijwel nooit een probleem. Op Martin Zandvoort kon ik altijd een beroep doen voor een vlot vervoer van kweker naar het lab. Dank je wel Martin.
Janneke Abbas en Rina Kleinjan hebben geholpen bij de opmaak van het proefschrift, waarvoor mijn hartelijke dank.
Gedurende de afgelopen jaren ben ik door financiële ondersteuning van de afdeling Plantenfysiologie van het AB-DLO maar vooral de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) in de gelegenheid geweest om een aantal congressen in binnen- en buitenland te bezoeken, een voorwaarde om verantwoord wetenschappelijk onderzoek te verrichten.
Veel mensen zijn mij op hun eigen wijze dierbaar en hebben mij in de afgelopen jaren op diverse manieren steeds weer doen beseffen dat de alstroemeria-wetenschap ook een heel gezonde bijzaak kan zijn. Toch was er ook altijd belangstelling voor het onderzoek: groene en gele bladeren, glibberdingen en andere ongrijpbare zaken waren voor velen op z'n tijd interessant. Wat is er veel afgepraat en gelachen. Jan en Henny, Gerard, Erik-Jan en Matthijs, Dieuwke, Salvatore, Ate en Clara, Michiel en Hanneke, Judith en Thom, Lieke, Saskia en Johan, Godelieve en Mathee, Barbara, Wouter, Lies, Jeroen, Marijn en Willemijn, Claudia, Jeroen en Pieter-Kees, Hester, Riet en Wil en anderen, dank je wel. Het blijft onbegrijpelijk dat vier van jullie er niet meer zijn.

Het proefschrift is geschreven, het praten nog niet gedaan.
Tot op 30 september

Wageningen – Celleno – Ede – Ermelo, voorjaar 1998