Gibberellins
and the cold requirement
of tulip

Gibberellinen
en de koudebehoefte
van tulpen

CENTRALE LANDBOUWCATALOGUS

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M. Rebers

Gibberellins and the cold requirement of tulip

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in de landbouw- en milieuwetenschappen
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dr. C.M. Karssen
in het openbaar te verdedigen
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des namiddags te half twee in de Aula
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Stellingen

1. Bij tulpebollen is er geen directe correlatie tussen gibberelline-gehalten aan het eind van een koude bolbewaring en de daaropvolgende stengelstrekking na het planten van de bollen.
   Dit proefschrift.

2. Voor de koude-gestimuleerde stengelstrekking van tulpen is GA_4 belangrijker dan GA_1.
   Dit proefschrift.

3. Niet alleen de koudebehandeling maar ook de bewaarperiode zelf, doet de gevoeligheid van tulpespruiten voor gibberellinen toenemen.
   Dit proefschrift.

4. Aan het eind van het bewaarseizoen zijn zowel de gevoeligheid voor gibberellinen als de GA-synthese capaciteit niet beperkend voor de *in vitro* groei van tulpespruiten uit ongekoelde bollen.
   Dit proefschrift.

5. Een uitspraak over de functie van een verhoogd gibberelline-gehalte aan het eind van de koudebehandeling van bloembollen is zinloos, wanneer ongekoelde controlebollen van dezelfde leeftijd niet geanalyseerd zijn.
   L.H. Aung and A.A. De Hertogh (1967) Plant Cell Physiol. 8: 201-205.
   T. Takayama, T. Toyomasu, H. Yamane, N. Murofushi and H. Yajima (1993) J. Japan Soc. Hort. Sci. 62: 189-196.
   Dit proefschrift.

6. De inspanningen die verricht worden om mensen zuiniger met natuurlijke grondstoffen om te laten gaan, staan in contrast met de verspilling van kennis en talent die ontstaat door werknemers slechts voor een beperkte periode aan te stellen.

7. Het is vreemd dat het voor- of dankwoord van een proefschrift het enige onderdeel is dat zonder tussenkomst van promotor of rector magnificus vermenigvuldigd mag worden, hoewel het de meest gelezen pagina's zijn.

8. Een tijdelijk werkverband buiten Nederland zou het relativerend vermogen van veel ontevreden Nederlanders aanzienlijk vergroten.

9. Het grote aantal criteria dat tegenwoordig gehanteerd wordt bij sollicitatie-procedures voor academici, staat in geen verhouding tot de geringe eisen die gesteld worden om aan een universitaire studie te mogen beginnen.

10. Het toenemende privé gebruik van de auto in Nederland kenmerkt de toenemende individualisering.

11. De bevolkingsexplosie als de oorzaak van armoede is een westers argument om de werkelijke oorzaken te verdoezelen.
Pakistan Times, 11 oktober 1994.

12. Het zou beter gaan met het milieu wanneer er in de economie naast producenten en consumenten, ook sprake zou zijn van "reducenten", zoals in de biologie.

13. De bewering dat altviolisten gedesillusioneerde violisten zouden zijn, wordt niet ondersteund door het grote aantal verzoeken dat ze krijgen om mee te spelen in muziekgezelschappen.
E. Heimeran en B. Aulich. (1936) In: Das stillvergnügte Streichquartett. Heimeran Verlag, München, p. 40.

14. Musici streven naar eenzame hoogten, muzikanten naar saamhorigheid.

Stellingen behorende bij het proefschrift 'Gibberellins and the cold requirement of tulip' door Mariken Rebers.

Wageningen, 16 december 1994

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Voorwoord

In 1989 ben ik begonnen bij de vakgroep Plantenfysiologie, om onderzoek te doen met onder andere de nieuw verworven GC-MS. Niet alleen dit onderzoek heb ik met plezier uitgevoerd, ook de omgevingsfactoren heb ik als heel plezierig ervaren. Er zijn hier veel mensen bij betrokken geweest en ik wil allen, ook degenen die ik hieronder niet met naam noem, hartelijk bedanken voor hun bijdrage.

Om te beginnen, de aanschaf van de kostbare GC-MS is vooral te danken geweest aan de inzet van prof. J. Bruinsma. Samen met Kees van Loon heeft hij een belangrijke rol gespeeld aan het begin van dit project.

Erik Knegt en Evert Vermeer hebben mij daarna zorgvuldig wegwijs gemaakt in de wereld van de chromatografie. Het was voor mij als veldbioloog een hele ontdekking dat ik me in de kelder, waar echt geen plantje wil groeien, uitstekend thuis voelde. Daarnaast heeft Erik mij veel bijgestaan bij allerlei zaken rondom het onderzoek; zelfs uren bollen pellen werd zo een plezierige bezigheid. Dank je wel! Ik heb ook veel geleerd van je fraaie woordkeuzes en zorgvuldige wijze van formuleren. Bovendien was je tot en met het laatste figuur in het proefschrift hoop in uitzichtloze computerdagen.

Evert heeft steeds de GC-MS aan de praat gehouden en verschrikkelijk veel gibberellines voor me gemeten. Daarnaast was hij ook altijd in voor het uitwisselen van culinaire wetenswaardigheden. Ruth van der Laan en Ben van der Swaluw, technische steunpilaren en mede-paling eters, hebben eveneens bijgedragen aan het goede werkklimaat.

Na anderhalf jaar kreeg ik een nieuwe hoogleraar als promotor: Linus van der Plas. Linus, je hebt heel veel tijd vrijgemaakt om mee te denken over het onderzoek

en de daarbij horende problemen. Je begrip en hulpvaardigheid leken onafhankelijk van je agenda en de zomerse buitentemperatuur. Het heeft mij bijzonder gestimuleerd om door te zetten en optimistisch te blijven.

De bij dit projekt betrokken medewerkers van het Laboratorium voor Bloembollen Onderzoek in Lisse wil ik noemen voor hun bijdrage aan verhelderende discussies. Vooral Hanneke Franssen heeft veel praktische problemen opgelost en theoretische moeilijkheden helpen onderzoeken.

Siep Massalt legde de groei van tulpespruiten in buizen fotografisch vast en Allex Haasdijk tekende de omslag van dit proefschrift, wat zeker een vermelding waard is. De studenten Dick van der Wal en Gaea Romeijn hebben een groot aantal tulpebollen in hun handen gehad. Ook al zijn veel van hun experimenten niet of nauwelijks meer in dit proefschrift terug te vinden, toch hebben zij op hun manier bijgedragen aan het onderzoek.

I am especially grateful to Yuji Kamiya and prof. Nobutaka Takahashi for their hospitality during my stay at RIKEN institute in Japan. In addition, thanks to their financial support I could join the symposium 'Frontiers of Gibberellin Research' in Japan. The discussions during these visits have substantially contributed to this thesis.

Daarnaast heb ik de afgelopen 5 jaar een grote variatie aan collega's gehad. Jullie wil ik bedanken voor jullie doorzettingsvermogen, om steeds weer op deskundige wijze de voor mij broodnodige afleiding te verzorgen. Ook het salonorkest 'Passepartout' liet mij steeds weer op tijd beseffen dat de tulpenwetenschap ook een heel gezonde bijzaak kan zijn. Midden in de werkweek maakten we muziek, in de wetenschap dat je eveneens kunt zoeken naar de correlatie tussen de kwaliteit van de piano en het geserveerde buffet.

Tenslotte bedank ik mijn ouders, Paul, Saskia en de familie Verheijen voor de belangstelling en gezelligheid. En Leon, dank je wel voor je geduld en nog veel meer!

Mariken

$A_{210\ nm}$	absorbance at 210 nm
ABA	abscisic acid
BX-112	prohexadione
BuOH	n-butanol
cv.	cultivar
dpm	disintegrations per minute
E.C.	enzyme commission number
ELISA	enzyme-linked immunosorbent assay
EtOAc	ethyl acetate
FW	fresh weight
GA, GA_n	gibberellin, gibberellin n
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
HPLC	high-performance liquid chromatography
IAA	indolyl-3-acetic acid
KRI	Kováts retention index or indices
$[M]^+$	molecular ion, parent ion
Me	methyl derivative
MeOH	methanol
MeTMSi	methyl trimethylsilyl derivative
MS medium	Murashige and Skoog medium
m/z	mass/charge ratio
P	paclobutrazol
PB	petroleum benzin 40-70
PVP	polyvinylpyrrolidone
QAE	quaternary ammonium ether
RIA	radioimmunoassay
SD	standard deviation of series
SIM	selected ion monitoring
TIC	total ion current
w/v	weight/volume

1 General introduction

Tulip

The year 1994 is special for people working in tulip cultivation and research in the Netherlands, because 400 years ago the first tulips were flowering in the Botanical Garden in Leiden, the Netherlands. This was the start of a period of 'tulipomania' in the beginning of 17th century, in which tulip bulbs were bought as speculation object at extremely high prices. In 1637 this tulipomania suddenly came to an end, although tulips continued to be popular.

The tulip is a monocotyledonous plant and is a member of the Liliaceae family. The genus *Tulipa* includes about 125 species, which are distributed in two sub-genera, Eriostemones and Leiostemones. The present day garden tulips, which belong to the Leiostemones, originate from types introduced into Europe from Turkey after the middle of the 16th century.

To date, the Netherlands is the main tulip bulb producer in the world, growing in 1983 about 86% of the total world tulip bulb production (Le Nard and De Hertogh, 1993).

Lifecycle

Tulip bulbs originate from seeds, which require low temperatures for proper development of the embryo. The embryo produces a plant with a cotyledonary leaf, one primary root and a hollow diverticulum called a "Dropper". This organ grows into the soil and produces a small bulb, which needs four to five additional growth

909 51

cycles before a flowering-size bulb is produced.

Seedlings are only used for breeding purposes. Commercially, tulips are vegetatively propagated, based on the production of daughter bulbs by the vegetative buds of the bulbs.

The tulip bulb has an annual replacement cycle, in which a key aspect is the continuous change that occurs in the various organs over time. At any time period, there are organs that are being initiated, developing or senescing. The growth cycle can be divided into three main phases (Le Nard and De Hertogh, 1993):

(1) At the end of spring, the aerial organs of the mother bulb are senescing and the growth of the daughter bulbs ceases. Shortly after bulb harvest in June or July, the flower bud and the root primordia of the daughter bulbs are initiated and formed. Flower bud differentiation is complete at about the end of July (stage "G", Cremer *et al.*, 1974). By the end of summer, all the organs of the daughter bulb plant are present, including granddaughter bulbs in the axils of the scales.

(2) After planting these daughter bulbs in autumn, root growth occurs rapidly. The already differentiated shoot elongates slowly and there is slight growth of the granddaughter bulbs. In contrast, the scales are slowly senescing and the basal plate is relatively unchanged.

(3) In early spring the greatest biomass increase occurs. Rapid shoot growth leads to flowering. Granddaughter bulb growth continues and is especially rapid after flowering. In contrast, the daughter bulb scales shrivel and progressively disappear.

Cold requirement

An important factor affecting the growth and development of tulip is temperature. The tulip has an obligatory warm-cool-warm cycle (De Hertogh *et al.*, 1983). The flower is initiated at relatively high temperatures (17°-20°C or higher). Following flower initiation, a cold period (2°-9°C) is required. At this low temperature physiological changes occur which prepare the shoot for adequate floral stalk elongation and flower development at the subsequent higher temperatures (17°-20°C). In a temperate climate as in the Netherlands, these phases can take place during the seasons under natural conditions. However, in horticultural practice, flowering is programmed by simulating the temperature conditions required in nature (forcing). The winter season in particular, can be replaced by dry-storing the bulbs in cooled ventilated chambers, prior to planting the bulbs. This cold temperature treatment can be applied after flower bud differentiation is complete (stage "G", Cremer *et al.*, 1974).

The duration of this cold treatment is quite critical to achieve adequate floral stalk extension, synchronous and rapid flower development and high flower quality after planting the bulbs. The number of days from planting to flowering and the final floral stalk length is a function of the number of weeks of low temperature that the bulbs have received. Both temperature-dependence and duration of the required dry-storage period, are cultivar specific. For bulbs of tulip cv. Apeldoorn, the relation between the duration of the cold treatment, and the subsequent floral stalk elongation and flower development, is shown in Figure 1.1. A dry-storage treatment of 12 weeks at 5°C prior to planting, will lead to proper floral stalk elongation and flower development. Shorter periods at 5°C usually lead to slower shoot elongation and delayed flower development. When bulbs are planted without any cold treatment, the growth of the terminal bud is strongly reduced and flower abortion often occurs.

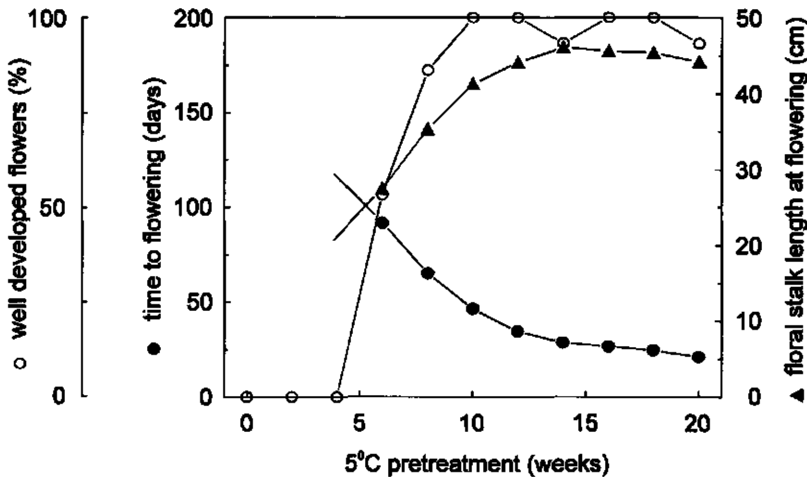

Figure 1.1 The development of the tulip sprout cv. Apeldoorn after planting bulbs at 20°C, in relation to the duration of the cold treatment (5°C) before planting.

For economic reasons, a practical assay to test whether a particular bulb has received a proper cold treatment is desirable. In order to identify parameters that are indicative for a proper cold treatment of tulip bulbs, there is a search for cold-dependent parameters which are related both to a proper cold treatment and to the subsequent floral stalk elongation and flower development (Boonekamp *et al.*, 1990). For that reason, a better knowledge of the physiological changes occurring in the

bulbs during the cold period is needed. In related projects research is directed towards carbohydrate and amino acid metabolism (Kollöffel *et al.*, 1992; Lambrechts *et al.*, 1992a; 1992b; 1993; 1994); respiration characteristics of isolated mitochondria and ethylene production (Kanneworff and van der Plas, 1990; 1992; 1994a; 1994b) and membrane composition and characteristics during the cold bulb treatment (Walch and van Hasselt, 1990).

These processes might be connected with hormonal changes. There are indications that gibberellins (GAs) in particular are involved in the cold requirement of tulip: application of GAs could partly replace the cold treatment (van Bragt and Zijlstra 1971; van Bragt and van Ast, 1976; Bylov and Smirnova, 1979; Hanks and Rees, 1980a; Hanks, 1982; Hanks and Rees, 1983). In addition, cold-stimulated floral stalk elongation was suggested to require GA biosynthesis (Shoub and De Hertogh, 1974; Hanks and Rees, 1977; Suh *et al.*, 1983a; Okubo *et al.*, 1986; Saniewski, 1989).

Gibberellins

In 1965, gibberellins were defined as compounds having an *ent*-gibberellane skeleton and biological activity in stimulating cell division, cell elongation or other biological activity as may be specifically associated with this type of naturally occurring substance (Paleg, 1965). To date, more than 90 different GAs have been characterized by their chemical structure in higher plants and fungi (Pearce *et al.*, 1994), their numerical order approximating the chronological order of their discovery. In general, GAs play an important role in growth and development of plants, particularly in dormancy breaking, germination, shoot extension and induction of flowering (Graebe, 1987).

The GAs can be divided into two main groups, the C20-GAs with a 20-carbon skeleton such as GA_{12}, and the C19-GAs with a 19-carbon skeleton such as GA_9. Within each of these groups, there is considerable variation in the degree and position of oxidation, which is usually hydroxylation. The major positions of hydroxylation are indicated in Figure 1.2, which also shows the structures of the non-hydroxylated GA_{12} and GA_9.

From this large group of structures, the number of bioactive GAs is limited. The numerous GAs present in a plant or plant organ may represent only a few bioactive GAs, the others being either precursors or inactivation products.

Gibberellins also exist as conjugates, linked to a limited number of low molecular weight molecules. The most common naturally occurring GA conjugates are GA-glucose conjugates (Hedden, 1987). Conjugated GAs themselves have low or no

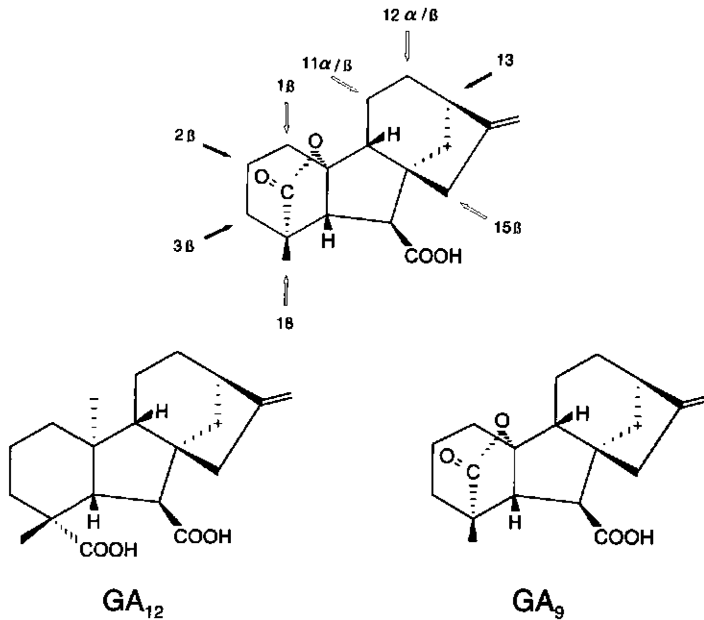

Figure 1.2 The main positions of GA hydroxylation in higher plants and the structures of the non-hydroxylated GA_{12} and GA_9. The closed arrows indicate the most common positions.

biological activity. They may yield free GAs after chemical or enzymic hydrolysis (Rood *et al.*, 1983). The function of conjugated GAs in plants is uncertain. Conjugation might convert GAs to inactive, water soluble molecules suited for transport or storage. It is possible that reversible conjugation is involved in the regulation of the active hormone levels (Rood *et al.*, 1983; Schneider and Schmidt, 1990).

Gibberellin biosynthesis

In higher plants there are several sites of GA biosynthesis: developing fruits or seeds, elongating shoot apical regions, roots and young leaves (Graebe and Ropers, 1978; Sponsel, 1987). The proper functioning of GA biosynthesis is essential for the normal development of plants. GA biosynthesis is activated and inactivated during the life cycle of a plant; the pathways are different in different species and even in different organs of the same species (Graebe, 1987). In the biosynthesis of GAs three stages can be distinguished (Figure 1.3): (1) the biosynthesis of *ent*-kaurene, (2) the reactions

from *ent*-kaurene to GA_{12}-aldehyde, and (3) the conversions after GA_{12}-aldehyde. The pathway until GA_{12}-aldehyde is identical in all plants (Sponsel, 1987). In contrast, the steps after GA_{12}-aldehyde, leading to the formation of bioactive GAs, can vary between different species.

Three routes can be distinguished, differing in positioning and timing of hydroxylation (MacMillan, 1984), as shown in Figure 1.4. In many higher plants, such as maize (Phinney, 1984; Fujioka *et al.*, 1988) and pea (Kamiya and Graebe, 1983), the early-13-hydroxylation pathway leads to GA_{20}, GA_{29} and GA_1. GA_1 is probably the active GA of this series in the control of stem growth (Phinney, 1984). In pea embryos, a parallel non-3,13-hydroxylation pathway leading to GA_9 and GA_{51} was found (Kamiya and Greabe, 1983). In addition, there is an early 3-hydroxylation pathway leading to GA_4 and GA_{34}, as found in *Arabidopsis* (Talon *et al.*, 1990). However, the observed pathways are rarely discrete. They may converge or diverge to form a metabolic grid, as shown in Figure 1.4 (adapted from Kamiya *et al.*, 1985 and Sponsel, 1987).

Among the physiologically active hormones, in particular the 3ß-hydroxylation of the GA molecule, as in GA_1 and GA_4, is considered to be an essential feature for promoting stem elongation (Talon *et al.*, 1990; Evans *et al.*, 1994). In contrast, 2ß-hydroxylation leads to inactivation of the GA molecule, e.g. the formation of GA_8 and GA_{34}. Other GAs show intermediate or low activity, depending on their conversion into active structures, or their structural resemblance to an active form (Sponsel, 1987).

Analysis of gibberellins

Gibberellins lack physical properties for selective physico-chemical detection, and until the introduction of gas chromatography-mass spectrometry (GC-MS) for GA analyses in 1967, GAs were analysed using bioassays. These procedures yielded at best only semiquantitative results and they provided hardly any information on individual GAs. Although bioassays are still in use for establishing the presence of GA-like substances in plant extracts, it is now generally acknowledged that they lack precision. Moreover, they provide no reliable data on GA levels since plants may contain inhibitors that can interfere with the bioassays.

In GC-MS, the mass spectrometer serves as a highly versatile GC detector. GC-MS is a suitable method to analyse the endogenous GA content, since the mass spectrum together with its retention time is a unique property for each GA. In

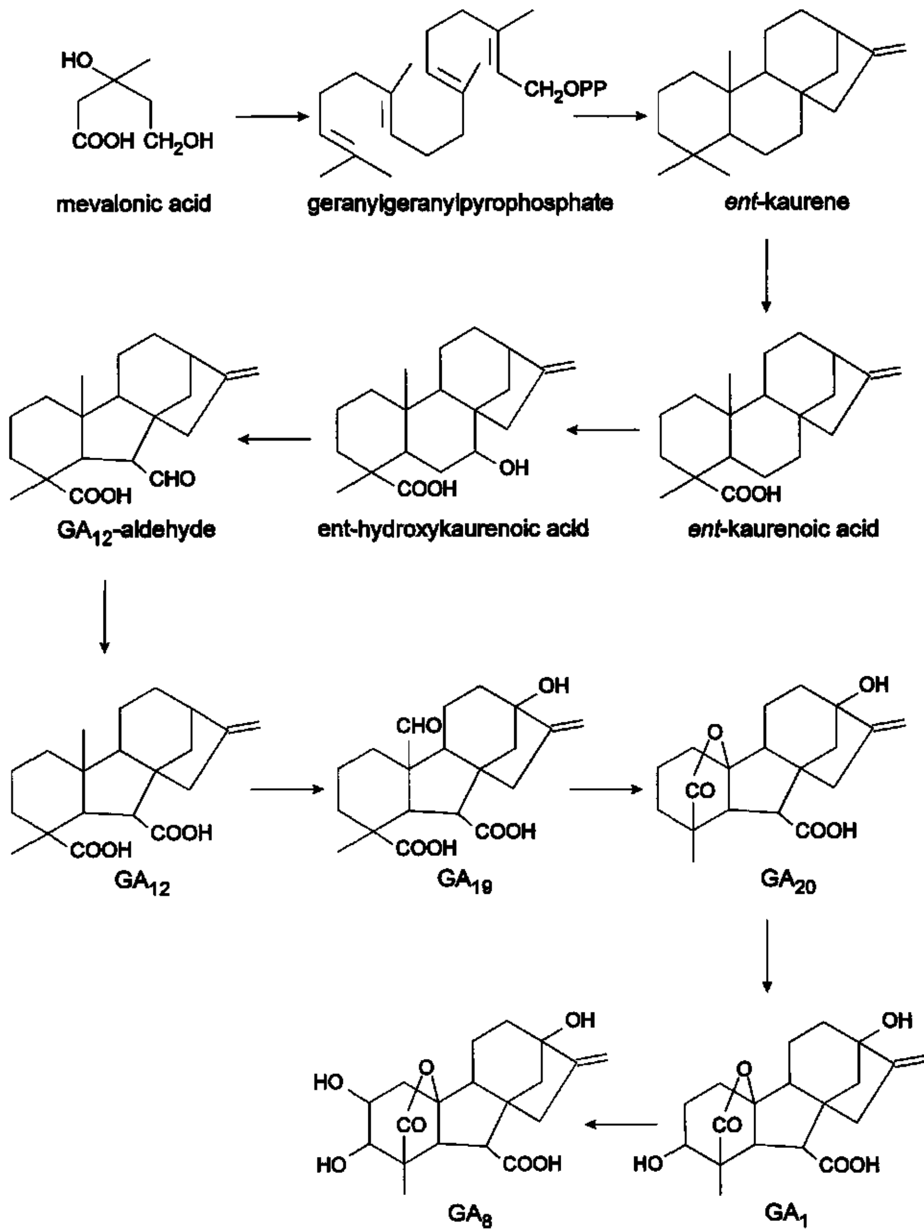

Figure 1.3 Principal reactions involved in the biosynthesis of gibberellins. Adapted from Rademacher (1991).

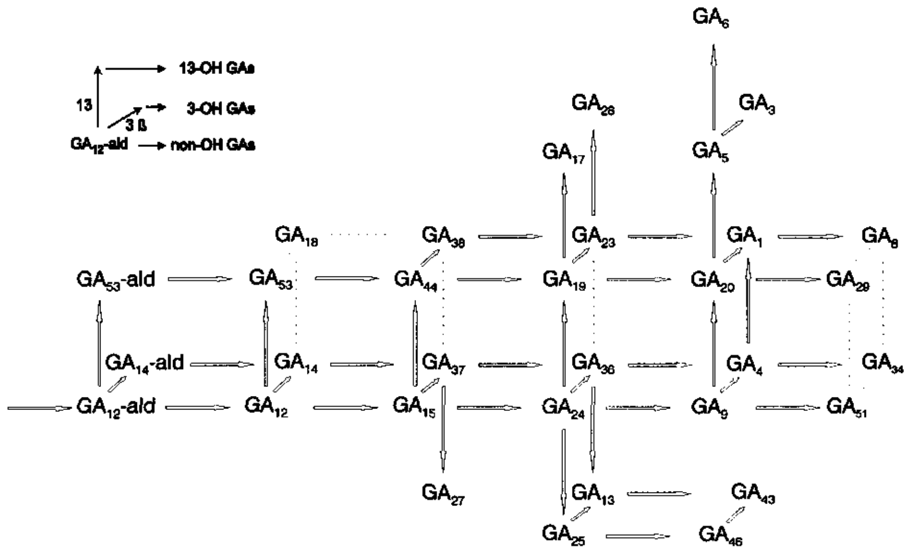

Figure 1.4 Metabolic grid of gibberellins in higher plants, showing known conversions. Dotted lines indicate hypothetical conversions. Adapted from Kamiya *et al.*, (1985) and Sponsel (1987).

addition, selected ion monitoring (SIM) provides a highly sensitive and selective method which, using isotope labelled GAs, enables quantification of the endogenous GA levels.

Alternatively, immunoassay techniques were developed for the analysis of GAs. The application of immunoassays to GA analyses is more complex than for other classes of plant hormones, because of the many qualitative differences in GA structures. Several sensitive RIA and ELISA methods are available for the detection of GAs. However, due to the cross reactivity of the antibodies, their applications are limited.

Inhibitors of GA biosynthesis

Methods to investigate the relationship between GAs and their physiological response, are the use of chemicals to block the GA biosynthesis, and genetic mutants which are blocked in the GA biosynthesis pathway or have a reduced sensitivity to GAs. For

tulip, no GA mutants are available.

GA biosynthesis inhibitors are known for their growth retarding ("dwarfing") effect on plants. They act by blocking specific steps in the biosynthetic pathway which lead to biologically active GAs. Three different groups can be distinghuished, inhibiting GA biosynthesis (Figure 1.3) at different stages (Rademacher, 1991): (1) "onium" compounds, (2) compounds with a nitrogen-containing heterocycle and (3) cyclohexanetriones

"Onium" compounds as chlormequat chloride (CCC), AMO-1618, chlorphonium chloride and mepiquat chloride, have a positively charged ammonium, phosphonium, or sulphonium moiety. These compounds interfere with the biosynthetic steps directly before *ent*-kaurene (Rademacher, 1991).

Several growth retardants with a nitrogen-containing heterocycle, e.g. ancymidol, paclobutrazol, tetcyclasis, uniconazole and inabenfide, inhibit the oxidative reactions leading from *ent*-kaurene to *ent*-kaurenoic acid (Rademacher, 1991).

Cyclohexanetriones block late reactions in the GA biosynthetic pathway. The primary action of e.g. prohexadione (BX-112) is the inhibition of 3ß-hydroxylations in GA biosynthesis, e.g. the conversion of GA_{20} to GA_1 (Nakayama *et al.*, 1990a; Nakayama *et al.*, 1992). To a lesser extent, prohexadione also interferes with 2ß-hydroxylations (Nakayama *et al.*, 1990b).

Gibberellins, stem elongation and flower development

Application of GAs to plants can influence a variety of processes, including stem elongation, dormancy and flowering (Crozier and Turnbull, 1984). In many plants that require either an inductive low temperature or long day treatment, exogenous GAs can substitute for the environmental stimulus (Pharis and King, 1985). There is considerable evidence that photoperiodic control of stem elongation in rosette plants is mediated by GAs (Metzger and Zeevaart, 1980). Although a long day treatment of spinach plants did not lead to an increase of the total GA content of the plants, the levels of individual GAs were altered. The level of GA_{19} decreased while the levels of GA_{20} and GA_{29} increased during the same period. These observations led to the suggestion that the conversion of GA_{19} to GA_{20} is under photoperiodic control (Metzger and Zeevaart, 1980). Following the detection of GA_1 and the use of the GA biosynthesis inhibitor prohexadione (BX-112), it was reported that the 3ß-hydroxylation of GA_{20} to GA_1 is necessary for stem elongation in spinach (Zeevaart *et al.*, 1993)

Some plants require a period of low temperature before flowering can be

induced. In *Brassica* species, flowering is initiated during the cold period, but in many plants flowers appear only after transfer to higher temperatures. The crucifer *Thlaspi arvense* requires a cold treatment for the induction of stem elongation and flowering. Longer cold treatments result in greater maximum stem length and reduce the lag period for the onset of flowering. It was shown that the cold-induced stem growth of *T. arvense* is a GA-dependent process. Applied GA_3 was able to replace the cold treatment completely, leading to stem elongation and flowering. In addition, cold-induced stem growth was shown to require continued GA biosynthesis (Metzger, 1985).

The effects of GAs on stem elongation have been studied in most detail. Many bioassays are based on the growth-stimulating effect of applied GAs. Yet, in spite of various studies on the mode of action of GAs, it has not been elucidated yet how GAs actually promote stem elongation. GAs can stimulate both cell division and cell elongation. Following GA treatment of *Samolus parviflorus*, a rosette long-day plant, the number of cell divisions in the subapical meristem increased considerably (Sachs, 1965). There are indications that the site of GA action is located in the cell wall (Crozier and Turnbull, 1984 and references in there). Applied GA_3 increased cell wall plasticity of lettuce hypocotyls. Changes in cell wall composition occurred during GA-induced extension growth but they did not appear to be related to the growth process itself. It appeared unlikely that the regulation is affected at the level of DNA or RNA synthesis. Experiments with colchicine indicated that the process is independent of microtubule or microfibril orientation patterns (Crozier and Turnbull, 1984).

It has been suggested that Ca^{2+} ions are involved in the hypocotyl response to GA_3. The GA-induced promotion of growth might be mediated by a movement of Ca^{2+} ions from the cell wall into the protoplast, facilitating increases in cell wall plasticity and hence growth (Crozier and Turnbull, 1984).

Exogenous GAs induce or promote flowering in many species, being particularly effective on long day plants (Pharis and King, 1985). GA application could replace the long day requirement for flower initiation in *Lolium temulentum* and the endogenous GA status changed with inductive treatments (Pharis *et al.*, 1987). In several long day requiring plants, GA biosynthesis inhibitors could inhibit both the increase in GA content and the stimulating effect of long day on flowering, while applied GA_3 reversed the inhibiting effect (Pharis and King, 1985). However, in *L. temulentum* the growth retardant CCC did not prevent flowering (Pharis *et al.*, 1987), and also in *Silene armeria*, the GA biosynthesis inhibitor AMO 1618 had no effect on flowering (Cleland and Zeevaart, 1970).

Remarkably, the structural GA requirements for inducing flowering in *L.*

temulentum were quite different from those for promoting stem elongation (Pharis *et al.*, 1987; Evans *et al.*, 1990). For stem elongation 3ß-hydroxylation of the GA molecule was essential, whereas florigenicity was reduced by it. Apparently, stem elongation and flower development might show different sensitivities to structural changes in the GA molecule. These results suggest that the GA receptor for stem elongation is different from that for flowering (Evans *et al.*, 1994).

Gibberellins and tulip

Research has been done on the effects of applied GAs on the development of tulips, with the aim of reducing or eliminating the lengthy cold requirement. GA appeared to substitute for the cold period in many, but not all tulip cultivars. GAs had the least effects on cultivars with the shortest cold requirement and more effect on those with longer cold requirements (Hanks, 1982). In addition, there was a partial rather than a complete effect of GA. Table 1.1 gives a survey of reports on the effects of applied GAs on tulip growth and development. In general, applied GA decreased the number of days from planting to flowering and could substitute partly for the cold treatment in several studies. All of the different application techniques used could yield effect, although bulb injection appeared the most effective method in most studies. For commercial purpose, however, this method is not practical. In many studies, floral stalk length was affected by GA application. Several studies reported shorter plants on GA application, but also longer plants or no effect on floral stalk length have been reported. Differences in response are probably due to different application techniques, times of application, and forcing treatments of the bulbs. In addition, most studies used GA_3, that when compared with several other GAs, appeared to be the least active GA for promoting floral stalk elongation or flower development in tulips (Hanks and Rees, 1980b).

Early studies investigated the endogenous GA content in tulip bulbs, during and after storage at different temperatures. These studies used low resolution chromatographic techniques and bioassays to determine the levels of GA-like substances. Table 1.2 a and b present a survey of these results. To allow the comparison of the data, the results were recalculated to express as ng GA_3-equivalents g^{-1} FW. The controversies in the literature with regard to the amount of GA present and the changes in GA content during the cold period, are clearly illustrated.

Table 1.1 Literature survey of reports on the effects of gibberellins applied by different methods and to different tulip cultivars.

cultivar	ref.[1]	cold treatment[2]	application		GA_n		result
			time	method	n	amount/conc.	
Apeldoorn	(1)	none or partly	at planting or during growth	injection	3, 4/7	1, 10, 50 mg	partly substituted for cold treatment, earlier flowering, most effective when applied before planting, final floral stalk length not affected, $GA_{4/7}$ more effective than GA_3
Apeldoorn	(2)	partly or full	during growth	drops on apex, absorption basal plate, injection	3, 4/7	0.4 mg/ml ?	each method effective, earlier flowering, longer plants, reduced flower blasting, more effect after partial cold treatment
Apeldoorn	(3)	none, partly or full	before, during and after cold treatment	injection	3	1 mg	earlier flowering, partly substituted for cold treatment, shorter plants
Apeldoorn	(4)	full	during growth	lanolin paste	3	0.1%	did not reverse effect of flower bud removal
Apeldoorn	(4)	full	during growth	soil drench	3	0.1-10 mg	reversed effect of ancymidol
Apeldoorn	(4)	full	during growth	injection	3	0.02-2 mg	reduced flower abortion
Apeldoorn, Emmy peeck	(5)	full	after planting	injection spraying soil drenching	3, 4/7 4/7 4/7	0.05 mg 0.3 mg/3ml 1.2 mg/12 ml	injection most effective, prevented ethylene-induced flower blasting, earlier flowering

Apeldoorn, Paul Richter	(6)	full	at planting	injection	4/7	0.2 or 2 mg	prevented flower abortion due to poor ventilation, earlier flowering, shorter plants, longer first internode
Apeldoorn	(7)	full	at planting	injection	3	1 mg	earlier flowering, shorter plants
Apeldoorn	(8)	partly	at planting	injection	1, 3, 4, 4/7, 5, 9, 13	0.1-10 mg	earlier flowering, partly substituted for cold treatment, shorter plants, GA_3 least effective, GAs 7, 4, 1 and 9 most effective
Apeldoorn	(8)	partly	during cold and during growth after planting	repeated injections	3	1 mg	earlier flowering, extremely effective
Paul Richter	(8)	partly	at planting	injection	3	0.1-10 mg	only high dose effective, earlier flowering
					4/7	1 mg	earlier flowering
Apeldoorn, Oxford	(9)	full	after planting	lanolin paste	3	2%	effect ambiguous
Apeldoorn, Paul Richter	(10)	none to full	at planting	injection	3, 4/7	1, 5, 10, 100 or 1000 mg	injection most effective, earlier flowering, partly substituted for cold treatment, shorter plants, GA_3 not effective in P. Richter
				immersion		1, 10 or 100 mg/l	
				soil drenching		1, 10 or 100 mg/l	

cultivar	ref.[1]	cold treatment[2]	application		GA_n		result
			time	method	n	amount/conc.	
Apeldoorn	(11)	none to full	at planting	vacuum infiltration	3, 4/7	0-1500 mg/l	earlier flowering, partly substitued cold treatment, final floral stalk length not affected
Apeldoorn	(12)	full	at planting	vacuum infiltration	3	150-250 mg/l	reduced flower losses due to vacuum infiltration, earlier flowering, shorter plants
Apeldoorn	(13)	partly or full	before, during or after cold	injection	3	1 mg	earlier flowering, application before cold least effective
Apeldoorn	(13)	partly	before, during or after cold	repeated injections	3	1 mg	increased effectiveness, except for fully cooled bulbs
Apeldoorn, Rose Copland	(14)	none to full	before, during or after cold	injection	3, 4/7	1 mg	earlier flowering, partly substituted for cold treatment, final floral stalk length not affected, $GA_{4/7}$ more effective than GA_3
Apeldoorn	(15)	none to full	at planting	injection	4/7	1 mg	earlier flowering, more effect after shorter cold treatments, shorter plants
Golden Apeldoorn, Red Matador	(16)	full	after planting	soil drenching	3	250-500 mg/l	reversed effect of ancymidol, earlier flowering
Gudoshnik	(17)	full	after planting	drops leaf sheath	3	1 mg	partly reversed effect of paclobutrazol

Gudoshnik	(18)	full	during growth	absorption basal plate	3	250 mg/l	partly reversed effect of root removal
London	(19)	none, partly or full	during cold treatment, at planting	injection immersion spraying	3	0.1, 1 and 5 mg/ml	all methods effective, partly substituted for cold treatment, earlier flowering, longer plants
Paul Richter	(20)	full	after planting	soil drenching	3, 4/7	25 mg/100 ml	reversed effect of ancymidol, $GA_{4/7}$ more effective than GA_3, flowering not affected
Paul Richter	(21)	full	after planting	drops on flower bud	3	0.4 mg/1 ml	reversed effect of ancymidol, did not reverse effect of flower bud removal
Mirjoran, Feu Superbe, Gudoshnik, Oxford, Red Shine, Miracle, Red Matador, Orange Favourite, Spring Song	(22)	partly to full	before, during and after cold treatment, at planting	injection	3	1 and 10 mg	earlier flowering
		partly to full	before, during and after cold treatment, at planting	lanolin paste	3	0.5 or 2%	not highly effective

[1](1) van Bragt and Zijlstra, 1971
(2) Murai *et al.*, 1975
(3) van Bragt and van Ast, 1976
(4) Hanks and Rees, 1977
(5) de Munk and Gijzenberg, 1977
(6) Moe *et al.*, 1978
(7) van Bragt and van Gelder, 1979
(8) Hanks and Rees, 1980b
(9) Saniewski and de Munk, 1981
(10) Hanks, 1982
(11) Hanks and Rees, 1983
(12) Jones and Hanks, 1984
(13) Hanks, 1984
(14) Hanks, 1985
(15) Suh *et al.*, 1992
(16) Suh *et al.*, 1983a
(17) Saniewski, 1989
(18) Kawa-Miszczak *et al.*, 1992
(19) Bylov and Smirnova, 1979
(20) Shoub and De Hertogh, 1974
(21) Okubo *et al.*, 1986
(22) Rudnicky *et al.*, 1976

[2]none: bulbs did not receive a cold treatment prior to planting
full: bulbs received a full cold treatment prior to planting
partly: bulbs received a partial cold treatment prior to planting

Table 1.2a Literature survey of reports on the endogenous gibberellin levels in tulip during cold or noncold bulb treatment, measured by bioassays. Only free GA levels in sprouts, whole bulbs or first internodes are included. To allow for comparison of the different data, results are recalculated to express as ng g^{-1} FW sprout, bulb or first internode. The conclusions are based on the changes in relation to the start of the cold treatment, or occurring during the cold treatment.

cultivar	ref.[1]	bulb treatment			tissue	bioassay[2]	GA_3 equiv.	conclusion
		storage condition	temperature (°C)	period (weeks)			(ng g^{-1}) FW	
Apeldoorn	(1)	dry	20	0	sprout	a	22.9	
		dry	5	12	sprout	a	6.3	
		dry	20	12	sprout	a	6.4	decrease
Apeldoorn	(2)	wet	field	0	sprout	b	72.2	
		wet	field	5.5	sprout	b	23.6	
		wet	field	9.5	sprout	b	182	
		wet	field	13.5	sprout	b	21.9	
		wet	field	17	sprout	b	10.1	fluctuation
Elmus	(3)	wet	non cold		sprout	c	0.43	
		wet	9	4	sprout	c	40	increase
Elmus	(4)	wet	non cold	0	sprout	a	0.47	
						c	0.37	
		wet	then 9	4	sprout	a	0.27	
						c	0.07	
		wet	then 5	4	sprout	a	0.08	
						c	0.06	
		wet	then 2	7	sprout	a	0.07	
						c	0.10	decrease
Paul Richter	(5)	wet	9	15	sprout	c	0.02	
		wet	9	16	sprout	c	14.4	increase
Purrissima	(6)	?	5	7	sprout	d	500	
		?	5	13	sprout	d	3200	increase
Ralph	(7)	dry	17	0	bulb	c	0.0003	
		dry	5	9	bulb	c	0.001	
		wet	9	13	bulb	c	0.12	increase

Table 1.2b As Table 1.2a; GA levels after the cold bulb treatment at subsequent higher temperatures. Conclusions are based on the changes in relation to the start of the increase in temperature.

cultivar	ref.[1]	growing conditions			tissue	bioassay[2]	GA_3 equiv.	conclusion
		light/dark	temperature (°C)	period (weeks)			(ng g^{-1}) FW	
Apeldoorn	(1)	light	15	0	sprout	a	6.3	
		light	15	2	sprout	a	1.2	decrease
Apeldoorn	(2)	light	field	13.5	sprout	b	21.9	
		light	field	17	sprout	b	10.1	
		light	field	20.5	sprout	b	27.3	
		light	field	23	sprout	b	20.1	fluctuation
Elmus	(4)	light	18	0	sprout	a	0.07	
						c	0.10	
		light	18	1	sprout	a	0.71	
						c	0.31	
		light	18	2	sprout	a	0.24	
						c	0.26	increase
Paul Richter	(5)	dark	9	0	sprout	c	0.02	
		dark	9	1	sprout	c	14.4	
		dark	17	1	sprout	c	19.4	increase
		light	9	1	sprout	c	0	
		light	17	1	sprout	c	0.0001	decrease
Paul Richter	(8)	light	20	0.5	first	d		no change
		dark	20	0.5	internodes	d		increase
Paul Richter	(9)	light	20	3	first	d		increase
		dark	20	3	internodes	d		increase
Ralph	(10)	light	13	0	bulb	a	0.42	
		light	13	1	bulb	a	0.07	decrease
		light	18	1	bulb	a	0.62	
		light	18	4	bulb	a	0.70	increase

[1](1) van Bragt, 1971
(2) Hanks and Rees, 1980a
(3) Aung and De Hertogh, 1967
(4) Einert *et al.*, 1972
(5) Alpi and De Hertogh, 1975
(6) Suh *et al.*, 1983b
(7) Aung and De Hertogh, 1968
(8) Okubo and Uemoto, 1985
(9) Okubo and Uemoto, 1986
(10) Aung *et al.*, 1969

[2]a dwarf pea
b lettuce hypocotyl
c barley half-seed
d dwarf rice

In several studies, it was reported that during the cooling period the amount of free GA increased (Aung and De Hertogh, 1967; 1968; Hanks and Rees, 1980b; Suh *et al.*, 1983b). However, van Bragt (1971) concluded that no clear relationship existed between the level of endogenous GAs and the floral stalk elongation after cold storage. A second hypothesis was suggested by Hanks (1982). To explain the only partial substitution of the cold treatment by applied GAs, Hanks (1982) supposed an increase in GA sensitivity during the cooling period.

Other hormones

Numerous plant growth regulators have been applied to tulips to determine the effects on sprout growth and flower development. Kinetin could reduce the incidence of flower abortion (Hanks and Rees, 1977). Exogenous ethylene induced flower bud blasting and injections of kinetin and $GA_{4/7}$ made the tulips less susceptible to ethylene (de Munk and Gijzenberg, 1977; Moe *et al.*, 1978). However, the presence of increased ethylene production due to wounding of bulbs, did not cause flower abortion. In contrast, it appeared to enhance floral stalk growth and flower development (Saniewski and Kawa-Miszczak, 1992).

When the flower bud was removed before the rapid elongation of the floral stalk, floral stalk extension was reduced considerably. Application of indoleacetic acid (IAA) reversed the effect of flower bud removal (Hanks and Rees, 1977; Saniewski and de Munk, 1981; Okubo *et al.*, 1986; Saniewski, 1989), but GA_3 was ineffective (Hanks and Rees, 1977; Okubo *et al.*, 1986). This auxin-induced floral stalk elongation could be inhibited by application of the GA biosynthesis inhibitor paclobutrazol (Saniewski, 1989), suggesting that GA biosynthesis was involved in the auxin-mediated stimulation of floral stalk growth. When the effect of root removal was investigated, GA_3 application restored the inhibiting effects of root removal (Kawa-Miszczak *et al.*, 1992).

Hanks and Rees (1977) suggested that an auxin-mediated system depending on auxin produced in the gynoecium, regulates the elongation of the upper internodes. A gibberellin-mediated system was suggested to control especially the elongation of the lower internodes. Okubo and Uemoto (1985; 1986) and Saniewski *et al*, (1990) suggested that elongation of all internodes is controlled by interactions of auxins and gibberellins. It was suggested that GA stimulates the auxin-biosynthesis, -transport or -response system (Saniewski and de Munk, 1981). Yet, on the basis of the experiments described sofar, a complete model comprising the various hormone effects

and explaining the regulation of floral stalk elongation in tulip, cannot be presented.

To investigate hormonal control of growth processes, *in vitro* studies appear to be a very useful tool, because influences from other organs are eliminated and direct effects of growth substances can be observed. The effects of different growth regulators on growth of isolated floral stalks, leaves and pistils from tulip was widely investigated by Saniewski and Gabryszewska (1983) and Kawa and Saniewski (1990a,b,c). Isolated floral stalks from cooled bulbs elongated more than floral stalks from noncooled bulbs. In addition, GA treatment stimulated growth in floral stalks from cooled bulbs only. Auxin induced growth in floral stalks from both bulb treatments, but only when the floral stalks were cultured in the inverted position, enabling polar basipetal transport of auxin. Experiments with isolated floral stalk pieces, suggested an effect of GA on the auxin-sensitivity of tulip floral stalk tissue (Rietveld *et al.*, 1994). In addition, results of Kawa and Saniewski (1990b) indicated that GAs were involved in leaf growth. Abscisic acid (ABA) alone and in the presence of auxin or GA inhibited growth of isolated floral stalks (Kawa and Saniewski, 1990c).

Auxin, ABA, cytokinins and ethylene have been identified in tulip. It was reported that low temperature increased the level of cytokinins in tulip bulbs (Rakhimbaev *et al.*, 1978) and decreased the level of ABA (Syrtanova *et al.*, 1973; Rakhimbaev *et al.*, 1978). However, Franssen and Voskens (1992) reported that no significant increase or decrease occurred in the amount of ABA per g FW sprout during bulb storage at 5°C, neither during storage at 17°C. For IAA, preliminary results of Franssen and Voskens (1992) showed a lower amount of IAA per g FW in cooled sprouts then in sprouts from noncooled bulbs.

Outline of thesis

Hypothesis

In spite of numerous studies implicating the involvement of GAs in the cold requirement of tulip, their physiological role is still inconclusive. Two hypotheses have been put forward to explain the role of GAs in the cold requirement of tulip:
1) during the cooling period of the bulbs the amount of free GAs increases (Aung and De Hertogh, 1967; 1968; Hanks and Rees, 1980b; Suh *et al.*, 1983b). Alternatively, due to a block in the conversion to active GAs, inactive GAs or GA precursors may accumulate in noncooled bulbs (cf. Metzger, 1990).

2) during the cooling period the sensitivity to GAs increases (cf. Trewavas, 1981; Hanks, 1982).

Approach

The experiments in this study, were directed towards qualitative and quantitative analyses of endogenous GAs, and the biological activity and metabolism of applied GAs. The tulip cultivar investigated was "Apeldoorn", which has a relatively long cold requirement of 12 weeks at 5°C. The endogenous GA content was analysed using GC-MS. The biological activity and metabolism of applied GAs was studied using an *in vitro* sprout system.

Chapters 2 to 4 deal with the endogenous GA content in tulip bulbs. The level of endogenous GAs in vegetative material can be very low (1 - 10 ng g^{-1} FW, Bearder, 1980). For the analyses of GAs in tulip bulb material, an extended purification was required prior to analyses by GC-MS. In chapter 2 this method is described. The identification by GC-MS of GAs and GA-related compounds in sprouts of tulip bulbs is described in chapter 3. In chapter 4 the changes in endogenous gibberellin levels in tulip bulb sprouts and basal plates during storage and subsequent growth are reported. The results are discussed with regard to floral stalk elongation after planting cooled and noncooled bulbs.

Chapter 5 and 6 deal with the effect and the metabolism of applied GAs. In chapter 5 the effect of applied gibberellins and a GA biosynthesis inhibitor on isolated sprouts from cooled and noncooled tulip bulbs is described. The metabolism of [^{3}H]- and [$^2H_3$]GA_9 in isolated tulip sprouts is reported in chapter 6.

In chapter 7 the results are discussed and compared with other studies concerning tulip. The results are evaluated with regard to the cold requirement and the possibility to develop a practical bulb test, to predict the ability of tulip bulbs for adequate floral stalk elongation and flower development after planting .

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2 Extraction, purification and analysis of gibberellins in tulip material

Introduction

The analysis of gibberellins (GAs) in plant material is associated with a number of technical difficulties. Because the concentration of GAs can be extremely low especially in vegetative material (Bearder, 1980), a highly sensitive detection method is required. Furthermore, the detection method must be able to discriminate between the large number of different GAs that may be encountered. Finally, the absence of characteristic physical properties restricts the number of available detection methods.

Combined gas chromatography-mass spectrometry (GC-MS) is the method of choice for the analysis of GAs, being highly sensitive as well as suited for the identification and quantification of GAs. Still, prior to GC-MS analysis, a high degree of sample purity is required, to prevent overloading the system and to minimize interference by contaminating compounds. Many purification procedures have been reported, their complexity depending on the tissue under investigation. Hedden (1987) and Pearce *et al.* (1994) reviewed methods of GA analyses. For the analysis of GAs in tulip material, the method of Croker *et al.* (1990) was chosen and adapted to suit the requirements of this type of material. The general procedure is described in this chapter. Further improvements and/or refinements are included in the chapters 3 and 4.

The analysed material consists of sprouts and basal plates from tulip bulbs during the cold storage period. The bulb parts are isolated and stored at -75°C until extraction. The extraction and purification are performed within the pH range 2.5-8.5 and below 40°C, to prevent degradation of the GAs (Hedden, 1987). Additionally,

during the purification procedure prolonged storage in aqueous solutions is avoided wherever possible.

Extraction

Methanol, having a low boiling point and both polar and apolar properties, is an appropiate solvent for the extraction of GAs from plant tissue. The material (50 g) is homogenized in liquid N_2. Then, 200 ml methanol (4:1 v/w) is added to the frozen material, together with ascorbic acid (0.25 g) to prevent oxidation reactions during the extraction. The homogenate is stirred overnight at 4°C, after which insoluble material is removed by centrifugation (*ca.* 27,000x*g*). Residual insoluble matter is removed from the supernatant by filtration (glass filter no. 4).

Purification

Within the 90 different GAs that may be encountered in an extract, there is considerable variation in the number and positions of in particular hydroxyl functions, leading to a broad range of polarities. Yet, in order to minimize the final number of fractions to be analysed, it is desirable to purify the GAs as one group as far as possible. Therefore, as in most other purification procedures, the first part of the procedure is directed towards reducing sample weight by several group separation steps, in order not to overload the more subtle procedures at later stages of sample purification. The general procedure is outlined in Figure 2.1. To monitor recoveries during purification, tiny amounts of tritiated GA_1, GA_4 and GA_9 (25,000 dpm each) are added to the aqueous methanol extract. At several points in the procedure the recoveries are determined, which is indicated in Figure 2.1.

The methanol and part of the plant water is removed by evaporation under reduced pressure and at a temperature not exceeding 40°C. The volume of the aqueous residue is adjusted to 20 ml with H_2O and adjusted to pH 7.5-8.0 with 1 N KOH. The sample is washed with petroleum benzin 40-60 (3 x equal volume) to remove compounds such as lipids, fats and pigments. Subsequently, a PVP column is used to remove phenolic compounds. Sieved PVP (*ca.* 1 g, mesh size 40-70) is used in order to ensure reasonably fast flow rates. The aqueous GA extract is poured on the column and eluted with 2 x 2.5 ml of H_2O at pH 8 and the eluates are combined. At this stage, already a substantial reduction in sample weight is achieved, allowing more efficient partitioning at later stages.

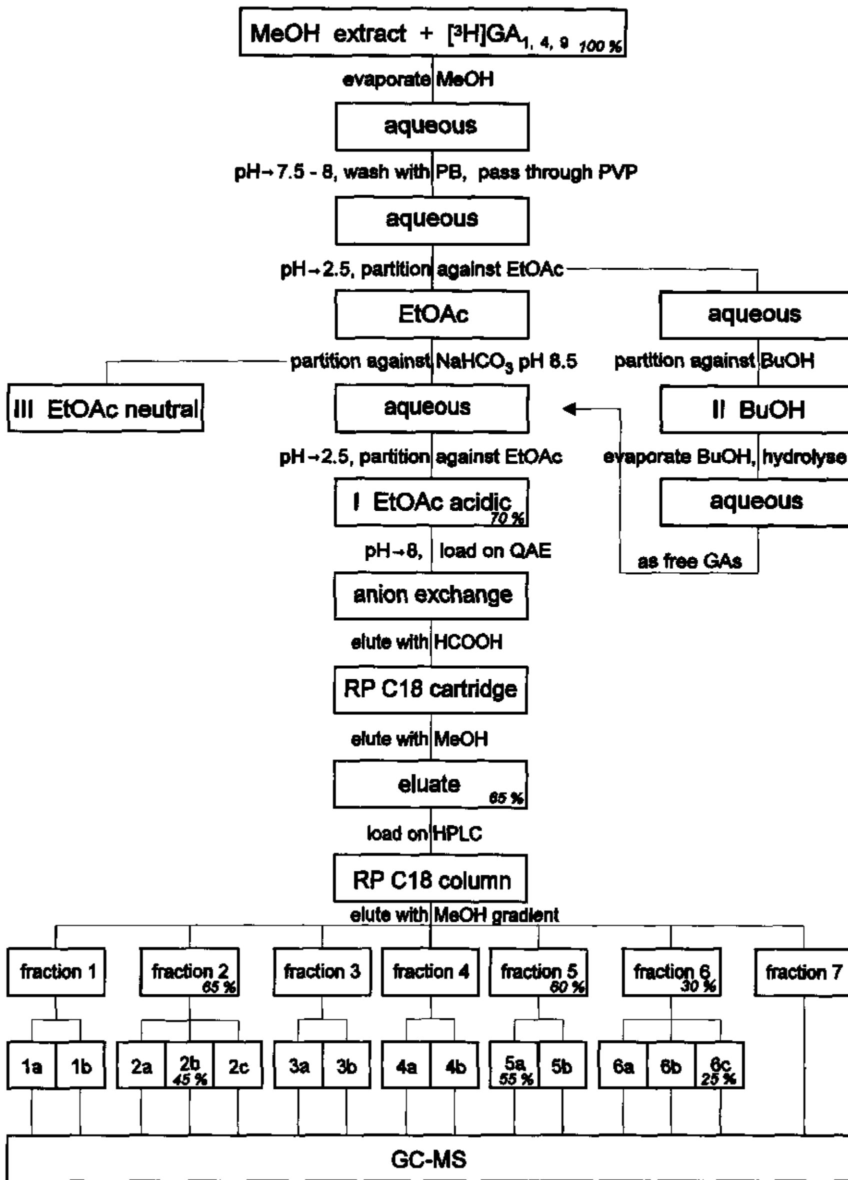

Figure 2.1 General purification procedure for the analysis of free and conjugated gibberellins (GAs) from tulip bulb material. Indicated are the combined recoveries of $[^3H]GA_1$, $[^3H]GA_4$ and $[^3H]GA_9$ after solvent partitioning and anion exchange chromatography. The individual recoveries of $[^3H]GA_1$, $[^3H]GA_4$ and $[^3H]GA_9$ after HPLC are given in the corresponding HPLC fractions: $[^3H]GA_1$ and $[^3H]GA_1Me$ in fractions 2 and 2b; $[^3H]GA_4$ and $[^3H]GA_4Me$ in fractions 5 and 5a; $[^3H]GA_9$ and $[^3H]GA_9Me$ in fractions 6 and 6c respectively.

Solvent partitioning

Since all GAs are weak organic acids (pKa ± 4.0; Hedden, 1987), changing the pH of the solvent will change their solubility. This property is used in solvent partitioning, to further reduce sample weight and also to separate the conjugated GAs from the free acids. The crude separation of the extract by solvent partitioning is included in Figure 2.1. The majority of the free GAs, including GA_1, GA_4 and GA_9, will be recovered in the ethyl acetate-soluble acidic fraction (I). The butanol-soluble fraction (II) will contain most GA conjugates, such as GA-glucosyl ethers and some glucosyl esters. Some very polar GAs may also be recovered in this fraction. The third, neutral ethyl acetate fraction (III), may contain some less polar glucosyl esters. In this study only the ethyl acetate acidic and the butanol soluble fractions are analysed (fractions I and II).

For the solvent partitioning procedure, the aqueous sample is adjusted to pH 2.5 with 6 N HCl to protonate the GAs, and partitioned against ethyl acetate (EtOAc, 3 x equal volume). For the acidification, HCl is preferred because this gaseous acid is removed during evaporation of the solvent and will not become concentrated. Some very polar and most of the conjugated GAs remain in the aqueous phase, from which they are extracted with *n*-butanol and saved for later analysis.

The combined organic phases are partitioned against 5% (w/v) sodium bicarbonate (3 x 1/5 volume). At its slightly alkaline pH of 8.5, the GA molecules will be dissociated and hence partition into the aqueous fraction, leaving neutral compounds in the ethyl acetate fraction. Then, to enable easy concentration of the extract, the combined aqueous phases are adjusted to pH 2.5 with HCl and partitioned against ethyl acetate (3 x 1/3 volume), which is subsequently concentrated to dryness by evaporation at 40°C. At this point the [^{3}H]GA recovery is determined and is usually *ca.* 70% of the total initial amounts.

After the solvent partitioning some major remaining impurities are fatty acids and sugars (Hedden, 1987). In tulip sprouts especially sugars may be present in such high concentrations (up to 0.2 g g^{-1} DW, Moe and Wickstrom, 1979), that although neutral and water soluble, small amounts may spill over into the ethyl acetate soluble acidic fraction.

Enzymatic hydrolysis of GA conjugates.

The aqueous phase left after ethyl acetate partitioning, contains conjugated GAs. This phase is repartitioned with *n*-butanol (3 x equal volume). The combined butanol phases

are reduced to dryness *in vacuo* at 40°C, with H_2O added to remove final traces of butanol. The residue is dissolved in 10 ml 0.5 M sodium acetate at pH 4.8. Small amounts (*ca.* 2 mg) of cellulase (EC 3.2.1.4; Sigma C-7377) and ß-glucosidase (EC 3.2.1.21; Sigma G-8625) are added and the sample is incubated for 24 h at 35°C. After hydrolysis, the pH is adjusted to 2.5 and the buffer phase is extracted with ethyl acetate (3 x equal volume). The ethyl acetate phases are taken to dryness *in vacuo* at 40°C. The sample residue is dissolved in 10 ml of H_2O, for QAE anion exchange chromatography as described for free GAs.

Anion exchange chromatography

Anion exchange chromatography exploits the similarity in acid strengths of the GAs and will further reduce the amount of neutral impurities in the extract.

In this study the anion exchanger QAE-Sephadex A-25 (Pharmacia) is used. A column of 5 cm long x 1 cm inner diameter is made, and equilibrated with 1% sodium formate at pH 8.0-8.5. The sample residue is dissolved in 10 ml of H_2O, adjusted to pH 8.0 (0.1 N KOH) in order to deprotonate the GAs, and subsequently applied onto the column in 4 x 10 ml. GAs, being negatively charged, are retained and neutral impurities are washed of with H_2O pH 8.0-8.5 (3 x 5 ml).

The GAs are eluted with 0.2 M formic acid (4 x 5 ml), at which acidity the GAs are neutral and will not be retained any longer. The eluate is loaded on-line onto a C18 Sep-Pak cartridge (Waters Associates, Milford, MA, USA) prewashed with methanol (5 ml) and H_2O (5 ml). This short cartridge allows quick reversed-phase separation of the extract. Moreover, it is a convenient way to reduce the sample volume. After washing with 2mM acetic acid + 1% methanol (2 x 5 ml), GAs are eluted with 80% aqueous methanol (5 ml), which is then evaporated to dryness at 40°C. The [^{3}H]GA recovery at this point is *ca.* 65%.

Reversed phase HPLC

Until this stage, the GAs are purified as a group. The final purification steps usually involve chromatographic procedures that result in separation of groups of GAs. Especially for the final stages of sample purification, high-performance liquid chromatography (HPLC) is the method of choice, because of its speed, high resolution power and reproducibility.

A Chromspher C18 column (Chrompack, Bergen op Zoom, The Netherlands, 250

mm long x 10.0 mm inner diameter) is used for the reversed-phase fractionation of the extract. The sample is applied dissolved in 1 ml 30% methanol and injected on to the column using a 1 ml loop. It appeared necessary to first dissolve the sample in 300 μl methanol and then make it up to 1000 μl with H_2O. Using the given system, this methanol concentration will not interfere with the separation and is sufficiently high to dissolve the sample. In order to fractionate the range of GAs, the sample is fractionated using a gradient of increasing methanol, containing 0.01% acetic acid to ensure protonation of the GAs: 0-5 min. 10%, 5-30 min. 10% to 70%, 30-40 min. 70 %, 40-50 min. 80 % aqueous methanol (column wash), at a flow rate of 4 ml min^{-1}. Detection occurs with an UV absorbance monitor at 210 nm.

The GAs elute in order of decreasing polarity, first described by Jones *et al.* (1980). Extensive lists with precise retention times in isocratic HPLC, were presented by Koshioka *et al.* (1983) and Jensen *et al.* (1986). By injections of available GA standards and with interpolation of data from Koshioka *et al.* (1983) and Jensen *et al.* (1986), a list of 48 GAs is compiled, which is included in Table 2.1.

For the qualitative analyses, seven putative GA-containing fractions of 8 - 36 ml are collected taking into account the retention times given in Table 2.1; 1: 12-19, 2: 19-22, 3: 22-28, 4: 28-31, 5: 31-33, 6: 33-36 and 7: 36-45 min. Figure 2.2A shows the fractionation by HPLC of an extract of tulip bulb sprouts, containing $[^3H]GA_1$, $[^3H]GA_4$ and $[^3H]GA_9$ as tracers.

At this stage, the individual recoveries of $[^3H]GA_1$, $[^3H]GA_4$ and $[^3H]GA_9$ can be determined. Typical values are 65, 60 and 30% respectively, of the initial amounts added.

Repetition of the HPLC procedure, using a different column or different liquids, will further fractionate the GA-containing fractions and improve the purity. Alternatively, the GA molecules can be modified in order to change their retention properties when using the same column and gradient. This second option is used in this study and has the advantage of the minimal adjustments and investments required. For this purpose, the carboxy functions of the GAs are converted to methyl (Me) esters with diazomethane. The methylation changes the polarity of the GAs, consequently changes the retention properties at the C18 column. Using the column and conditions as described above, this results in shifts of the retention times of 2 to 3 minutes compared with the free acids. Several C20-GAs have more than one carboxy function and will elute 3 to 10 minutes later. Other compounds (impurities) without carboxy functions, will not be methylated and retain their original elution time, or compounds become methylated but shift different than GAs.

Table 2.1 HPLC and GC retention times, Kováts retention indices (KRI) and characteristic ions for selected ion monitoring of Me and MeTMSi derivatives of gibberellins. HPLC retention times are shown both for the free acids and a number of methylated GAs. Indicated (bold) are data measured in this study; remaining data are obtained by interpolation of data from Koshioka *et al.* (1983), Jensen *et al.* (1986) and Gaskin and MacMillan (1991).

	HPLC		GC		
	R_t Free	R_t Me	R_t	KRI	principal ions
GA	(min.)	(min.)	(min.)		(*m/z*)
55	12.5-15		**24.0**	**2766**	**594 535 448 375**
8	**15.0**	**17.2**	**24.8**	**2823**	**594 535 448 379**
29	**16.0**		**22.9**	**2686**	**506 491 447 375**
39	16-17.5			·	**565 488 430 398**
33	16-17.5		**22.9**	**2684**	**520 430 383 358**
30	16-17.5		**22.4**	**2646**	**504 414 369**
23	17.5-20				**550 522 463 432**
28	20-21				**580 565 371 208**
38	20-21		**26.2**	**2942**	**520 505 430**
41	20-21		**24.7**	**2821**	**567 526 492 400**
26	20-21		**25.0**	**2847**	**520 402 343**
3	**20.5**	**22.8**	**23.2**	**2705**	**504 489 370**
1	**21.0**	**23.6**	**22.8**	**2677**	**506 491 448 313**
6	21-24				**432 417 373 302**
18	22-24		**22.2**	**2648**	**536 521 477 319**
35	22-24		**22.5**	**2653**	**506 416 390 326**
22	22-24		**23.0**	**2700**	**504 489 414 401**
21	22-24		**23.2**	**2715**	**462 447 430 403**
2	22-24		**24.0**	**2764**	**508 493 418 289**
31	22-24		**21.0**	**2556**	**416 385 326 222**
43	24-28				**580 431 371 349**
5	**26.4**	**29**	**20.1**	**2490**	**416 401 357 343**
10	26-28				**420 405 363 331**
16	26-28		**22.2**	**2645**	**506 416 390 360**
20	**27.2**	**29.7**	**20.1**	**2495**	**418 403 375 359**
27	24-28		**25.6**	**2900**	**520 430 343 223**
47	28-30		**22.3**	**2640**	**506 459 313**
36	28-30		**21.8**	**2605**	**462 430 402 312**
13	27-30	**42.5**	**21.8**	**2605**	**477 460 400 310**
40	27-30		**20.8**	**2539**	**371 343 299 225**
44	28-30				**432 417 373 238**
42	**28.5**		**23.5**	**2723**	**523 448 416 376**
19	30-32		**21.7**	**2607**	**462 434 402 374**
54			**21.8**	**2613**	**506 416 390 375**
34	**30.5**	**32.9**	**22.8**	**2672**	**506 416 372**
51	**30.6**	**33**	**20.6**	**2531**	**418 386 328 284**
17	**31.0**	**37**	**21.3**	**2584**	**492 460 433 401**
37	30-31		**24.2**	**2777**	**432 342 310 284**
7	**30.5**	**33.5**	**20.4**	**2511**	**416 384 356 298**
iso-7	**32.0**	**34.2**	**20.8**	**2537**	**416 384**
4	**32.7**	**35.2**	**20.6**	**2522**	**418 289 284 225**
53	**34.0**		**20.3**	**2506**	**448 389 251 208**
14	**34.8**	**44.5**	**20.1**	**2501**	**448 416 388 298**
24	**35.0**	**40-42**	**19.5**	**2461**	**342 314 286 226**
9	**35.5**	**39.9**	**17.7**	**2333**	**330 298 270 243**
25	36-40	38-42			**372 312 284 225**
15	36-42		**22.3**	**2628**	**344 312 284 239**
12	**45.0**		**18.0**	**2355**	**360 328 300 285**

Thus, the putative GA-containing fractions are evaporated to dryness, dissolved in 100 μl methanol and an ether solution of diazomethane is added until the solution remains yellow. After a few minutes at room temperature, excess diazomethane is removed with a stream of nitrogen. The fractions 1 to 6 are each refractionated using the same HPLC system and gradient as described before. For each fraction, 2 or 3 putative methylGA-containing fractions are collected, as shown in Table 2.2.

Table 2.2 Time intervals of the fractions collected during HPLC of a tulip bulb sprout GA extract. Presented are the retention times of collected fractions after separation of the original extract and after chromatography of the methylated fractions.

HPLC fraction nr.	free acids time interval (min.)	Me esters time interval (min.)
1	12-19	a 14-21
		b 21-29
2	19-22	a 20-22
		b 22-24.5
		c 24.5-32
3	22-28	a 24-30
		b 30-38
4	28-31	a 30-32
		b 32-35
5	31-33	a 33-35.5
		b 35.5-39
6	33-36	a 35-38
		b 38-41
		c 41-46
7	36-45	not refractionated

[^{3}H]GA$_1$Me, [^{3}H]GA$_4$Me and [^{3}H]GA$_9$Me elute in fractions 2b, 5a and 6c, respectively, their recoveries are usually about 45, 55 and 25%. Figure 2.2B shows an example of the refractionation of methylated fraction 5. Although further analysis demonstrated the presence of GA$_4$ in this fraction, no absorption peak representing GA$_4$ can be observed in this chromatogram.

In this way, a substantial reduction in the amount of contaminating compounds is achieved for the fractions 1 to 6. For fraction 7 the second fractionation appeared not necessary. This fraction is ready for GC-MS without further purification.

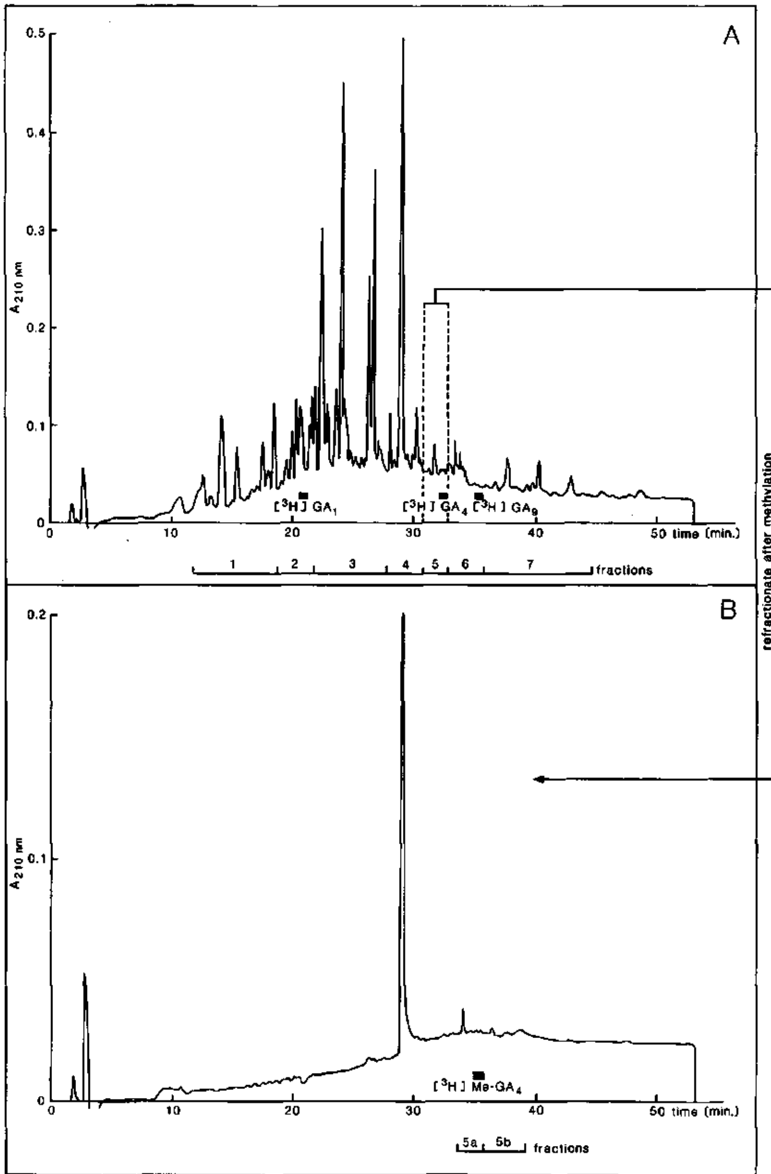

Figure 2.2 Fractionation by HPLC of an extract of tulip bulb sprouts, containing [^{3}H]GA$_1$, [^{3}H]GA$_4$ and [^{3}H]GA$_9$ as tracers. (A) HPLC profile after initial fractionation, and fractions collected as shown. [^{3}H]GA$_1$, [^{3}H]GA$_4$ and [^{3}H]GA$_9$ elution times are indicated. (B) fraction 5 of A refractionated after methylation, and fractions collected as indicated. The [^{3}H]GA$_4$Me elution time is indicated.

Qualitative analyses

To date, gas chromatography-mass spectrometry (GC-MS) is the most widely used method for the identification of GAs. The mass spectra together with retention indices provide sufficient evidence to distinguish and identify unequivocally all of the known GAs.

The mass spectrometer serves as a highly versatile GC detector. After GC separation, the compounds entering into the ion source of the mass spectrometer are ionized, resulting in a characteristic fragmentation of the molecule. Full mass spectra are obtained by scanning a mass range of interest, in this study from 200 to 600 atomic mass units (amu). The mass spectra are a plot of the relative abundance of fragment ions against their mass/charge ratio (*m/z*). Cyclic scanning of the mass range throughout a GC run generates hundreds of spectra, which are handled by computer. The stored data can be processed in several ways. Summing the ion currents for each scan yields a reconstructed total ion current chromatogram (TIC) which is equivalent to a flame ionisator detector (FID) output. When a reference compound or a reference spectrum is available for comparison, a reliable identification can be made on the basis of the two spectra. In practice, ions at low *m/z* values occur with high frequency and are common to many spectra. Consequently, in comparisons heavier ions are more important than light ones. The GC retention times are additional parameters and in some cases comparisons of retention times are essential. Certain epimers such as GA_{34} and GA_{47} give virtually identical mass spectra and can be distinguished using GC-MS only on the basis of their retention times.

The sensitivity of a mass spectrometer can be improved about 100-fold, if used to monitor only a limited number of ions. Furthermore, taking into account the retention times of the possible GAs in the sample, the selectivity can be enhanced by scanning sets of specific masses for selected time intervals only. This selective ion monitoring (SIM) method can be used for identification of GAs present in amounts too small to yield full mass spectra. Of greater interest, however, is its use for quantitative analyses, as described in a later section.

Prior to GC analysis, derivatization of the GAs is required to increase their volatility. For this purpose, the carboxylic acid groups are usually converted to methyl esters and hydroxyl groups are converted to trimethylsilyl (TMSi) ethers. In this study the GAs are already methylated during the HPLC procedure. The trimethylsilylation further decreases the polarity of the GA methyl esters, but, more important, it improves their mass spectral characteristics. The TMSi ethers are more likely than the

Me esters to give intense molecular ions, and they give characteristic fragmentation patterns that may help in GA identification.

In this study, for trimethylsilylation the fractions are heated in 15 μl Derivasil at 70°C for 10 min. This silylating agent (Chrompack, Bergen op Zoom, The Netherlands) is a mixture of bis-trimethylsilyltrifluoroacetamide : trimethylchlorosilane : trimethylsilylimidazole : pyridine (3:2:3:10). Before adding the reagent, it is important that the sample is thoroughly dry since both the reagent and derivative are decomposed by moisture.

Derivatized samples are analyzed using a Hewlett Packard 5890 GC coupled to a HP 5970 mass selective detector (Hewlett Packard, Amstelveen, The Netherlands).

Samples (4 μl) are injected splitless into a fused silica capillary column (CPSil 5CB, Chrompack, Bergen op Zoom, the Netherlands; 50m x 0.25 mm x 0.4 μm film thickness) and separated in a temperature gradient: 0-2 min. 70°C, 2-11 min. 70°C to 250°C, 11-31 min. 250°C to 300°C. Carrier gas is He at 1.2 ml min^{-1}. The injector- and interface temperature are 250°C and 290°C respectively.

The gas chromatographic behaviour of GA Me esters and Me esters TMSi ethers has been published extensively. Table 2.1 includes the retention times of 48 GAs, obtained on a CPSil5B column. Absolute retention times will differ from system to system, depending on temperature program, gas flow, column coating and condition. In the course of this study different columns and of different sizes have been used, such as a CPSil 8CB (Chrompack, 50 m x 0.22 mm x 0.12 μm film thickness) and an Ultra-1 column (Hewlett Packard, 25 m x 0.20 mm x 0.33 μm film thickness). Consequently, the temperature programs were adapted and different retention times were obtained. However, relative retention times can usually be predicted with reasonable accuracy. The Kováts Retention Index (KRI) is a convenient parameter for comparing retention times when different systems or conditions are used. This value is calculated from the retention times of alkanes co-injected with the sample. The retention index is related to retention time by

$$RI = 100 \frac{R_x - R_n}{R_{n+1} - R_n} + 100n$$

where R_x is the retention time of the compound of interest. R_n and R_{n+1} are the retention times of the alkanes with respectively n and n+1 carbon atoms, which elute respectively immediately before and after the compound of interest.

In this study the KRI values were determined by coinjecting available standards with paraffin wax (0.5 μg, congealing point about 55°C). These values are included in Table 2.1. A comprehensive list of KRI values and spectra of the methyl esters of

non-hydroxylated GAs and the MeTMSi derivatives of hydroxylated GAs, is published by Gaskin and MacMillan (1991). The spectra and KRI of the tulip extracts were compared with the measured values and to published data to establish the identity of the endogenous GAs in tulip extracts.

An example of the GC fractionation and MS detection of a tulip sprout extract is presented in Figure 2.3. Figure 2.3A displays a TIC trace and in 2.3B the full-scan spectrum at the expected retention time of GA_4MeTMSi is shown. For comparison, the full-scan spectrum of authentic GA_4MeTMSi is shown in Figure 2.3C. A useful application is to reconstruct chromatograms of individual ion currents from the sample spectra, which may reveal the presence of GAs hidden in co-eluting impurities. Figure 2.3D presents a series of mass chromatograms for ions characteristic in the mass spectrum of GA_4MeTMSi, reconstructed from the TIC trace of Figure 2.3A. Although GA_4 is poorly distinguishable in the TIC trace and can only be tentatively identified based on the spectrum in 2.3B, the mass chromatograms of the characteristic *m/z* values clearly indicate its presence at the expected retention time. In chapter 3 the identification of endogenous GAs in tulip sprouts is described.

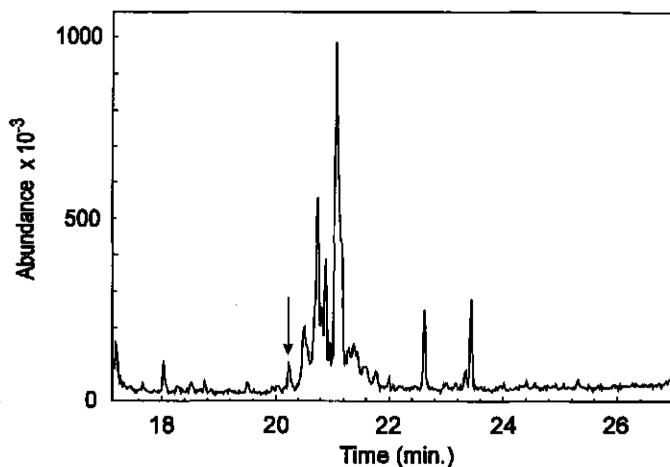

Figure 2.3 GC-MS of a methylated and trimethylsilylated extract of tulip sprouts (fraction 5a of Table 2.2). (A) Total ion current (TIC) trace. The expected retention time of GA_4MeTMSi is indicated by the arrow.

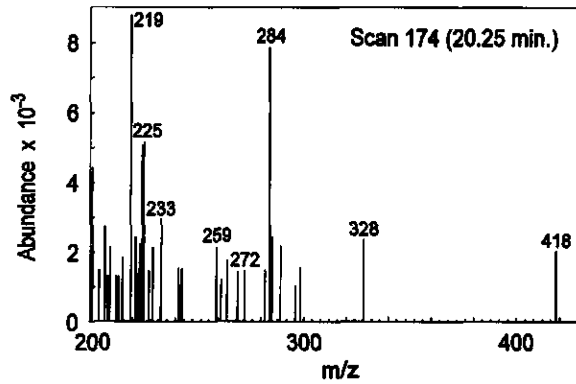

Figure 2.3 (B) Full-scan mass spectrum at the indicated retention time in (A)

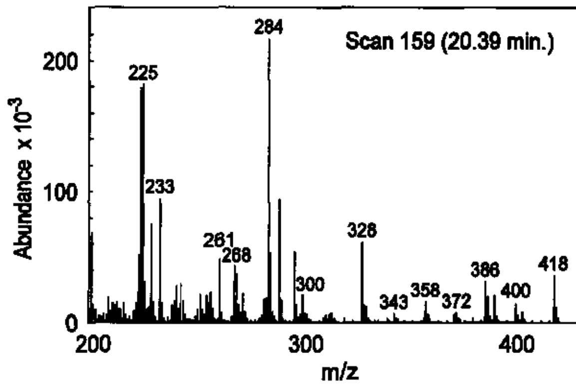

Figure 2.3 (C) Full-scan mass spectrum of authentic GA_4MeTMSi.

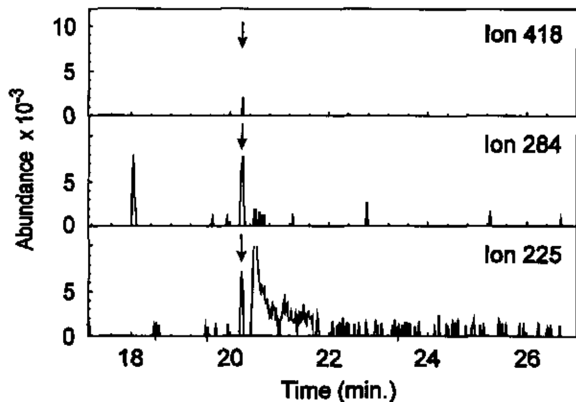

Figure 2.3 (D) Mass chromatograms for ions characteristic of the mass spectrum of GA_4MeTMSi, reconstructed from the TIC of (A). The arrows indicate the retention time of GA_4MeTMSi.

Quantitative analyses

Quantitative analysis are performed using SIM. Using isotopically-labelled GAs as internal standards, GC-SIM provides a highly sensitive and selective quantification method (Hedden, 1987). The mass spectrometer is set to monitor specific masses rather than to scan the entire mass range.

The use of [$^2H_2$]GAs as internal standards which are added at the start of the extraction, corrects for sample losses during purification and chromatography. [$^2H_2$]GAs are heavier than the nonlabelled GAs. Figure 2.4 shows the mass spectra of GA_4MeTMSi and [$^2H_2$]GA_4MeTMSi.

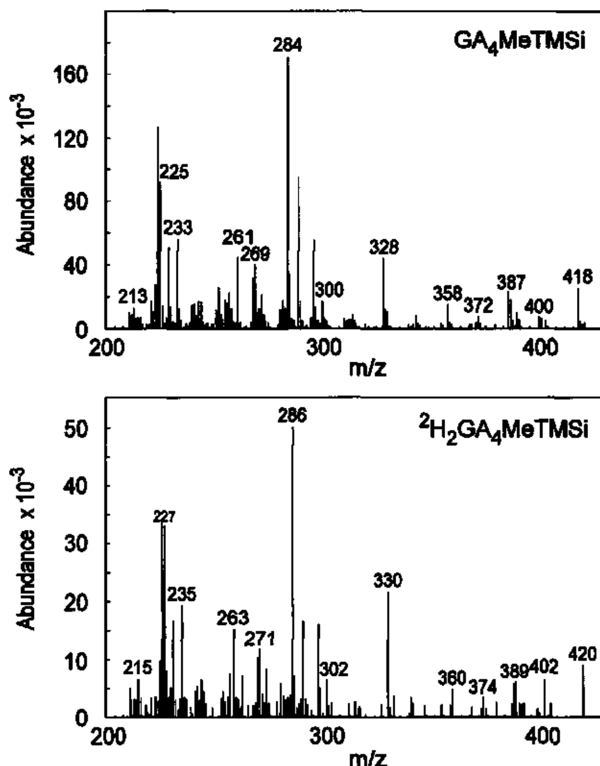

Figure 2.4 Mass spectra of GA_4MeTMSi and [$^2H_2$]GA_4MeTMSi.

A calibration curve is constructed by mixing GA_4 and [$^2H_2$]GA_4 in different proportions and measuring peak areas from the SIM traces. For GA_4 and [$^2H_2$]GA_4 the

m/z values 284, 286, 418 and 420 are monitored and separate curves are made for *m/z* 284/286 and 418/420. A possible small intercept on the y-axis represents the minor contribution of e.g. deuterated GA_4 to the ion at *m/z* 284, due to *m/z* 282 in the spectrum of GA_4MeTMSi. Of more importance, however, is the correction for the contribution from natural isotopes: the contribution from e.g. non-labelled GA_4 to the ion at *m/z* 420 is 6.8%. An example of a corrected calibration curve for the ions at *m/z* 418 and 420 is presented in Figure 2.5.

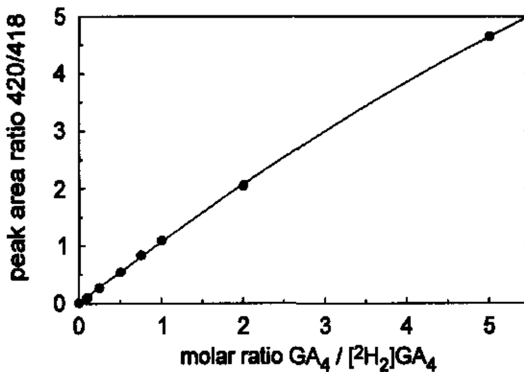

Figure 2.5 Corrected calibration curve for isotope dilution analyses of GA_4MeTMSi by GC-MS using [$^2H_2$]GA_4MeTMSi as an internal standard, after subtracting 6.8% of the intensity of the *m/z* ion 418 from that of the *m/z* ion 420.

In the present study, at the extraction [$^2H_2$]GA_1, [$^2H_2$]GA_4, [$^2H_2$]GA_9, [$^2H_2$]GA_{24} and [$^2H_2$]GA_{34} (100 ng each) are added together with the methanol for quantification of their endogenous levels. They were obtained from L.N. Mander, Research School of Chemistry, Canberra, Australia. The amounts of internal standards to be added were estimated in preliminary experiments.

When the identity of the endogenous GAs has been established, the purification procedure may be simplified. In this study, the HPLC procedure is refined by decreasing the number and size of the collected fractions: the HPLC effluent is collected in 4 fractions of 4.8 - 8.8 ml (1: 20.7-21.9, 2: 29.5-31.5, 3: 32-33.2 and 4: 34-36.2 min.) for respectively GA_1, GA_{34}, GA_4, and GA_9 + GA_{24}. All fractions are methylated with excess ethereal diazomethane and taken to dryness. The fraction containing both GA_9 and GA_{24} is ready for GC-MS without further purification. The other fractions are each fractionated again, using the same RP HPLC system and gradient.

Small fractions of 4.8 - 6.4 ml containing GA_1Me (23.2-24.4 min.), $GA_{34}Me$ (32.4-34 min.) and GA_4Me (34.7-35.9 min.), are collected and evaporated to dryness.

In the GC-SIM analyses, two or four characteristic ions are monitored for each GA with dwell times of 100 ms:

$GA_1MeTMSi$	and	$[^2H_2]GA_1MeTMSi$	: *m/z* 506 and 508
$GA_4MeTMSi$	and	$[^2H_2]GA_4MeTMSi$	: *m/z* 284, 286, 418 and 420
GA_9Me	and	$[^2H_2]GA_9Me$	: *m/z* 298 and 300
$GA_{24}Me$	and	$[^2H_2]GA_{24}Me$	: *m/z* 314 and 316
$GA_{34}MeTMSi$	and	$[^2H_2]GA_{34}MeTMSi$	: *m/z* 506 and 508

The ion chromatograms of the ions monitored for the internal standard and endogenous GA are integrated. After correction for the contribution of the natural isotopes, the peak area ratios are entered into the calibration curves. In the present study the limit of detection of the analysed GAs is of the order of *ca.* 1 ng per injection in GC-MS and *ca.* 0.01 ng in GC-SIM. The results of the quantitative analyses of this study, are described in chapter 4.

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3 Identification of gibberellins in tulip bulb sprouts during storage[1]

Mariken Rebers, Evert Vermeer, Erik Knegt, Cathryn J. Shelton[2] and Linus H.W. van der Plas

Summary

The involvement of gibberellins in the regulation of floral stalk elongation and flowering has been implicated in cold-requiring plants, including tulip (*Tulipa gesneriana* L.). To investigate their role in tulip, an inventory was made of GAs, including conjugated forms, in sprouts of cooled and noncooled bulbs of cv. Apeldoorn. Using gas chromatography-mass spectrometry GA_4, GA_9, GA_{12}, GA_{24}, GA_{34} and three GA-related compounds were detected. All detected GAs and GA-related compounds were found in the free, as well as in the conjugated form. They occurred in sprouts of both cooled and noncooled bulbs.

Introduction

Tulip bulbs, with terminal buds containing a complete flower, require a period of low temperature for floral stalk elongation and adequate flowering. In this cold requirement the involvement of gibberellins has been implicated (van Bragt and Zijlstra, 1971; van Bragt and van Ast, 1976; Hanks and Rees, 1977; Hanks, 1982; Saniewski, 1989).

A general role of GAs has been suggested in the regulation of bolting in cold-

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requiring plants (Pharis *et al.*, 1987). In tulip, it has been shown that application of GAs could partly replace the cold treatment (van Bragt and Zijlstra, 1971; van Bragt and van Ast, 1976; Hanks, 1982), whereas inhibitors of GA biosynthesis inhibited floral stalk elongation of cooled tulip bulbs (Shoub and De Hertogh, 1974; Suh *et al.*, 1992). The inhibiting effect of ancymidol could be reversed by simultaneous application of GA_{4+7} (Shoub and De Hertogh, 1974).

Previous studies, using bioassay procedures, have revealed the presence of GA-like substances in tulip bulbs (Aung and De Hertogh, 1967). It was shown that the quantitative and qualitative nature of these substances was altered by subjecting the bulbs to either varying periods of cold or by different temperature regimes (Aung and De Hertogh, 1968; Aung *et al.*, 1969; Syrtanova and Rakhimbaev, 1973). Terminal buds contained higher levels of extractable GA-like substances than the scales (van Bragt, 1971). Partial characterization of GAs in tulip bulbs has been obtained. By co-chromatography of purified extracts with available standards, the presence of GA_1, GA_5, GA_8, GA_9 and GA_{13} was suggested (Aung *et al.*, 1971). However, precise data on the identitities of GA-like substances were still lacking.

Unequivocal identification of endogenous GAs is essential before trying to interpret any physiological role of endogenous or applied GAs. The purpose of the present work was to identify conclusively the GAs in tulip sprouts as part of an extensive study of the role of GAs in the cold requirement of tulip. The application of gas chromatography-mass spectrometry (GC-MS) and GC-selected ion monitoring (SIM) provides an advanced method for GA analyses. In the present paper, the identification by GC-MS of five GAs and three GA-related compounds in sprouts of tulip bulbs is described.

Material and methods

Plant material

Field grown tulip bulbs (*Tulipa gesneriana* L. cv. Apeldoorn) were harvested in July 1990 and stored at 20°C until October. Then, one group of bulbs was transferred to 5°C, while another group was stored at 17°C. After selected storage durations, the sprouts were isolated from the bulbs and stored at -75°C until extraction.

Extraction and purification procedures

The purification procedure was adapted from Croker *et al.* (1990). Sprouts (50 g) were homogenized in liquid N_2, and methanol (MeOH) (4:1 v/w) was added together with ascorbic acid (0.25 g). The homogenate was stirred overnight at 4°C. After centrifugation (*ca.* 27,000 *g*) and filtration (glass filter no. 4), small amounts of tritiated GA_1, GA_4 and GA_9 (25,000 dpm each) were added to monitor recoveries during purification. MeOH was removed under reduced pressure at 40°C. The aqueous residue was adjusted to pH 7.5-8.0 (1 N KOH) and washed with petroleum benzin 40-60 (3 x equal volume). The aqueous phase was loaded on to a PVP column (*ca.* 1 g) prewashed with H_2O. The column was eluted with H_2O (2 x 2.5 ml) at pH 8 and the eluates were combined, adjusted to pH 2.5 (6 N HCl) and partitioned against ethyl acetate (EtOAc) (3 x equal volume). The aqueous phase contained conjugated GAs and was saved for later analysis. The combined organic phases were partitioned against 5% (w/v) sodium bicarbonate (3 x 1/5 volume). The combined aqueous phases were acidified to pH 2.5 (6 N HCl) and partitioned against EtOAc (3 x 1/3 volume), that was taken to dryness *in vacuo* at 40°C.

The residue was dissolved in 10 ml of H_2O, adjusted to pH 8.0 (0.1 N KOH), and loaded in 4 x 10 ml onto a QAE Sephadex A-25 anion exchange column (5 cm long, 1 cm inner diameter) preequilibrated with 1% sodium formate pH 8.0-8.5. The column was washed with H_2O pH 8.0-8.5 (3 x 5 ml) and GAs were eluted with 0.2 M formic acid (4 x 5 ml) and loaded directly onto a C18 Sep-Pak cartridge prewashed with MeOH (5 ml) and H_2O (5 ml). After washing with 2mM acetic acid + 1% MeOH (2 x 5 ml), GAs were eluted with 80% aqueous MeOH (5 ml), which was then evaporated to dryness *in vacuo* at 40°C.

Samples were fractionated by reversed-phase (RP) HPLC using a Chromspher C18 column (250 mm long x 10.0 mm inner diameter) and a gradient of increasing MeOH containing 0.01% acetic acid (0-5 min. 10%, 5-30 min. 10% to 70%, 30-40 min. 70 %, 40-50 min. 80 % aqueous MeOH), at a flow rate of 4 ml min^{-1}. Samples were dissolved in 300 μl MeOH, made up to 1 ml with H_2O, and injected on to the column using a 1 ml loop. Eight putative GA containing fractions of 8 - 24 ml were collected and evaporated to dryness (1: 12-19, 2: 19-22, 3: 22-28, 4: 28-31, 5: 31-33, 6: 33-36, 7: 36-39 and 8: 39-45 min.). All fractions were methylated with excess ethereal diazomethane and evaporated to dryness.

Fractions 7 and 8 were ready for GC-MS without further purification. Fractions 1 to 6 were each fractionated again, using the same RP HPLC system and gradient.

Putative methyl-GA containing fractions of 8 - 12 ml were collected and taken to dryness.

Enzymatic hydrolysis of GA conjugates

The original aqueous phase following EtOAc partitioning contained conjugated GAs. This phase was repartitioned with *n*-butanol (3 x equal volume). The combined butanol phases were reduced to dryness *in vacuo* at 40°C with H_2O added to remove final traces of butanol, and enzymatically hydrolysed with cellulase (EC 3.2.1.4) and ß-glucosidase (EC 3.2.1.21) at 35°C in 10 ml 0.5 M sodium acetate (pH 4.8) for 24 h. After hydrolysis, the pH was adjusted to 2.5 and the buffer phase was extracted with EtOAc (3 x equal volume). The EtOAc phases were taken to dryness *in vacuo* at 40°C. The extract was dissolved in 10 ml of H_2O, for QAE anion exchange chromatography as described for free GAs.

GC-MS Analyses

Fractions were trimethylsilylated by heating in bis-trimethylsilyltrifluoroacetamide:trimethylchlorosilane:trimethylsilylimidazole:pyridine (3:2:3:10) (15 μl) at 70°C for 10 min. Samples (4 μl) were injected splitless into a WCOT fused silica capillary column (25 m x 0.20 mm x 0.33 μm film thickness) at an oven temp of 70°C. After 2 min. the oven temp was increased at 30°C $min.^{-1}$ to 250°C and then at 2.5°C $min.^{-1}$ to 310°C. Carrier gas was He at 1.2 ml min^{-1}. The injector- and interface temp were 275°C and 310°C respectively. Mass spectra were acquired at 70 eV after 13 min., scanning from 200 to 600. The amounts of some GA-like compounds were too small to give full scan mass spectra, these were rerun using selected ion monitoring (SIM). The Kováts retention indices (KRI) were determined by coinjecting the samples with paraffin (0.5 μg). Spectra and KRI were compared to pure standards or to published data (Gaskin and MacMillan, 1991).

Results and Discussion

An inventory was made of GAs including the conjugated forms, in sprouts from bulbs cooled for different periods of time. The results of HPLC and GC-MS for authentic and identified GAs are summarized in Table 3.1.

The presence of GA_4, GA_9, GA_{12} and GA_{24} could be demonstrated by comparing

full scan mass spectra and KRI with available standards. GA_{34} was detected by GC-SIM. Additionally, three GA-related compounds were detected.

Table 3.1 Comparison of HPLC and GC retention times, Kováts retention indices (KRI) and relative intensities of characteristic ions for MeTMSi derivatives of GAs in tulip bulb sprouts, with those of authentic GAs. HPLC retention times are shown both for the free and methylated GAs and GA containing fractions. For free GAs and conjugated GAs after hydrolysis, the same ions and abundances were observed.

		HPLC		GC		
		R_t Free	Me	R_t	KRI	principal ions and relative abundance
GA		(min.)	(min.)	(min.)		*m/z* (% base peak)
34	Ref.	30.5	32.9	17.3	2700	506 (100), 416 (8), 288 (19), 229 (24), 223 (31), 217 (36)
	Sample	28-31	32-35	17.3	2699	506 (100), 416 (6), 288 (19), 229 (11), 223 (16), 217 (37)
4	Ref.	32.7	35.2	15.6	2557	418 (27), 328 (39), 289 (48), 284 (100), 233 (31), 224 (65)
	Sample	31-33	32-35.5	15.6	2557	418 (25), 328 (30), 289 (55), 284 (100), 233 (52), 224 (94)
24	Ref.	35	41-46	15.1	2510	374 (4), 342 (35), 314 (100), 286 (97), 226 (97), 254 (50)
	Sample	33-36	41-46	15.1	2510	374 (4), 342 (40), 314 (100), 286 (97), 226 (97), 254 (57)
9	Ref.	35.5	39.9	13.7	2381	330 (9), 298 (100), 270 (61), 243 (46), 226 (50), 217 (20)
	Sample	33-36	38.5-41	13.7	2381	330 (10), 298 (100), 270 (60), 243 (40), 226 (35), 217 (17)
12	Ref.	44.5	n.d.	13.9	2404	360 (2), 328 (30), 300 (100), 285 (19), 241 (25), 225 (14)
	Sample	39-45	n.d.	13.9	2404	360 (3), 328 (25), 300 (100), 285 (20), 241 (22), 225 (21)
12OH-12	Sample	22-28	n.d.	15.9	2609	448 (7), 416 (50), 388 (32), 326 (30), 298 (100), 239 (65)
OH-12	Sample	33-36	n.d.	15.2	2546	448 (20), 416 (10), 388 (24), 345 (60), 298 (25), 239 (40), 207 (100)
?	Sample	22-28	n.d.	18.5	2812	594 (12), 504 (32), 491 (100), 401 (47), 360 (22), 342 (36)

n.d. = not determined

Based on their molecular ions, two of these were hydroxylated derivatives of GA_{12}. One of them has been detected previously and is probably 12-OH-GA_{12} (Gaskin and MacMillan, 1991). The third GA-related compound contained three hydroxyl groups based on *m/z* 594, but could not be identified.

All detected GAs and GA-related compounds were also detected after hydrolysis of the aqueous phase containing putative conjugated GAs. Apparently they were present in the free as well as in the conjugated form. They occurred in sprouts of both cooled and noncooled bulbs. By comparison with the abundances of mass ions of known amounts of standards, the endogenous amounts were estimated to be less than 10 ng g^{-1} fresh weight.

In previous studies (Aung *et al.*, 1971), co-chromatography of purified tulip extracts by GC suggested the presence of GA_1, GA_5, GA_8, GA_9 and GA_{13}. GA_1, GA_5, GA_8 and GA_9 were detected in the scales, GA_1 in the shoot, and GA_5, GA_9 and GA_{13} in the roots. In the present study only sprouts were analysed and GA_1 was not detected. GA_1 is generally suggested to be the main endogenous GA active in many plants. Yet, GA_1 was not detected in the sprouts analysed in the present study, although [^{3}H]GA_1 was clearly recovered in the corresponding HPLC fraction (data not shown).

In the present study GA_4, GA_9, GA_{12}, GA_{24} and GA_{34} were identified. Except for GA_9, these GAs have not been identified in tulip before (Aung *et al.*, 1971). However, in that report the complete identification of GAs in the bulb extracts was prevented by a lack of a more extended set of authentic GA standards. Many of the biologically active GA-like substances did not correspond with any of the gibberellin standards available. Therefore, a more complete identification of the tulip GAs had to await the availability of known GA standards and/or the use of mass spectrometry. Considering the GLC retention times, the 'bound' compound in shoots eluting at 8.6 min. in the previous study (Aung *et al.*, 1971), might have been GA_{24} and the compound in the scales eluting at 8.0 min. possibly was GA_{12}. The latter however was not detected in the shoots. One of the retention times 37.0 and 40.5 min. (Aung *et al.*, 1971), might originate from the unidentified GA-related compound with probably three hydroxyl groups, detected in the present study.

Besides different results due to detection and identification methods, another explanation might be sought in the material analysed. In the earlier study, developing shoots from rooted bulbs after wet-storage at 9°C were analysed. In the present study, sprouts were analysed after dry bulb storage at 5°C, conditions where no rooting occurs and the sprouts only double in size.

The detected GAs belong to two families of GAs with different hydroxylation patterns: the non- and the 3-hydroxylated GAs. The conversion of GA_9 to GA_4 has been demonstrated in higher plants (Sponsel, 1987), suggesting that the major pathway operating in tulip sprouts during storage is $GA_{12} \rightarrow GA_{15} \rightarrow GA_{24} \rightarrow GA_9 \rightarrow GA_4 \rightarrow GA_{34}$ (Figure 3.1). The amount of GA_{15} present might have been below the detection level. Comparable results have been obtained in related plant families. In extracts from dormant bulbs of *Lilium elegans*, GA_4, GA_9, GA_{12}, GA_{15}, GA_{24} and GA_{34} were identified in addition to GA_1, GA_{19}, GA_{20}, GA_{44}, GA_{51} and 3-epi-GA_{34} (Takayama *et al.*, 1993). In dormant bulbils of *Dioscorea opposita,* GA_4, GA_9, GA_{12} and GA_{24} were detected as well as were GA_{19}, GA_{20}, GA_{36} and GA_{53} (Tanno *et al.*, 1992).

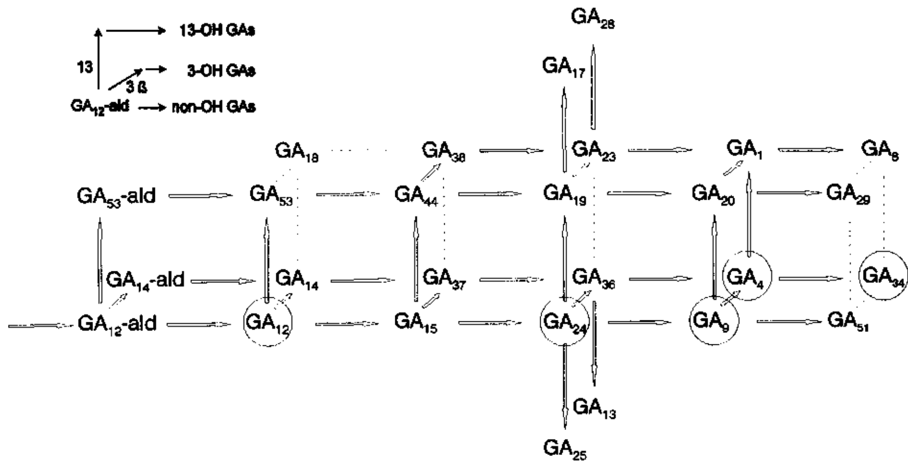

Figure 3.1 Metabolic grid of GAs in higher plants showing known conversions. The GAs detected in sprouts of tulip bulbs are encircled. Dotted lines indicate hypothetical conversions. Adapted from Kamiya *et al.* (1985) and Sponsel (1987).

Remarkably, 13-hydroxylated GAs, present in many plant species and identified in *Lilium* and *Dioscorea*, were not detected in any of the tulip sprout extracts. These GAs possibly were not present in tulip sprouts, or present in amounts below the detection limits of the system used. Apparently, in tulip sprouts during storage, 13-hydroxylated GAs are not the major occurring gibberellins. In *Thlaspi arvense*, a cold-requiring winter annual, it was suggested that two pathways for GA metabolism function in the regulation of different processes. The non-hydroxylation route leading

to GA_9 may regulate thermoinduced floral stalk growth, while the 13-hydroxylation pathway leading to GA_1 possibly controls processes such as petiole growth, that proceed in noninduced plants (Metzger, 1990). In the case of tulip, this would mean that GA_1 or other 13-hydroxylated GAs may be detected when analysing other parts of the plant, but are not involved in the cold-induced floral stalk elongation and flowering of tulip[3].

The occurrence of conjugated GAs in extracts of tulip bulbs has been established before (Aung and De Hertogh, 1968), but they were not characterized individually.

Although GA-like compounds have previously been detected in tulip sprout extracts, this is the first report of the unequivocal identification of GAs in this species.

Acknowledgements

We acknowledge the gift of $[^3H]GA_9$ from A. Crozier, University of Glasgow, UK and of GA_{12} and GA_{24} from N. Murofushi, University of Tokyo, Japan. GA_9 was a gift from the Department of Plant Cytology and Morphology, Agricultural University Wageningen, The Netherlands. GA_{34} was synthesized with the assistance of B. Twitchin, Research School of Chemistry, Australia. This research was supported by the Bulb Research Centre, Lisse, The Netherlands.

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4 Gibberellins levels and floral stalk elongation in tulip[1]

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Summary

To investigate the role of gibberellins (GAs) in the cold requirement of tulip (*Tulipa gesneriana* L. cv. Apeldoorn), bulbs were dry-stored at 5°C or at 17°C for 12 weeks prior to planting at 20°C. Only cooled bulbs showed rapid sprout growth and developed a full-grown flower. Changes in endogenous GA levels were measured in sprouts and basal plates during bulb storage as well as after planting, by GC-SIM using deuterated internal standards. GA_4 and GA_{24} were the major gibberellins, with levels up to *ca.* 10 ng per sprout, floral stalk or basal plate. GA_1, GA_9 and GA_{34} were present in much lower amounts. During bulb storage the level of GA_4 per sprout increased, especially in noncooled bulbs. After 12 weeks, these sprouts contained more GA_4 and GA_1 than cooled sprouts. After planting cooled bulbs, the level of GA_4 in the floral stalks tended to increase, whereas in planted noncooled bulbs this increase did not occur.

There was no direct correlation between cold-stimulated growth and a change in the endogenous GA status in sprouts or basal plates during cold bulb storage.

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Introduction

Tulip bulbs (*Tulipa gesneriana*), with terminal buds containing a complete flower, require a period of low temperature for rapid floral stalk elongation and adequate flower development at subsequent higher temperatures. In this cold requirement the involvement of gibberellins has been implicated (Aung and De Hertogh, 1967; Hanks, 1982; Saniewski, 1989). Application of GAs could partly substitute for the cold treatment (van Bragt and Zijlstra, 1971; Hanks, 1982). In addition, cold-stimulated floral stalk elongation was suggested to require GA biosynthesis (Shoub and De Hertogh, 1974; Saniewski, 1989). Floral stalk elongation in isolated sprouts could be inhibited by a GA biosynthesis inhibitor and this effect was reversed by simultaneous application of GA_4 or GA_9 (Rebers *et al.*, 1994b, chapter 5). Since GA sensitivity (Trewavas, 1982) appeared not to be the limiting factor for noncooled bulbs (Rebers *et al.*, 1994b), research is directed towards the endogenous GA content.

Previous studies, using bioassay procedures, reported an increase of the endogenous levels of GA-like substances during the cold treatment (Aung and De Hertogh, 1967; Aung and De Hertogh, 1968; Hanks and Rees, 1980). However, van Bragt (1971) concluded that no clear relationship existed between the level of endogenous GA-like substances at the end of bulb storage and the cold-stimulated floral stalk elongation at subsequent higher temperatures.

Evidence for increased activity of gibberellin-like substances in relation to cold-stimulated floral stalk elongation is inconclusive. We investigated the endogenous GA content in tulip bulbs as part of a study about the role of GAs in the cold requirement of tulip. In previous work, the presence of GA_4, GA_9, GA_{12}, GA_{24} and GA_{34} was demonstrated in both cooled and noncooled tulip bulb sprouts (Rebers *et al.*, 1994a, chapter 3). Because no qualitative changes in the GA pattern were observed, we investigated the quantitative changes using deuterated internal standards and GC-SIM.

Since basal plates (Aung, 1971) and bulb roots (Kawa-Miszczak *et al.*, 1992) have been suggested as sites of GA biosynthesis, the basal plates comprising the root primordia were analysed as well, next to the sprouts. The changes in endogenous GA levels in tulip bulb sprouts and basal plates during bulb storage are reported, as well as those in floral stalks and basal plates including roots after planting, at subsequent higher temperatures. The results are discussed with regard to the role of GAs in the floral stalk elongation of tulip.

Materials and methods

Plant material

Experiments were performed with field grown bulbs (*Tulipa gesneriana* L. cv. Apeldoorn) harvested in July 1990, 1991 and 1992. Bulbs were stored in a dark, ventilated room at 20°C until the treatments began in October of each year. After an initial sampling of 250 bulbs, the bulbs were transferred to dark ventilated rooms at either 5°C for the cold treatment, or 17°C for the noncooled controls. After 12 weeks, in January 1991, 1992 and 1993, sprouts and basal plates (250 each) were isolated and stored at -75°C until extraction for analyses of GAs before and after storage.

For GA analyses during subsequent growth, in January 1993 5°- and 17°C-pretreated bulbs were planted in soil in a greenhouse at 20°C. Irradiance was 30 W m^{-2} (Philips HPI-T 400 W lamps, Eindhoven, The Netherlands). Sprout growth was monitored during 5 weeks. Flowering, defined as full-red colouration of the perianth, could be observed after four weeks. Samples for GA analyses were taken 4, 8, and 11 days after planting. At each sampling date, plants were divided into floral stalk, flower, leaves and basal plate including roots (100 each). The plant parts were stored at -75°C until extraction.

GA extraction and purification

The GAs were extracted and purified as described in detail previously (Rebers *et al.*, 1994a), with some adaptations for the quantitative analyses. Samples consisted of 15 to 100 sprouts, floral stalks or flowers (15-50 g), or 7 to 50 basal plates or basal plates including roots (50 g). The material was homogenized in liquid N_2, and MeOH (4:1 v/w) was added together with ascorbic acid (0.1 % w/v) and deuterated standards of GA_1, GA_4, GA_9, GA_{24} and GA_{34} (100 ng each) for quantification of their endogenous levels. The gibberellin standards GA_1, GA_4, GA_9, GA_{34}, [17,17-$^2H_2$]GA_1, [17,17-$^2H_2$]GA_4, [17,17-$^2H_2$]GA_9, [17,17-$^2H_2$]GA_{24} and [17,17-$^2H_2$]GA_{34} were obtained from L.N. Mander, Research School of Chemistry, Canberra, Australia. GA_{24} was provided by N. Murofushi, University of Tokyo, Tokyo, Japan. The amounts of internal standards to be added were estimated in preliminary experiments. Solvent partitioning, anion exchange chromatography and reversed phase HPLC were performed as described before (Rebers *et al.*, 1994a). The HPLC effluent was collected in four fractions of 4.8 to 8.8 ml (1: 20.7-21.9, 2: 31-33, 3: 33-34.2 and 4:

35-37.2 min.) for respectively GA_1, GA_{34}, GA_4, and GA_9 + GA_{24}. All fractions were methylated with excess ethereal diazomethane and taken to dryness. The fraction containing both GA_9 and GA_{24} was ready for GC-MS. The other fractions were each fractionated again, using the same HPLC system and gradient. Fractions of 4.8 to 6.4 ml containing GA_1Me (24.2-25.4 min.), GA_{34}Me (33.5-35.1 min.) and GA_4Me (36.2-37.4 min.), were collected and evaporated to dryness.

Fractions were trimethylsilylated with Derivasil (bis-trimethylsilyl trifluoroacetamide : trimethylchlorosilane : trimethylsilylimidazole : pyridine [3:2:3:10] Chrompack, Bergen op Zoom, The Netherlands). Derivatized samples were analysed using a HP 5890 GC coupled to a HP 5970 mass selective detector (Hewlett Packard, Amstelveen, The Netherlands). Samples (4 μl) were injected splitless into a Hewlett Packard Ultra-1 fused silica capillary column (25 m x 0.20 mm x 0.33 μm film thickness) as described previously (Rebers *et al.*, 1994a).

For the quantitative analyses, two or four characteristic ions were monitored for each GA with dwell times of 100 ms ([$^2H_2$]GA_1MeTMSi standard/GA_1MeTMSi, *m/z* 508 and 506; [$^2H_2$]GA_4MeTMSi standard/GA_4MeTMSi, *m/z* 420, 418, 286 and 284; [$^2H_2$]GA_9Me standard/GA_9Me, *m/z* 300, 298; [$^2H_2$]GA_{24}Me standard/GA_{24}Me, *m/z* 316 and 314; [$^2H_2$]GA_{34}MeTMSi standard/GA_{34}MeTMSi, *m/z* 508 and 506). The endogenous amounts of GA_1, GA_4, GA_9, GA_{24} and GA_{34} were calculated from the peak area ratios 508/506, 420/418 and/or 286/284, 300/298, 316/314 and 508/506, respectively, using calibration curves produced by mixing internal standards and unlabelled compounds in known ratios and taking into account the abundance of the natural isotopes (Hedden, 1987). Quantification of GAs generally was performed in two to six independent analyses in which equal amounts of plant material were analysed.

Results

Growth and development

The changes in fresh weight of sprouts and basal plates are shown during dry bulb storage (0-12 weeks), and the first 11 days after planting (Figure 4.1). During cooled and noncooled dry storage of the bulbs, sprouts continued to grow slowly. At the end of storage, sprouts in noncooled bulbs were larger then sprouts in cooled bulbs. However, while sprout weight in noncooled bulbs hardly increased after planting, sprout growth was rapid in cooled bulbs. After 11 days sprouts of cooled bulbs weighed twice as much as those of noncooled bulbs. The changes in fresh weight of

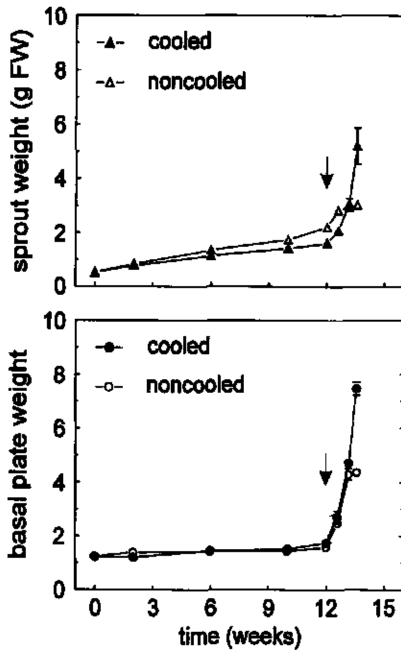
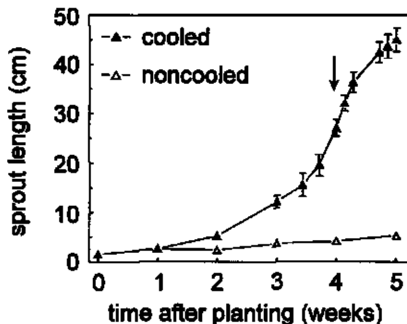


Figure 4.1 Development of sprouts and basal plates during 12 weeks of cooled (5°C) and noncooled (17°C) bulb storage and shortly after planting at 20°C of the pretreated bulbs. Averaged data from 1991, 1992 and 1993. The arrow indicates the planting time. Each point represents the mean of 2-4 samples of 50-60 sprouts or basal plates. The developing roots were included with the basal plates. Error bars indicate SD when they exceed the size of the symbol.

basal plates which comprised the root primordia, were negligible during bulb storage. Root development occurred in both cooled and noncooled bulbs the first days after planting. A week after planting, the basal plates including the roots of noncooled bulbs did not further increase in weight, whereas those in cooled bulbs continued growing.

For the bulbs stored at 5°C prior to planting, sprout growth proceeded rapidly (Figure 4.2). A full-grown flower was observed 4 weeks after planting. Each plant flowered at the same date and after flowering the floral stalk continued growing. In the noncooled bulbs, the flower stalk did not elongate up to 5 weeks after planting and full flower development did not occur.

Figure 4.2 Sprout growth of cooled (12 weeks 5°C) and noncooled (12 weeks 17°C) bulbs, planted January 1993 and cultivated at 20°C. The arrow indicates the time that flower development of the cooled bulbs was complete. Error bars indicate SD (n = 5).



GA levels before and after dry storage, and during subsequent growth

The endogenous GA levels were measured before and after dry bulb storage, and during subsequent growth. In addition to GA_4, GA_9, GA_{24} and GA_{34}, previously identified in tulip sprouts (Rebers *et al.*, 1994a), the presence of GA_1 was demonstrated in concentrations less than 3.5 ng g^{-1} FW. GA_4 and GA_{24} were the major gibberellins, with levels up to 10 and 15 ng g^{-1} FW in basal plates and floral stalks, respectively. The general levels of GA_1, GA_9 and GA_{34} were lower, as well as were the GA levels in flowers (results not shown). Since it is difficult to judge the role of changes in GA levels from the data per g FW as the plant parts continued to grow (Figure 4.1), the results are presented on organ basis (sprout, floral stalk or basal plate). Comparable results for harvests 1990, 1991 and 1992 prompted us to combine the data of the analyses of the GA levels during dry bulb storage (Figure 4.3 and 4.5). The GA levels after planting of cooled or noncooled bulbs were analysed in 1993 only (Figure 4.4 and included in Figure 4.5).

During bulb storage (Figure 4.3), the level of GA_4 in noncooled sprouts increased significantly (Student's t-test, $p < 0.05$). The level of GA_9 significantly increased during cooled as well as during noncooled storage ($p < 0.05$), whereas the levels of GA_{24}, GA_1 and GA_{34} tended to decrease during storage. After 12 weeks of storage, the amounts of GA_4 and also GA_1 in noncooled sprouts were significantly higher than in cooled sprouts ($p < 0.05$).

After planting the bulbs at 20°C, GA levels were analysed in floral stalks, flowers and basal plates including the roots, after 4, 8, and 11 days. In the floral stalks (Figure 4.4), the levels of GA_4 and GA_{24} changed after planting. The level of GA_4 in floral stalks of cooled bulbs tended to increase gradually, whereas this increase did not occur in floral stalks of noncooled bulbs. For both cooled and noncooled bulbs, the level of GA_{24} in floral stalks was higher at day 4 (Figure 4.4) than the level in the total sprout before planting (Figure 4.3, cooled and noncooled sprouts). In noncooled bulbs the GA_{24} level further increased and then decreased 11 days after planting. In cooled bulbs the level of GA_{24} already decreased between 4 and 8 days. The low level of GA_1 further decreased in the noncooled floral stalks; in the cooled ones the level of GA_{34} showed some increase. In the flowers of planted noncooled bulbs, the GA levels remained at the low level of less than 1 ng per flower. In cooled bulbs, the levels of GA_{24}, GA_9, GA_1 and GA_{34} showed the same picture, but the GA_4 level increased 5-fold to 2.5 ng per flower 11 days after planting, although the growth of the flower was negligible (results not shown).

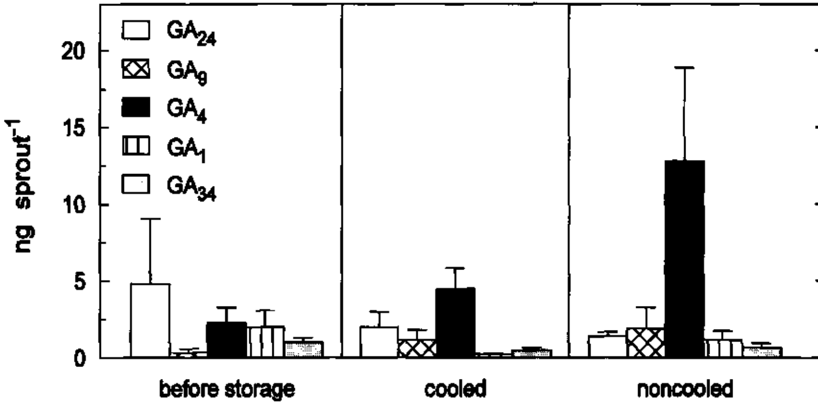


Figure 4.3 Endogenous levels of GA_{24}, GA_9, GA_4, GA_1 and GA_{34} in sprouts before storage and after cooled (12 weeks 5°C) or noncooled (12 weeks 17°C) bulb storage. The results of three harvest years were taken together. Each bar represents the mean of 3-6 measurements, except for GA_1 (n = 2-5). Error bars indicate SD.

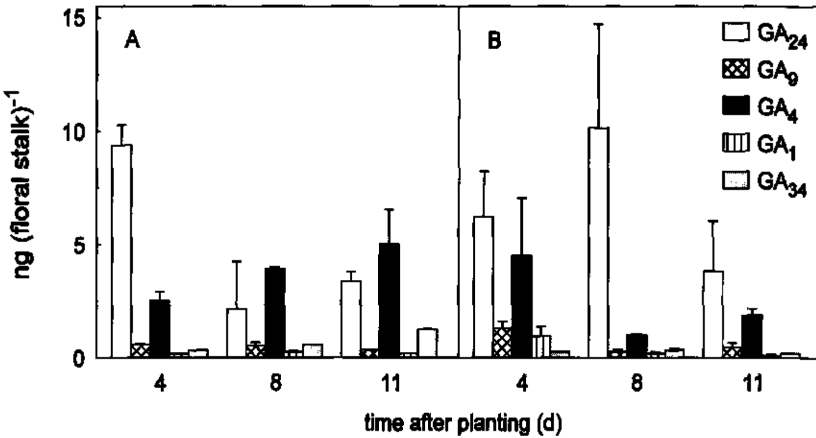


Figure 4.4 Endogenous levels of GA_{24}, GA_9, GA_4, GA_1 and GA_{34} in floral stalks shortly after planting cooled (A) or noncooled bulbs (B) at 20°C. Error bars indicate SD (n = 2).

The GA levels in the basal plates during bulb storage and after planting are presented together (Figure 4.5). Since after planting the bulbs, the GA levels in the basal plates including the roots remained at a constant level for 11 days, it seemed justified to take these data together. During storage and after planting of both cooled and noncooled bulbs, the levels of GA_4 and GA_9 in basal plates tended to decrease. The decrease in the level of GA_4 after planting noncooled bulbs was significant ($p < 0.05$). The levels of GA_1 and GA_{34} were low and remained more or less the same.

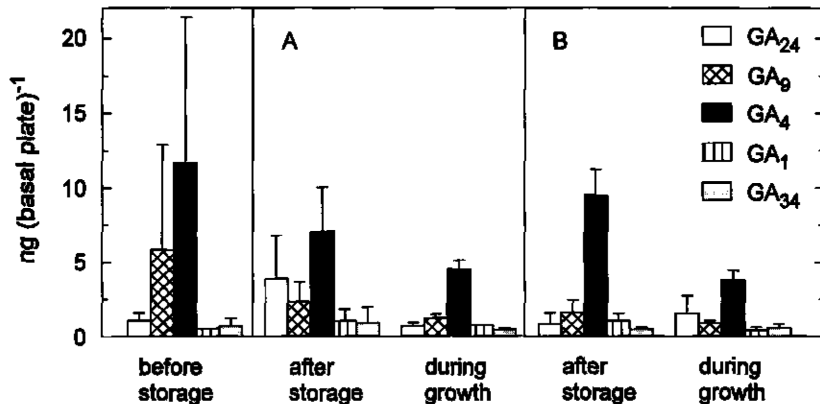


Figure 4.5 Endogenous levels of GA_{24}, GA_9, GA_4, GA_1 and GA_{34} in basal plates before and after cooled (12 weeks 5°C; A) or noncooled (12 weeks 17°C; B) bulb storage and shortly after planting at 20°C ("during growth"). For the results at the start and end of bulb storage, the data of three harvest years were taken together and each bar represents the mean + SD of 3-5 measurements, except for GA_1 (n = 2). For the results per basal plate (including roots) after planting, the GA levels at day 4, 8 and 11 were taken together and the mean values + SD are presented (n = 3).

Discussion

In a previous study (Rebers *et al.*, 1994a), GA_1 was not detected in tulip. Reexamination of the endogenous GAs with more purified HPLC fractions, revealed the presence of GA_1 in both bulb sprouts and basal plates. Nevertheless, with concentrations below 2 ng per sprout or basal plate, GA_1 was not a major component in any of the extracts.

In earlier studies, using bioassays, a cold treatment of tulip bulbs was reported to lead to an increase in the levels of GA-like substances per g FW in sprouts (Aung and

De Hertogh, 1967; Hanks and Rees, 1980) or in whole bulbs (Aung and De Hertogh, 1968). In contrast, van Bragt (1971) and Einert *et al.* (1972), reported a decrease in the level of free GAs per g FW sprout during cooled storage. In *Lilium elegans*, a related cold-requiring bulb species, the levels per g FW bulb of GA_4 and GA_{24} measured by GC-SIM, were reported to increase, whereas the levels of GA_1, GA_{19} and GA_{20} decreased during cooled bulb storage (Takayama *et al.*, 1993).

To correlate an increase or decrease in GA level during storage, with the cold-stimulated growth and flowering at subsequent higher temperatures, a change in GA level should be limited to either cooled or noncooled bulbs only. In several other studies, noncooled control bulbs of the same age were not included in the GA analyses (Aung and De Hertogh, 1967; Aung and De Hertogh, 1968; Hanks and Rees, 1980; Takayama *et al.*, 1993). In the present study, an increase was observed for the amount of GA_4 per sprout (Figure 4.3). This increase, however, especially occurred during noncooled bulb storage and resulted in a significant higher amount of GA_4 in these sprouts than during cooled storage. However, little development of noncooled sprouts was observed after planting (Figure 4.2). Therefore, it is unlikely that an increased level of GA_4 is correlated with floral stalk elongation and flowering after planting.

Using bioassays, van Bragt (1971) reported comparable total levels of gibberellins per g fresh weight in bulb sprouts after 12 weeks of bulb storage at either 5 or 20°C (both 6 ng g^{-1} FW). In the present study, sprouts had almost identical total GA levels per g fresh weight at the end of the 17 or 5°C bulb treatments (7 and 5 ng g^{-1} FW respectively). When expressed as ng per sprout, the total GA level in noncooled sprouts tended to be even higher than in cooled sprouts (Figure 4.3; 18 and 8 ng per sprout). This higher GA content in sprouts from noncooled bulbs might be connected with higher metabolic activity at 17°C than at 5°C; at the end of storage, sprouts from bulbs stored at 17°C were considerably larger than those from bulbs stored at 5°C (Figure 4.1).

Alternatively, GA_4 may be an accumulating inactive precursor, due to a block in the conversion to active GAs, possibly GA_1. However, a decrease in the level of GA_4 and a concomitant increase in GA_1 was not observed in the developing sprouts of cooled bulbs after planting (Figure 4.4). On the contrary, the GA_4 level tended to decrease in sprouts of noncooled bulbs, which hardly showed any growth. Hence, although local differences within the sprouts cannot be ruled out, changes in GA content occurring during cooled or noncooled bulb storage might be more related to bulb age and metabolic activity during storage itself than to cold-stimulated growth and development at subsequent higher temperatures.

In a previous study (Rebers *et al.*, 1994b), it was demonstrated that GA biosynthesis is involved in sprout growth after bulb storage at subsequent higher temperatures. Therefore, it is possible that the changes in GA levels during storage are of minor importance and that the major changes occur after planting when rapid sprout growth of cooled bulbs occurs (Figure 4.2).

After planting cooled bulbs at 15 to 18°C in light, a decrease in GA content of sprouts per g FW was reported by van Bragt (1971) and Alpi and De Hertogh (1975). On the other hand, an increase in the content of GA-like substances per g FW was reported by Aung *et al.* (1969) analysing whole bulbs, and Einert *et al.* (1972), analysing sprouts.

In the present study, in floral stalks of planted bulbs the low levels of GA_1 and GA_9 were maintained in both cooled and noncooled bulbs, suggesting that these GAs presumably do not have important roles in floral stalk elongation. On the other hand, in floral stalks of cooled bulbs (Figure 4.4), the level of GA_4 tended to increase after the first week, at the time that sprout growth started as well (Figures 4.1 and 4.2). In noncooled bulbs the increase in the level of GA_4 did not occur. Remarkably, in both planted cooled and noncooled bulbs, the levels of GA_{24} in the floral stalks at day 4 were higher than the levels in the total sprouts after storage, immediately before planting. Apparently, GA biosynthesis to GA_{24} occurred in both cooled and noncooled bulbs. In cooled bulbs the level of GA_{24} per floral stalk decreased again after the transient increase. Since GA_{24} is a precursor of GA_4, the decreasing level of GA_{24} and the increase of GA_4 in floral stalks of cooled bulbs might indicate the conversion of GA_{24} to GA_4 via GA_9. *In Lilium elegans* too, after planting cooled bulbs at higher temperatures, the levels of GA_4 and GA_{24} measured in whole bulbs showed changes, whereas the levels of GA_1 and GA_9 remained at a constant level (Takayama *et al.*, 1993)

Although the basal plates (Aung, 1971) or the roots (Kawa-Miszczak *et al.*, 1992), have been suggested as site of GA biosynthesis, no significant increases in GA levels were observed in these bulb organs during storage or after planting the pretreated bulbs (Figure 4.5). The general GA levels in the basal plates and roots seemed to decrease after planting. However, it is possible that GA biosynthesis occurred and the free GAs were converted to GA conjugates, which were not monitored in the present study.

In *Thlaspi arvense*, a cold-requiring winter annual, the major difference between cooled and noncooled plants was observed for a GA precursor: kaurenoic acid, and the conversion of kaurenoic acid to GA_{12}-aldehyde after the vernalization, was

suggested to be the principal step in the thermoinductive regulation of GA biosynthesis in *Thlaspi* (Hazebroek *et al.*, 1993). This mechanism might be considered for tulip as well. Yet, the cold requirement of tulip is distinct from that of *T. arvense*, to which cold is essential for both floral initiation and floral stalk elongation (Metzger, 1985). In tulip the flower is present already before the start of the cold treatment.

The presence of both GA_1 and GA_4 suggests that either GA_4 or GA_1 might be the major GA active in tulip, the other GAs present being either precursors to GA_4 (GA_{24} and GA_9) or an inactive GA metabolite (GA_{34}) (Sponsel, 1987). In *Lilium*, GA_4 was suggested to have a major role in the cold-stimulated sprouting of bulbs (Takayama *et al.*, 1993). Although cold-stimulated growth appeared not to be specifically related to a change in GA_4 level during bulb storage, the absence of a significant accumulation of GA_1 and the increase in the level of GA_4 only in growing floral stalks of cooled bulbs, support the idea that GA_4 is involved in the elongation of the floral stalk in tulip.

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5 Effects of exogenous gibberellins and paclobutrazol on floral stalk growth of tulip sprouts isolated from cooled and noncooled tulip bulbs[1]

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Summary

The biological activity of GA_1, GA_4 and GA_9, endogenous in tulip bulb sprouts, was tested *in vitro* on sprouts of cooled and noncooled tulip bulbs (*Tulipa gesneriana* L. cv. Apeldoorn), in the presence or absence of the GA biosynthesis inhibitor paclobutrazol.

At early starting dates of incubation, floral stalks from both cooled and noncooled bulbs did hardly show any elongation in the absence of exogenous GA. Paclobutrazol had no effect on floral stalk elongation, and the response to GAs of sprouts from cooled bulbs was greater than that of sprouts from noncooled bulbs. At later starts of incubation, considerable floral stalk elongation already occurred without GA application. Paclobutrazol inhibited this floral stalk elongation, and the growth of sprouts from both cooled and noncooled bulbs was stimulated by GA application. The effect of paclobutrazol was reversed by simultaneous application of GA. Application of GA with and without paclobutrazol resulted in the same elongation of the floral stalk, indicating the absence of substantial side effects of the inhibitor.

The isolated sprouts did not develop a full-grown flower without the addition of GA. GA_4 was more effective than GA_9 in stimulating this flower development. GA_1 was no more effective than GA_4.

The results demonstrate that sprouts from both cooled and noncooled bulbs are

[1] The principal part of this chapter will be published in Physiologia Plantarum

responsive to exogenous GAs *in vitro*, and may be a site of GA biosynthesis.

Introduction

In tulip bulbs, flowers are initiated at relatively high temperatures (17°C or higher). Following flower initiation, a cold period is required for adequate floral stalk elongation and flower development at subsequent higher temperatures (17-20°C). When nonrooted bulbs of *Tulipa gesneriana* L. cv. 'Apeldoorn' are stored at 5°C for 12 weeks prior to planting, floral stalk elongation and flower development proceed rapidly. Shorter periods at 5°C result in slower floral stalk elongation and delayed flower development. When noncooled (17°C) bulbs are planted, the growth of the flower stalk is strongly reduced and flower abortion often occurs (le Nard and De Hertogh, 1993).

In this cold requirement the involvement of GAs has been implicated (Aung and De Hertogh, 1967; Hanks, 1982; Saniewski, 1989). Gibberellin A_3 or $GA_{4/7}$ have been administered to whole bulbs, with the aim of reducing or eliminating the lengthy cold storage period. Using application methods such as bulb immersion, soil drenching, vacuum infiltration, drops on the apex, injection or absorption by the basal plate, these studies showed that GA could partly replace the cold treament of tulip cv. Apeldoorn (van Bragt and Zijlstra, 1971; Murai *et al.*, 1975; van Bragt and van Ast, 1976; Hanks and Rees, 1980a; Hanks, 1982; Hanks and Rees, 1983; Hanks, 1985; chapter 1). In addition, cold-stimulated floral stalk elongation was suggested to require GA biosynthesis (Shoub and De Hertogh, 1974; Hanks and Rees, 1977; Okubo *et al.*, 1986; Saniewski, 1989; Suh *et al.*, 1983).

Two hypotheses have been proposed to explain the role of GAs in the cold requirement of tulip. Using bioassays, it was shown that during a cold treatment of bulbs the amount of free GAs increases (Aung and De Hertogh, 1967; Aung and De Hertogh, 1968; Hanks and Rees, 1980b). However, van Bragt (1971a) concluded that no clear relationship exists between the level of endogenous GAs and the floral stalk elongation after cold storage. A second hypothesis suggested that during the cooling period, tissue sensitivity to GAs increases (Hanks, 1982).

As part of an extensive study about the roles of GAs in the cold requirement of tulips, we tested the biological activity of some crucial GAs. In previous work, we demonstrated by combined gas chromatography-mass spectrometry (GC-MS) the presence of GA_1, GA_4, GA_9, GA_{12}, GA_{24}, GA_{34} in tulip bulb sprouts (Rebers *et al.*, 1994a; 1994b, chapters 3 and 4). These GAs belong to the non-, the early 3- and the early-13-hydroxylation pathway. In this series, GA_1 or GA_4 might be the major active

GAs for floral stalk elongation and flower development (Sponsel, 1987). The other GAs are either precursors (GA_{12}, GA_{24} and GA_9) or inactive GA metabolites (GA_{34}). In the present study, the biological activities of GA_1, GA_4 and GA_9 were tested. The sensitivity to exogenous GAs was studied in sprouts from both cooled and noncooled bulbs. The growth retardant paclobutrazol was used to study the role of GA biosynthesis in floral stalk elongation. Paclobutrazol, applied to whole tulip bulbs either before or after cooling, was shown to inhibit floral stalk growth but allowed normal development of leaves and flowers (Saniewski, 1989).

For these experiments an *in vitro* system was used by growing isolated sprouts on a liquid medium. This system allowed for precise applications of GAs and inhibitor. The incubations were repeated with different starting dates in two successive years.

Materials and methods

Plant material

Experiments were performed with bulbs (*Tulipa gesneriana* L. cv. Apeldoorn) harvested in 1991 and 1992.

For both harvests, field-grown bulbs were lifted at the end of June and stored in a dark, ventilated room at 20°C until start of the treatment. Bulbs of both harvests reached stage 'G' (completion of flower bud differentiation, Cremer *et al.*, 1974) at the end of July and had circumferences of 12.5-13 cm.

All experiments were carried out starting with nonrooted bulbs, and during the (dry) temperature treatments, no rooting occurred. The starting dates of the different bulb treatments, the dates of sprout isolation and the GAs applied are presented in Table 5.1. For experiment 91A, only noncooled bulbs were used, and for experiment 92D, only cooled bulbs.

Bulbs were transferred to dark ventilated rooms at 5°C for the cold treatment, or 17°C for the control, noncold treatment. At the end of the bulb treatments, sprouts were isolated from the bulbs including a small part of the attached basal plate. The explants were surface-sterilized (1% NaClO, 30 min.) and washed. Just before starting the incubation, the remaining part of the basal plate was cut away and the sprouts were incubated in glass tubes with 1 ml of liquid medium. Per incubation treatment, 10 sprouts were used in experiments 91A, B and 92A, and 5 per treatment in experiments 92B, C and D. Initial floral stalk lengths were 8 mm for experiment 91A and 15 mm for the other experiments.

Table 5.1 Starting dates of 5°C or 17°C bulb treatments, dates of sprout isolation and the gibberellins applied. Prior to the 5°C or 17°C treatment the bulbs were stored at 20°C.

Experiment		Starting date of bulb treatments 5°/17°C	Date of sprout isolation/ start incubation	GA applied
91	A	[a]	Oct 17, 91	GA_1, GA_4, GA_9
	B	Oct 10, 91	Jan 10, 92	GA_1, GA_4, GA_9
92	A	Oct 22, 92	Jan 21, 93	GA_4, GA_9,
	B	Oct 28, 92	Jan 28, 93	GA_4
	C	Nov 4, 92	Feb 4, 93	GA_9
	D	Nov 18, 92	Feb 18, 93	GA_9

[a] sprouts isolated from 20°C stored bulbs

Growth medium

The *in vitro* procedure was adapted from van Bragt (1971b). Murashige and Skoog (1962) medium without agar was used, with 8% sucrose added. In the experiments of 1991 vitamins were added according to van Bragt (1971b): 2 mg l^{-1} of biotin, thiamine-HCl, nicotinic acid and pyridoxine. In the experiments of 1992 standard MS medium (Flow Laboratories, Irvine, UK) was used, containing respectively 0, 0.1, 0.5 and 0.5 mg l^{-1} of the vitamins mentioned above. The slight differences between the nutrient media used, are not relevant for the type of experiments performed.

Application of GAs and inhibitor

Pure GA_1 and GA_4 were obtained by HPLC isolation, from respectively commercially available GA_3 (Sigma, St. Louis, MO, USA; # G3250 Lot 12F-0335 containing 3% GA_1) and GA_{4+7} (ICI, Yalding, UK). GA_9 was obtained from L.N. Mander (Canberra, Australia). Solutions of the following concentrations were prepared: 0, 10 or 100 μg GA ml^{-1} for experiments 91A, B and 92A; 0, 0.1, 1.0, 10, 100 and 316 μg ml^{-1} for experiments 92B and C; and 0 and 10 μg ml^{-1} for experiment 92D. Precise amounts of GA were obtained by pipetting from a dilution series of GA in methanol. The methanol was evaporated under reduced pressure and medium was added. In order to dissolve the GAs in the aqueous MS media, the solutions were heated at 40°C for 5 min. and subsequently stirred overnight. During dissolving the pH was monitored and adjusted to 6.0, at which acidity the solubility of the tested GAs at

20°C was higher than 350 $\mu g\ ml^{-1}$. The solutions were sterilized using an 0.2 μm filter (Schleicher & Schuell, Dassel, Germany).

The maximum solubility of paclobutrazol (ICI, Yalding, UK) in H_2O is 35 mg l^{-1} at 20°C. In order to obtain this concentration for the incubation solutions, 50-100 mg l^{-1} paclobutrazol were suspended in MS medium. This suspension was heated to 40°C for 5 min. and then stirred overnight at 20°C. Filtering of this suspension over a 0.2 μm filter yielded a clear solution, thus containing 35 mg l^{-1} paclobutrazol. pH values of the incubation media were adjusted to 6.0.

Growing conditions

Sprouts were grown in a growth cabinet (Heraeus Vötsch, Germany) at 20°C with a 16 h light period. The irradiance (Philips TLD 58W/84, Eindhoven, The Netherlands) was 10 W m^{-2}. Used medium was replenished with fresh medium without GA or inhibitor.

Recording and analyses

Throughout this study, the term sprout is used for the undeveloped sprout inside the bulb as well as for the developing shoot, both including the floral stalk, leaves and flower. The term floral stalk is used only for the floral axis.

Sprout growth was monitored weekly. After 5 weeks growth rates declined. Flowering could be observed after 3 weeks and was defined as full-red colouration of the perianth. The floral stalks were generally hidden between the leaves and were not measured until termination of the experiments. In the experiments of 1991, all floral stalk length measurements were made 8 weeks after start of the last incubation, although sprout growth had stopped earlier. In the experiments conducted in 1992, measurements were made 5 weeks after the start of the incubation. Floral stalk lengths were measured to the base of the perianth segments.

Results

Floral stalk elongation in vitro

In Figure 5.1 the floral stalk lengths of sprouts incubated in the absence of exogenous GAs and incubated at different starting dates are presented. Floral stalk elongation of

sprouts incubated starting at October 17 or January 10, was very small, even after a cold treatment of the bulbs for 13 weeks at 5°C.

When sprouts were isolated later (January 21 through February 18), a considerably greater floral stalk elongation was observed. Floral stalks of sprouts from cooled bulbs elongated more than those from noncooled bulbs, but the difference was less for sprouts at later starts of incubation.

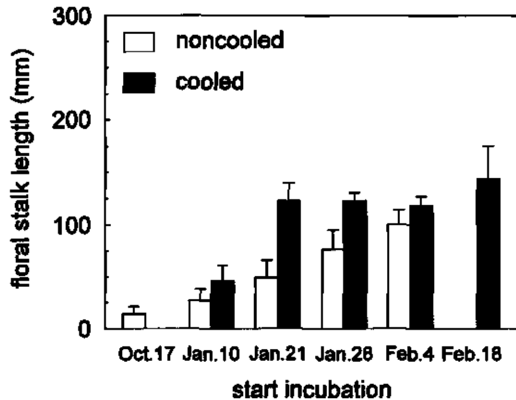

Figure 5.1 Floral stalk lengths of isolated sprouts grown in the absence of exogenous GAs, in relation to the starting date of sprout incubation. The sprouts were isolated from cooled or noncooled bulbs. The initial floral stalk lengths were 8 mm for the incubations started October 17, and 15 mm for the other starting dates. Error bars indicate SD (n = 5 or 10; for details see plant material).

Effect of GAs on floral stalk elongation

Figure 5.2 presents the individual effects of 10 μg GA_1, GA_4 or GA_9 on floral stalk elongation, in sprouts from cooled and noncooled bulbs, incubated at different starting dates. Gibberellin administration stimulated floral stalk elongation in sprouts from cooled bulbs. In sprouts from noncooled bulbs, the extent to which floral stalk elongation was stimulated by GA depended strongly on the starting date of incubation. Sprouts from noncooled bulbs incubated October 17 or January 10, hardly responded to applied GA_1, GA_4 or GA_9, whereas for incubations started January 21 or later, GA_4 or GA_9 application clearly stimulated floral stalk elongation. The final length of these stalks was comparable with that of the GA_4 or GA_9-treated ones from cooled bulbs.

In Figure 5.3 the effects of increasing amounts of GA_4 and GA_9 are compared.

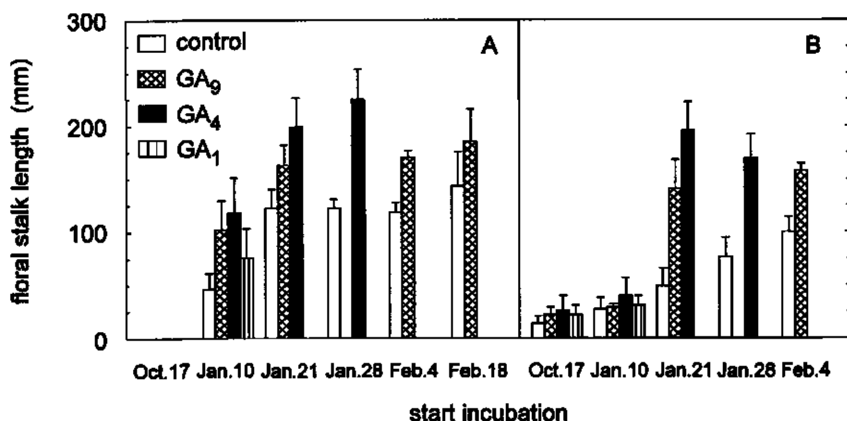

Figure 5.2 Effect of 10 μg GA_9, GA_4 or GA_1 application in relation to bulb treatment and starting date of sprout incubation. (A) sprouts isolated from cooled bulbs, (B) sprouts isolated from noncooled bulbs. The initial floral stalk lengths were 8 mm for the incubations started October 17, and 15 mm for the other starting dates. For details about the applied GAs, see Table 5.1. Error bars indicate SD (n = 5 or 10; for details see plant material).

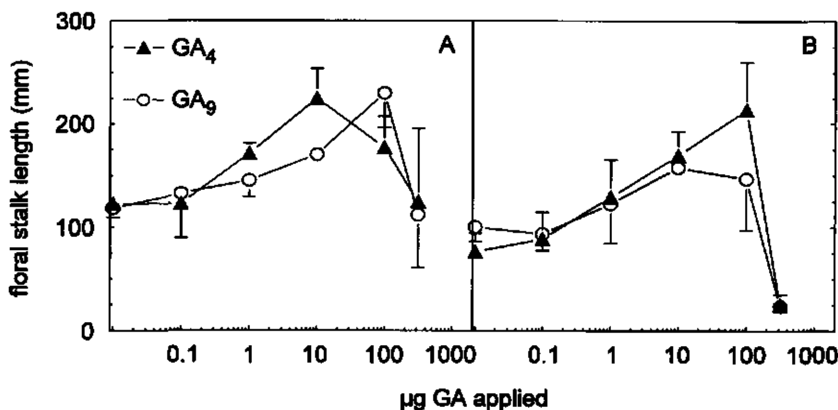

Figure 5.3 Effect of increasing amounts of GA_4 or GA_9 in relation to bulb treatment. (A) sprouts isolated from cooled bulbs, (B) sprouts isolated from noncooled bulbs. The incubations were started January 28 (GA_4, experiment 92B), or February 4 (GA_9, experiment 92C). The initial floral stalk lengths were 15 mm. Error bars indicate SD (n = 5).

The maximum stimulating effect of GA_4 and GA_9 was the same, although the maximum effect of GA_4 seemed to be reached at lower doses in sprouts from cooled bulbs. High concentrations of GA (more than 100 μg per sprout) led to side effects and negatively affected floral stalk elongation. Gibberellin A_1 was only tested in the incubations started at October 17 and January 10, and was no more effective than GA_4.

Effect of paclobutrazol on floral stalk elongation

The growth retardant paclobutrazol was used to study the role of GA biosynthesis in the floral stalk elongation of isolated sprouts. The effect of paclobutrazol alone and in combination with 10 μg GA is presented in Figure 5.4. Since the individual effects of GA_4 and GA_9 were not significantly different, it seemed warranted to combine the data of both GAs in order to obtain a more complete picture. For this purpose, the individual data of the treatments with GA_4 and GA_9 of the incubations started October 17, January 10 and January 21, were taken together and averaged. In the later starts of incubations, only GA_4 (January 28) or GA_9 (February 4) were used.

In the incubations started October 17 or January 10, there was no effect of paclobutrazol on floral stalk elongation of either sprouts from cooled or noncooled bulbs. The floral stalks in these sprouts hardly showed any growth in the presence or absence of the inhibitor. At later starts of incubation, in sprouts from both cooled and noncooled bulbs floral stalk elongation was inhibited and the effect was reversed by simultaneous application of GA_4 or GA_9. The increasing effect of paclobutrazol on sprouts from noncooled bulbs was remarkable (Figure 5.4B). Despite the increased floral stalk elongation of the control sprouts at later starts of incubation, the inhibitor reduced the stalk lengths for consecutive incubations to comparable low values.

To exclude interference of side effects of paclobutrazol, the combined effect of 10 μg GA_9 and paclobutrazol was compared with the effect of 10 μg GA_9 alone (Figure 5.5). The results show that 10 μg GA_9 completely reversed the growth inhibiting effect of paclobutrazol and that there was no significant difference between the stimulating effect of GA_9 with and without the inhibitor, for sprouts from both cooled and noncooled bulbs.

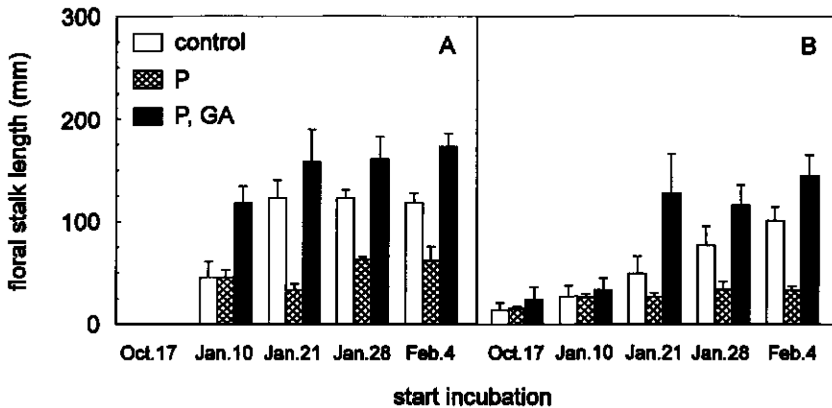

Figure 5.4 Effect of paclobutrazol (P) alone and in combination with 10 μg GA, in relation to bulb treatment and starting date of sprout incubation. (A) sprouts isolated from cooled bulbs, (B) sprouts isolated from noncooled bulbs. The initial floral stalk lengths were 8 mm for the incubations started October 17, and 15 mm for the other starting dates. For the experiments started October 17, January 10 and January 21, the individual data of the treatments with GA_4 and GA_9 were taken together and averaged. In the incubation started January 28, only GA_4 was applied and at February 4, only GA_9. Error bars indicate SD (n = 5 or 10; for details see plant material).

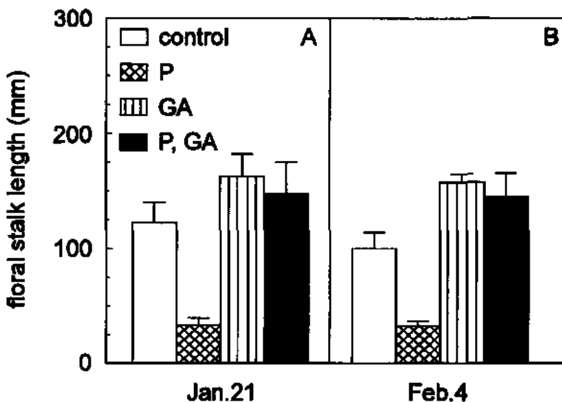

Figure 5.5 Effect of paclobutrazol, 10 μg GA_9 and the combination of 10 μg GA_9 with paclobutrazol, in relation to bulb treatment. (A) sprouts isolated from cooled bulbs, (B) sprouts isolated from noncooled bulbs. The incubations were started January 21 (A, experiment 92A), or February 4 (B, experiment 92C). The initial floral stalk lengths were 15 mm. Error bars indicate SD (n = 5).

Flower development and the effect of GAs and paclobutrazol

Without the addition of GAs, the isolated sprouts did not develop a full-grown, red-coloured flower in any of the experiments. GA application stimulated the appearance of well-developed flowers 3 weeks after the start of incubation. Between the successive experiments of different starting dates, there were no substantial differences in the percentages of well-developed flowers. In addition, no effect of paclobutrazol on flower development was observed (data not shown). The effect of GA application on flower development, seemed to be dependent only on the pretreatment of the bulbs and the kind of GA used.

Application of GAs could stimulate flower development in sprouts from both cooled and noncooled bulbs, but the percentage of well-developed flowers in sprouts from cooled bulbs (40% [n = 45], treatment with 10 μg GA_4) was larger than that in sprouts from noncooled bulbs (14% [n = 42], treatment with 10 μg GA_4). Gibberellin A_4 and GA_9 showed some difference in stimulating flower development; in most treatments GA_4 (40%, cooled bulbs, see above) was more effective than GA_9 (21% [n = 43], sprouts from cooled bulbs treated with 10 μg GA_9).

Discussion

For proper sprout development, tulip bulbs need a cold treatment. Bulbs stored continuously at higher temperatures show greatly reduced growth, whereas after a cold treatment rapid floral stalk elongation is observed, yielding stalk lengths for the Apeldoorn cv. of 40 cm or more. In this study, it was shown that isolated sprouts are also able to develop a fully grown floral stalk *in vitro*, although the maximum length was limited to 20-25 cm.

Floral stalk length and GA

At early starting dates of incubation, sprouts or floral stalks of both cooled and noncooled bulbs, did hardly show any elongation in the absence of exogenous GA (Figure 5.1). However, when the incubations were started later in the storage season, considerable floral stalk elongation occurred. The results suggest that delaying the start of incubation stimulated floral stalk elongation. Sprouts from cooled bulbs reached the phase of maximum floral stalk elongation earlier than sprouts from noncooled bulbs.

This corresponds with results obtained in earlier studies, when sprouts (van Bragt, 1971b) or stalk explants (Kawa and Saniewski, 1990) from cooled and noncooled bulbs were used, which were isolated at various dates. Using MS medium containing 0.8 % agar, their results also showed an increase in sprout or floral stalk elongation when the explants were isolated at later dates in the storage season.

The response to exogenous GAs appeared to be strongly dependent on pretreatment of the bulbs and starting date of the incubation (Figure 5.2). Comparable changes in GA response of tulips during the storage season, have also been reported for GA application to whole bulbs (Hanks and Rees, 1977; Moe *et al.*, 1978; Hanks and Rees, 1980a). To explain the different responses to applied GAs of intact cooled and noncooled bulbs, Hanks (1982) supposed an increase in GA sensitivity during the cooling period. Such an increase in GA sensitivity was indeed observed in this study: sprouts from noncooled bulbs incubated starting October 17, hardly showed any reponse to GA (Figure 5.2B). Treatment of these bulbs at 5°C for 13 weeks led to a considerable increase in GA response (Figure 5.2A; sprouts incubated starting January 10), while treatment at 17°C did not lead to a comparable increase in GA sensitivity (Figure 5.2B; sprouts incubated starting at January 10). However, when sprouts were incubated at later starting dates, the initial difference in GA sensitivity disappeared and sprouts from both bulb treatments were able to respond to GA, *in vitro* resulting in comparable floral stalk lengths (Figures 5.2 A and B; sprouts incubated starting January 21, 28 and February 4). The results indicate a change in GA sensitivity in sprouts from both cooled and noncooled bulbs. Yet, in sprouts from cooled bulbs the reponse to applied GAs developed more rapidly than in sprouts from noncooled bulbs.

Floral stalk length and paclobutrazol

The growth retardant paclobutrazol inhibits the oxidative reactions from *ent*-kaurene to *ent*-kaurenoic acid (Rademacher, 1991). Therefore, the presence or absence of an inhibitory effect gives information about the ability to perform GA biosynthesis reactions and/or about the availability of precursors for the synthesis of GAs. In previous studies, the growth retarding effect of paclobutrazol (Saniewski, 1989; Suh *et al.*, 1992) and also ancymidol (Shoub and De Hertogh, 1974; Hanks and Rees, 1977; Suh *et al.*, 1983) was demonstrated using intact, cooled bulbs. In the present *in vitro* study, it was additionally demonstrated that floral stalk elongation in sprouts from noncooled bulbs could be inhibited by paclobutrazol. This inhibitory effect appeared to be dependent not only on pretreatment of the bulbs, but also on the starting date of the

incubation (Figure 5.4).

Apparently, in sprouts from cooled bulbs incubated early in the storage season (starting before January 10), growth was negligible and therefore could not be inhibited by paclobutrazol (Figure 5.4A). In these sprouts GA biosynthesis apparently did not occur. The combined application of paclobutrazol with GA_4 or GA_9 indicated that they did have an ability to respond to applied GA. In sprouts isolated from cooled bulbs at January 21 or later, GA biosynthesis and GA sensitivity were clearly present. From these results it is obvious that the sensitivity for applied GAs developed before the GA biosynthesis capacity. In sprouts from noncooled bulbs (Figure 5.4B) incubated starting at October 17 and January 10, sensitivity appeared to be absent probably as well as GA biosynthesis. The ability to respond to applied GA was first seen for sprouts incubated starting at January 21, in which also some GA biosynthesis occurred. At later starts of incubation, the presence of GA biosynthesis and GA sensitivity was evident (Figure 5.4B; sprouts incubated starting January 28 and February 4).

In addition to the effect on GA biosynthesis, paclobutrazol has also been reported to affect sterol metabolism and the levels of abscisic acid, cytokinin and ethylene (Davis and Curry, 1991). In that case, exogenously applied GA would not be able to give a complete reversion of the growth inhibition. However, in the present study the growth inhibiting effect of paclobutrazol could completely be reversed by simultaneous application of GA (Figures 5.4 A and B) and there was no significant difference between the effect of GA_9 alone and with paclobutrazol (Figures 5.5 A and B). Moreover, the minor side effects of very high dosages of paclobutrazol reported by Suh *et al.* (1992), such as minor flower loss and slightly delayed flowering, were not observed in the present study. These results suggest that the possible side effects of paclobutrazol were negligible in tulip and that the primary action of paclobutrazol was the inhibition of GA biosynthesis. From these observations we infer that GA biosynthesis can occur in sprouts from cooled as well as from noncooled bulbs, thus providing GAs for floral stalk elongation.

GA and flower development

The cold requirement of tulip is distinct from that of many biennals, e.g. *Thlaspi arvense*, in which cold is essential for floral stalk elongation and floral initiation (Metzger, 1990). In tulip the flower is already formed before the start of the cold treatment. During either cold or noncold bulb storage, the floral stalk and this flower

bud continue to grow slowly. A cold bulb treatment guarantees rapid floral stalk elongation and the synchronous development to a full-grown, red-coloured flower when the intact bulb is subsequently moved to warmer temperatures. However, without cold treatment floral stalk elongation will be slow or absent, but a full-grown flower is sometimes possible, although delayed.

In this *in vitro* study, the isolated sprouts did not develop a full-grown flower in the absence of applied GAs. The flower bud apparently failed to complete its further development and blasted. As a consequence, inhibition of the GA biosynthesis by paclobutrazol could not have an additional inhibiting effect on flower development. Van Bragt (1971b), using isolated sprouts on solid medium, also reported flower bud blasting in sprouts without added GAs. Apparently, at any starting date of incubation, GAs were essential to prevent the flower bud from blasting. This may be explained by the *in vitro* conditions i.e. the absence of roots or of the basal plate, corresponding with results obtained by Kawa-Miszczak *et al.* (1992) where removal of roots from bulbs kept in water, caused reduced floral stalk growth and flower bud blasting. These effects were reversed by application of GA_3.

It is also possible that a shortage of other hormones, such as cytokinins, hinders the complete development of the flower in the isolated sprouts, since injections of kinetin in whole bulbs reduced the incidence of flower abortion due to poor growing conditions (Hanks and Rees, 1977; de Munk and Gijzenberg, 1977; Moe *et al.*, 1978).

The results of Kawa-Miszczak *et al.* (1992) indicated that the bulb roots may be site of GA biosynthesis. Results of Aung (1971) suggested the basal plate as the site of GA production in intact, rooted bulbs. The results in this study however, demonstrate that at least later in the storage season, the sprout may also be an important site of GA biosynthesis. Since the sprout consists of various organs, the exact localization of the sites of GA biosynthesis remains to be determined.

The results of the present study suggest that the capacity for GA biosynthesis in the sprouts apparently was not sufficient for complete flower development. As a consequence, paclobutrazol could not further affect flower development, although its effect on floral stalk elongation was evident and showed that paclobutrazol was transported into the sprouts in a concentration sufficient to affect GA biosynthesis. Although floral stalk elongation and flower development apparently both require GA to proceed, they are separate processes. Whether the sensitivity for GAs of these processes is different, or the transport of GAs to the organ site where it is needed is insufficient (i.e. for flower development) is unknown.

Effect of different GAs

Comparing the biological activities of GA_1, GA_4 and GA_9, the results indicate that GA_4 tended to be more effective than GA_9, especially in stimulating flower development. In the October 17 and January 10 experiments, also GA_1 was tested and appeared to stimulate flower stalk elongation and flower development, but was no more effective than GA_4. In several other studies, using intact bulbs and various application techniques, $GA_{4/7}$ appeared more effective than GA_3 in reducing the time to flowering of cooled bulbs (van Bragt and Zijlstra, 1971; Hanks and Rees, 1983). Gibberellins A_{4+7} but not GA_3, could reverse the dwarfing effect of the GA biosynthesis inhibitor ancymidol on the cold-stimulated floral stalk elongation (Shoub and De Hertogh, 1974). When different GAs were used to investigate the response of partly cooled, intact bulbs to GAs, the order of effectiveness in giving earlier flowering was $GA_4 > GA_1 > GA_9 >> GA_3$ (Hanks and Rees, 1980a). Although a conversion of GA_4 into GA_1 cannot be excluded, it is suggested that GA_4 is the major active GA in tulip sprouts. This is supported by the absence of a significant accumulation of GA_1 as endogenous GA in tulip (Rebers *et al.*, 1994b). The biological activity of GA_9 might be due to its conversion to GA_4, since GA_9 is a direct precursor of GA_4 in many plants (Sponsel, 1987).

In a preliminary experiment we used prohexadione (BX-112), a compound inhibiting 3ß-hydroxylations, such as the conversion of GA_9 to GA_4 (Nakayama *et al.*, 1990). The results indicated that in the presence of prohexadione, GA_9-stimulated flower development was inhibited or delayed, whereas flower development stimulated by GA_4 was not inhibited or even stimulated. These results suggest that the critical feature for flower development might be the 3ß-hydroxylation of GA_9 to GA_4.

Comparison of isolated sprouts and intact bulbs

Comparing sprout growth *in vitro* and in intact planted bulbs early in the storage season, isolated sprouts from noncooled bulbs as well as intact noncooled bulbs show poor floral stalk elongation and flower development. Intact cooled bulbs planted early in the storage season perform better, as well as isolated sprouts from cooled bulbs incubated at this time. Later in the storage season, the growth of isolated sprouts from noncooled bulbs gradually improves and the initial difference with the sprouts from cooled bulbs decreases. In addition, in the presence of exogenous GAs, floral stalk elongation and flower development can be similar for sprouts from both cooled and

noncooled bulbs. Yet, in intact noncooled bulbs planted later in the storage season, floral stalk elongation remains always extremely poor and often flower bud blasting occurs. This difference between the *in vivo* and *in vitro* sprout development, might be explained by considering the growing conditions of the sprouts. In intact, noncooled bulbs, the initially fleshy scales gradually dehydrate. This process is irreversible and probably hinders the essential transport of nutrients from scales to sprouts after planting. *In vitro* the uptake of nutrients by the isolated sprout is simplified by removing the scales and cutting away the basal plate. This suggests that the poor growth and development of noncooled, intact bulbs late in the storage season, is not caused by a low GA level, low GA sensitivity or low GA biosynthesis capacity, but that the supply of nutrients is limiting due to deterioration of the scales, as a consequence of dehydration at the high storage temperatures.

Alternatively, it is possible that dissecting the sprouts from the bulbs removed inhibitors, present in the basal plate or scales. The presence of abscisic acid (ABA) in tulip bulbs has been established, and the roots (Aung and De Hertogh, 1979) and basal plate (Aung and De Hertogh, 1979; Terry *et al.*, 1982) have been suggested as sites of ABA biosynthesis. Terry *et al.* (1982) suggested that the lack of extension growth of the floral shoot of noncooled bulbs, may be related to its high ABA content. A difference in ABA content between cooled and noncooled bulbs, however, has not been demonstrated yet (Franssen and Voskens, 1992).

Although a role of other plant growth substances cannot be excluded, the results in the present study clearly demonstrate the involvement of GAs in the floral stalk elongation and the flower development of tulip.

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6 Metabolism of GA_9 in isolated sprouts from cooled and noncooled tulip bulbs

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Summary

The metabolism of GA_9 was studied in sprouts from cooled (-2° and 5°C) and noncooled (17°C) tulip bulbs (*Tulipa gesneriana* L. cv. Apeldoorn). [^{3}H]GA_9 and [2,2,6-$^2H_3$]GA_9 were applied to isolated sprouts by injection into the floral stalk and the metabolites were analysed in the sprouts after 24 h. According to HPLC analyses, [^{3}H]GA_9 was converted to GA_4-like, and GA_{34}-like and/or GA_{51}-like compounds. The labelled metabolites of [$^2H_3$]GA_9 were identified by gas chromatography-mass spectrometry and the conversion of [$^2H_3$]GA_9 to [$^2H_3$]GA_4 and [$^2H_2$]GA_{34} was demonstrated. GA_4 and GA_{34} are native to this species and present in tulip sprout tissue. In the presence of prohexadione (BX-112), applied simultaneously with, or 24 h before [$^2H_3$]GA_9, the formation of [^{2}H] metabolites was less or absent. No evidence was found for the metabolism of GA_9 to GA_1, supporting the hypothesis that GA_4 is the major intrinsically active GA in the floral stalk elongation of tulip.

Introduction

Tulip bulbs, with terminal buds containing a complete flower, require a period of low temperature for rapid floral stalk elongation and adequate flowering at subsequent higher temperatures. After 12 weeks of bulb treatment at 5° (cooled) or 17°C

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(noncooled), cooled bulbs show rapid sprout growth and develop a full-grown red flower, whereas in noncooled bulbs floral stalk growth is strongly reduced and often flower abortion occurs (le Nard and De Hertogh, 1993). In this cold requirement the involvement of gibberellins has been implicated (Aung and De Hertogh, 1967; Hanks, 1982; Saniewski, 1989).

Gibberellins are required for, or at least promote, the elongation of the floral stalk of tulip. Application of GAs to intact bulbs (*Tulipa gesneriana* L.) could partly replace the cold treatment (van Bragt and Zijlstra, 1971; Hanks and Rees, 1980; Hanks, 1982; Hanks and Rees, 1983). In addition, floral stalk elongation of isolated sprouts could be inhibited by the GA biosynthesis inhibitor paclobutrazol and this effect was reversed by simultaneous application of GA_4 or GA_9 (Rebers *et al.*, 1994b, chapter 5). GA_1, GA_4, GA_9, GA_{12}, GA_{24} and GA_{34} were demonstrated to be endogenous in tulip bulb sprouts during storage (Rebers *et al.*, 1994a; 1994c, chapters 3 and 4). The conversions of GA_9 to GA_4 and of GA_4 to GA_1 have been demonstrated in higher plants (Sponsel, 1987; Kobayashi *et al.*, 1993), suggesting the metabolic route GA_{12} [$\rightarrow$ GA_{15}] $\rightarrow GA_{24} \rightarrow GA_9 \rightarrow GA_4 \rightarrow GA_{34}$ and $GA_4 \rightarrow GA_1$.

In this route, GA_4 or GA_1 might be the major active GAs, the others being precursors (GA_9, GA_{12}, GA_{15}, GA_{24}) or an inactivation product (GA_{34}) (Sponsel, 1987). Information concerning the GA biosynthetic pathway operating in tulip sprouts, is a prerequisite for an understanding of the origin and the regulation of biologically active GAs and their role in floral stalk elongation. The purpose of the present study was to investigate whether tulip sprouts are able to metabolize GA_9 to biologically active GA_4 or GA_1, and whether sprouts from cooled or noncooled bulbs show differences in GA_9 metabolism.

[^{3}H]GA_9 and [$^2H_3$]GA_9 were applied to isolated sprouts by injection into the floral stalk, in the presence or absence of prohexadione which is known to inhibit the conversion of GA_9 to GA_4 (Nakayama *et al.*, 1990a). The labelled metabolites were identified by HPLC and GC-MS. The metabolism of GA_9 and the effect of prohexadione were compared between sprouts from cooled and noncooled bulbs.

Materials and methods

Plant material

Experiments were performed with field-grown bulbs (*Tulipa gesneriana* L. cv. Apeldoorn) harvested in 1992 and 1993. For both harvests, bulbs were lifted at the

end of June and stored in a dark, ventilated room at 20°C until start of treatment. Depending on the time of the year, 5°C-cooled (available November-February) or -2°C-cooled (available March-October) bulbs were used.

In 1992, bulbs were transferred to a dark ventilated room at 5°C at December 1. After 4 weeks they were planted in moist peat and stored at -2°C for 15 weeks till April 12, 1993. During this treatment the bulbs slowly started rooting. Using this technique, 'ice tulips' are obtained, in which the proces of rapid sprout development and flowering can be retarded until the temperature is raised again to 17-20°C (de Jong *et al.*, 1990).

In 1993, bulbs were transferred to dark ventilated rooms at 5°C for the cold treatment, or 17°C for the noncold treatment at November 17. These bulbs were stored for 12 weeks until February 9, 1994. During these (dry) temperature treatments, no rooting occurred.

The two types of cooling treatments, both yielded adequate floral stalk elongation and full-grown flowers within 4 weeks after (planting and) raising the temperature.

At the end of the bulb treatments, sprouts were isolated from the bulbs including a small part of the attached basal plate. Just before starting the incubation, the remaining part of the basal plate was cut away and the sprouts were injected with substrate.

Chemicals

[^{3}H]GA_9 (1.2 TBq $mmol^{-1}$) was obtained from Prof. A. Crozier, University of Glasgow, Glasgow, UK. [2,2,6-$^2H_3$]GA_9 was a gift from Dr. H. Yamane, Department of Agricultural Chemistry, University of Tokyo, Tokyo, Japan. Prohexadione-Ca (BX-112) was a gift from Dr. W. Rademacher, BASF, Limburgerhof, Germany. For the experiments, the free acid was used, obtained by extraction with ethyl acetate from an acidic aqueous solution (pH 3) of prohexadione calcium.

Application of [^{3}H]GA_9, [$^2H_3$]GA_9 and BX-112

[^{3}H]GA_9 was injected into sprouts from cooled -2°C bulbs (400,000 dpm in 5 μl methanol each). [$^2H_3$]GA_9 was injected into sprouts from cooled (5°C) or noncooled (17°C) bulbs (10 sprouts, 50 ng each) with or without BX-112 (10 μg). A third group was injected with BX-112 24 h before [$^2H_3$]GA_9 application. In the experiments with deuterated GA_9, all injections were in 4 μl 50% aqueous methanol.

Sprouts were incubated in glass tubes with 0.5 ml H_2O at 20°C, under continuous white light (approximately 1.7 W m^{-2}). After 24 h the sprouts were frozen in liquid N_2 and stored at -75°C until analysis.

Analyses of metabolites of [^3H]GA_9 from -2°C bulb sprouts

The sprouts were extracted with methanol and the extracts were purified by solvent partitioning as described before (Rebers *et al.*, 1994a). After solvent partitioning, the sample residue was loaded on a C18 Sep-pak cartridge without preceding anion exchange chromatography. The cartridge was washed with H_2O and GAs were eluted with 10 ml 80% methanol. The eluate was evaporated to dryness and then fractionated by reversed-phase C18 HPLC, using a CAPCELL PAK column (4.6 x 250 mm) and a gradient of acetonitrile in H_2O containing 0.1% acetic acid at a flow rate of 1 ml $min.^{-1}$ (0-5 min. 20% acetonitrile, 5-25 min. 20-80% acetonitrile linear gradient). The radioactive metabolites obtained from the [^3H]-labelled substrates were monitored with an on-line radioanalyzer (Beckman 171 radio isotope detector), and the radioactivity of each HPLC fraction (1 ml) was analysed by liquid scintillation counter (Packard 2000 CA analyser).

Analyses of metabolites of [$^2H_3$]GA_9 from 5°C and 17°C bulb sprouts

The sprout extracts were purified by solvent partitioning, anion exchange chromatography and reversed phase HPLC as described before (Rebers *et al.*, 1994a), using a Chromspher column (C18, 250 x 10.0 mm) and a gradient of increasing methanol containing 0.01 % acetic acid (0-5 min. 10%, 5-30 min. 10-70%, 30-40 min. 70 %, 40-50 min. 80%). GC-MS analysis of the purified [$^2H_3$]GA_9 incubation products was carried out on an Hewlett Packard HP 5970 mass spectrometer connected to a HP 5890A gas chromatograph as described before (Rebers *et al.*, 1994a). For the detection of [$^2H_3$]GA_1, [$^2H_3$]GA_4, [$^2H_3$]GA_9 and [$^2H_2$]GA_{34}, their characteristic ions were monitored together with the characteristic ion of the endogenous GA, with dwell times of 100 ms ([$^2H_3$]GA_1MeTMSi/endogenous GA_1MeTMSi, *m/z* 509 and 506; [$^2H_3$]GA_4MeTMSi/endogenous GA_4MeTMSi, *m/z* 421 and 418; [$^2H_3$]GA_9Me/endogenous GA_9Me, *m/z* 301 and 298; [$^2H_2$]GA_{34}MeTMSi/-endogenous GA_{34}MeTMSi, *m/z* 509 and 506). The labelled metabolites were identified based on co-chromatography with the endogenous GAs, which had been identified previously using Kováts retention indices, full-scan mass spectra and selected ion

monitoring of 6 ions (Rebers *et al.*, 1994a). The integrated peak areas of the $[M+3]^+$ ions were corrected for the abundances of the natural isotopes. Although the ion 508 would have been a better choice for the detection of $[^2H_2]GA_{34}$MeTMSi, the ion 509 is usable. By the use of this *m/z* value, a natural isotope of 508, the relative abundances of the product $[^2H_2]GA_{34}$MeTMSi were underestimated by a factor of *ca.* 3.

Results

Application of $[^3H]GA_9$ to -2°C bulb sprouts

Figure 6.1 presents the HPLC radio chromatogram of the products from an incubation of sprouts with $[^3H]GA_9$. The data are presented as the percentages of the total recoverable $[^3H]$ label, which was more than 90% of the initially applied amount. The retention times of relevant authentic GAs are indicated. According to the HPLC analyses, GA_9 was converted to GA_4-like, and to GA_{34}-like or GA_{51}-like compounds. No radioactivity was detected that co-eluted with authentic GA_1.

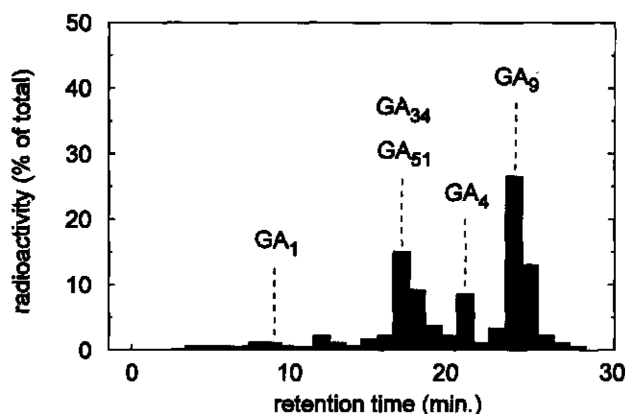

Figure 6.1 Radioactivity detection (percentage of the total recovered radioactivity) after reversed phase HPLC of $[^3H]GA_9$ metabolites in an extract of tulip sprouts. Indicated are the retention times of authentic GA_1, GA_4, GA_9, GA_{34} and GA_{51}.

Application of $[^2H_3]GA_9$ to cooled (5°C) and noncooled (17°C) bulb sprouts

After application as described above, the HPLC purified fractions corresponding to

GA_1, GA_4, GA_9 and GA_{34} were collected, methylated, silylated and analysed by GC-MS. Full-scan spectra of the remaining substrate $[^2H_3]GA_9$ were obtained in all extracts (data not shown). The amounts of the metabolites were insufficient to obtain full-scan mass spectra but the identities of the $[^2H]$GAs could be confirmed by co-chromatography with the endogenous GAs in the selected ion monitoring mode. The data obtained are summarized in Table 6.1.

Table 6.1 GC-SIM data on metabolites from the $[^2H_3]GA_9$ incubation with isolated sprouts from cooled and noncooled tulip bulb sprouts.

bulb treatment	products		
	$[^2H_3]GA_4$	$[^2H_2]GA_{34}$	$[^2H_3]GA_1$
	$[M+3]^+/[M]^+$ (% relative peak area ratio)		
	421/418	509/506	509/506
cooled	62	27	0
noncooled	36	32	0

The results indicate the presence of $[^2H_3]GA_4$ and $[^2H_2]GA_{34}$, which demonstrates the conversion of $[^2H_3]GA_9$ to $[^2H_3]GA_4$ and $[^2H_2]GA_{34}$. Endogenous GA_1 was detected, but the *m/z* 509 of $[^2H_3]GA_1$ was observed only in quantities corresponding with the natural abundance of the isotope. Comparing the results of sprouts from cooled and noncooled bulbs, both groups had metabolized $[^2H_3]GA_9$ to $[^2H_3]GA_4$ and $[^2H_2]GA_{34}$. The endogenous levels of GA_4 in sprouts of cooled and noncooled bulbs are significantly different (Rebers *et al.*, 1994c). When the relative peak area ratios of $[^2H_3]GA_4$ are related to the endogenous levels at time of application (4.5 and 12.8 ng per sprout respectively, Rebers *et al.*, 1994c), less $[^2H_3]GA_4$ was produced in cooled sprouts than in noncooled sprouts (2.8 ng and 4.6 ng produced per sprout respectively).

Table 6.2 presents the GC-SIM data on metabolites from the $[^2H_3]GA_9$ incubation in the presence of BX-112, applied simultaneously with or 24 h before the substrate. In cooled sprouts, some $[^2H_3]GA_4$ was formed when BX-112 was applied simultaneously with $[^2H_3]GA_9$. When BX-112 was applied 24 h before, $[^2H_3]GA_4$ was not detected, but also the endogenous GA_4 was not found. $[^2H_2]GA_{34}$ was not detected neither when BX-112 was applied simultaneously nor when it was applied 24 h before

$[^2H_3]GA_9$.

In noncooled sprouts, when BX-112 was applied 24 h before $[^2H_3]GA_9$, only small amounts of both metabolites were found. When BX-112 was applied simultaneously with the substrate, no $[^2H_3]GA_4$ and $[^2H_2]GA_{34}$ could be detected and also endogenous GA_{34} was not found.

Table 6.2 GC-SIM data on metabolites from the $[^2H_3]GA_9$ incubation with isolated sprouts from cooled and noncooled tulip bulb sprouts, in the presence of prohexadione (BX-112). BX-112 was applied simultaneously with or 24 h before the substrate.

bulb treatment	BX-112 addition	products	
		$[^2H_3]GA_4$	$[^2H_2]GA_{34}$
		$[M+3]^+/[M]^+$ (% relative peak area ratio)	
		421/418	509/506
cooled	simultaneous	17	0
	24 h before	0[a]	0
noncooled	simultaneous	0	0[a]
	24 h before	6	8

[a] neither $[M+3]^+$ nor $[M]^+$ (endogenous GA) detectable

Discussion

The metabolism of $[^3H]GA_9$ and $[^2H_3]GA_9$ was studied in isolated sprouts. GA_9 is endogenous to tulip sprouts at levels below 2 ng per sprout (Rebers *et al.*, 1994c), but was applied at levels which exceeded this concentration in order to obtain easy GC-MS identification of the metabolites. Substrate overloading in GA metabolism experiments may result in conjugation of excess substrate, or in metabolites that are not found as endogenous compounds (Hedden, 1987). In addition, an effect of the applied GA on the endogenous GA production cannot be excluded. In the present study, the amount of deuterated GA_9 applied per sprout exceeded the endogenous amount 10-25 fold. However, it is unlikely that the entire amount also reached the site of GA metabolism. In addition, after the application of tritiated GA_9, only less than

10% of the radioactivity was retained in the aqueous phase after solvent partitioning. This fraction, containing putative GA conjugates, was not further analysed.

Application of [^{3}H]GA_9 resulted in the formation of [^{3}H]-labelled GA_4 and a peak co-chromatographing with GA_{34}. The metabolites GA_4 and GA_{34} are native to tulip and present in sprout tissue (Rebers *et al.*, 1994a). A possible conversion of [^{3}H]GA_9 to [^{3}H]GA_{51} cannot be excluded because the HPLC procedure did not separate [^{3}H]GA_{34} and [^{3}H]GA_{51}, although endogenous GA_{51} has not been detected in tulip bulb sprouts (Rebers *et al.*, 1994a). The production of GA_{34} as [$^2H_2$]-metabolite from [$^2H_3$]GA_9 was demonstrated (Table 6.1), indicating that GA_{34} was a main metabolite.

A clear conversion of GA_9 to GA_4 and GA_{34} was observed in the isolated sprouts from cooled and noncooled bulbs (Table 6.1). The conversion of GA_9 to GA_4 has also been demonstrated in cell-free preparations of *Phaseolus vulgaris* seed (Takahashi *et al.*, 1986), in *Picea* shoots (Moritz, 1990) and in shoots of *Cucumis sativus* (Nakayama *et al.*, 1991). GA_{34} is a result of 2ß-hydroxylation of GA_4 and is relatively inactive (Reeve and Crozier, 1974; Sponsel, 1987).

In the presence of BX-112, applied before or simultaneously with [$^2H_3$]GA_9, the formation of [^{2}H]GA_9-metabolites was less or absent (Table 6.2). This corresponds with other studies, in which the inhibitory effect of BX-112 on the 3ß-hydroxylation of GA_9 to GA_4 was demonstrated (Nakayama *et al.*, 1990a). In addition, BX-112 was suggested to inhibit the 2ß-hydroxylation such as the conversion of GA_4 to GA_{34} (Nakayama *et al.*, 1990b). Therefore, the failure to detect endogenous GA_4 and GA_{34} in two experiments in the presence of the inhibitor (Table 6.2), might also be due to these inhibitory effects.

When GA_1, GA_4 or GA_9 were applied to isolated sprouts, all three of them could stimulate floral stalk elongation, while for stimulating full flower development, GA_9 tended to be the least effective GA (Rebers *et al.*, 1994b). It is possible that the activity of applied GA_9 is due to its conversion to GA_4. The results in this study clearly demonstrate that isolated sprouts are able to convert applied GA_9 to GA_4. Moreover, both cooled and noncooled sprouts were capable of this conversion, corresponding with our earlier findings that floral stalk elongation in both groups of sprouts could be stimulated by application of GA_9 (Rebers *et al.*, 1994b).

It is possible that GA_4 on its turn, might have to be converted to GA_1 before showing activity. Evidence is accumulating that GA_1 is the main endogenous GA active in the control of shoot elongation in several species of higher plants (Phinney, 1984; Graebe, 1987; Kobayashi, 1990). Kobayashi *et al.* (1993) and Junttila (1993)

have suggested that the biological activity of GA_4 in maize, rice and *Arabidopsis thaliana* shoots is due to its conversion to GA_1. The conversion of GA_4 to GA_1 has been demonstrated in vegetative tissues of *Phaseolus*, maize, rice and *Arabidopsis* (Kobayashi *et al.*, 1993) in pollen of *Pinus attenuata* (Kamienska *et al.*, 1976) and in cell cultures of carrot (Koshioka *et al.*, 1983). On the other hand, GA_4 has been suggested to be active per se for the control of stem elongation in shoots of *Cucumis sativus* (Nakayama *et al.*, 1991) and in the germination of *A. thaliana* seeds (Derkx *et al.*, 1994).

In tulip, no evidence was found for the conversion of GA_9 (via GA_4) to GA_1, neither in the HPLC analyses of [^{3}H]GA_9-metabolites nor in the GC-SIM analyses of the products of [$^2H_3$]GA_9. It is possible that the failure to detect [^{3}H]GA_1 or [$^2H_3$]GA_1, was due to the rapid turnover of this GA, which only occurs in small amounts in tulip bulb sprouts (less than 1.2 ng per sprout, Rebers *et al.*, 1994c). However, GA_8, which is the inactivation product of GA_1, has not been detected as endogenous GA in tulip bulb sprouts (Rebers *et al.*, 1994a), and was not found as metabolite of [^{3}H]GA_9. The suggested inability of sprouts to produce GA_1 from applied GA_9, implicates that the endogenous GA_1 originates from the early-13-hydroxylation pathway. Other GAs of this pathway, such as GA_{19} and GA_{20}, have not been detected but might have been present in amounts below detection (Rebers *et al.*, 1994a). Further, the increasing level of GA_4 in the growing floral stalk and the absence of a significant accumulation of GA_1 (Rebers *et al.*, 1994c), support the hypothesis that GA_4 is the major intrinsically active GA in the floral stalk elongation of tulip.

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7 General discussion

The cold requirement of tulip

In the life-cycle of tulip, an important factor affecting growth and development is temperature. In a year, three phases in development can be distinguished which differ in the temperature required (De Hertogh *et al.*, 1983). In the first phase the flower is initiated at relatively high temperatures. In the second phase a cold period is required to prepare the shoot for the third phase, in which rapid floral stalk elongation and flower development occurs at subsequent higher temperatures. In horticultural practice, flowering is programmed by simulating the temperature conditions required, in particular the cold period (forcing). After flower bud differentiation is complete (stage "G", Cremer *et al.*, 1974), bulbs are dry-stored in cooled ventilated chambers (2°-9°C), prior to planting at higher temperatures. The duration of this cold treatment is a major factor determining the development of floral stalk and flower after planting the bulbs (le Nard and De Hertogh, 1993).

For cv. Apeldoorn, a dry-storage treatment of 12 weeks at 5°C prior to planting will lead to proper floral stalk elongation and flower development. Shorter periods at 5°C usually result in slower shoot elongation and delayed flowering. Without any cold treatment, the growth of the terminal bud is strongly reduced and flower abortion often occurs (le Nard and De Hertogh, 1993).

In horticultural practice, there is a need for a practical assay to test whether a particular bulb has received a proper cold treatment. Suitable test parameters might be physiological changes, induced by the cooling treatment, which are related to a proper

cold treatment as well as to the subsequent floral stalk elongation and flower development (Boonekamp *et al.*, 1990). For that reason, a better knowledge of the physiology of the bulbs during the cold period and subsequent growth is essential.

More plants are known that need a period of low temperature for stimulating flower stalk elongation. The crucifer *Thlaspi arvense* requires a cold treatment for the initiation of stem elongation and flowering, and the mechanisms involved with this vernalization requirement have been studied in detail. As in tulip, longer cold treatments result in greater maximum stem length and reduce the lag period for the onset of flowering. It has been shown that the cold-induced stem growth of *Thlaspi* is a gibberellin-dependent process (Metzger, 1990), and the mechanism regulating the cold-induced stem elongation in *Thlaspi* might be considered for tulip as well. However, the cold requirement of tulip is distinct from that of *Thlaspi*. Without cold treatment, *Thlaspi* plants remain as vegetative rosettes (Metzger, 1990). In tulip, flower formation occurs at high temperatures, before the start of the cold treatment. Furthermore, during either cold or noncold bulb storage, the floral stalk and the flower bud continue to grow slowly (chapter 4, Figure 4.1). A cold bulb treatment guarantees rapid floral stalk elongation and synchronous full flower development when the intact bulb is subsequently moved to higher temperatures. Without cold treatment floral stalk elongation will be slow or absent. However, although many flowers will be aborted, a full-grown flower is still possible although much delayed (le Nard and De Hertogh, 1993). This indicates that, whereas in *Thlaspi* the quantitative effects of a cold treatment are the same for both flower induction and stem elongation (Metzger, 1990), in tulip flower initiation and floral stalk elongation are separate processes.

Cold treatment and gibberellins: two hypotheses

The physiological changes during the cold storage of tulip bulbs might be connected with hormonal changes. In particular, gibberellins have been implicated in the response of tulip to cold, since application of GAs could partly replace the cold treatment (chapter 1, Table 1.1). Furthermore, cold-stimulated floral stalk elongation was suggested to require GA biosynthesis (Shoub and De Hertogh, 1974; Hanks and Rees, 1977; Suh *et al.*, 1983; Okubo *et al.*, 1986; Saniewski, 1989). Moreover, using bioassay procedures, endogenous GA-like substances were measured and reported to increase during the cooling period (see also chapter 1, Table 1.2a).

These observations led to a first hypothesis to explain the role of GAs in the cold

requirement: during the cold period the amount of free GAs increases. This increased GA content is supposed to be responsible for the stimulation of floral stalk elongation after subsequent transfer to higher temperatures. This increased amount of free GAs, or of one particular GA, might provide a suitable parameter to test whether the duration of the cold treatment was sufficient to ensure adequate floral stalk elongation and full flower development.

A second hypothesis suggests that rather than the GA content, an increased sensitivity for GAs is responsible for the development at higher temperatures. Hence, a cooling period is needed to increase the GA sensitivity of the tulip sprout.

Hypothesis 1: cold treatment and GA content

The GAs endogenous to tulip were characterized and GA_1, GA_4, GA_9, GA_{12}, GA_{24}, GA_{34} and three GA-related compounds were detected (chapters 3 and 4). The detected GAs belong to three families of GAs with different hydroxylation patterns: the non-hydroxylation pathway (GA_{12}, GA_{24} and GA_9), the early-3-hydroxylation pathway (GA_4 and GA_{34}) and the early-13-hydroxylation pathway (GA_1). The conversions of GA_9 to GA_4 and of GA_4 to GA_1 have been demonstrated in higher plants (Sponsel, 1987; Kobayashi *et al.*, 1993), suggesting that the metabolic gibberellin route operating in tulip is GA_{12} [$\rightarrow GA_{15}$] $\rightarrow GA_{24} \rightarrow GA_9 \rightarrow GA_4 \rightarrow GA_{34}$ and $GA_4 \rightarrow GA_1$ (Figure 7.1).

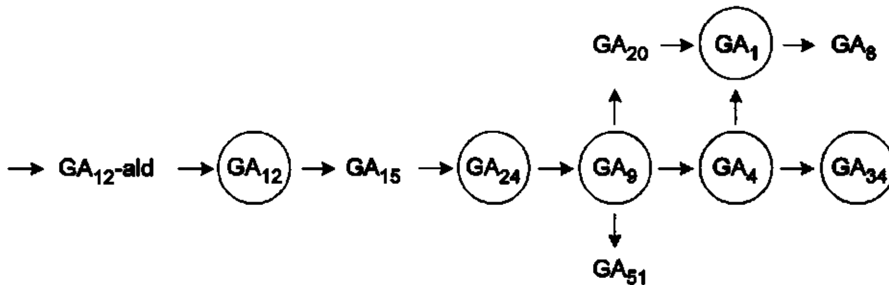

Figure 7.1 Metabolic pathway of GAs in higher plants showing known conversions. The GAs detected in tulip are encircled.

The levels of the free gibberellins GA_1, GA_4, GA_9, GA_{24} and GA_{34} during cooled or noncooled bulb storage were measured in bulb sprouts (chapter 4). To

correlate an increase or decrease in GA level during storage, with the cold-stimulated growth and flowering at subsequent higher temperatures, a change in GA level should be limited to either cooled or noncooled bulbs. The results showed that the levels of some GAs indeed changed during storage, but none of these changes seemed to occur in cooled or noncooled bulbs in particular. The largest change was observed for GA_4, which level increased in cooled as well as in noncooled sprouts. After 12 weeks sprouts of noncooled bulbs contained significantly more GA_4 per sprout than those of cooled bulbs. Yet, sprouts in noncooled bulbs did hardly show any development up to 5 weeks after planting. Therefore, it is unlikely that the increased level of GA_4 at the end of bulb storage is directly correlated with floral stalk elongation and flowering after planting, at subsequent higher temperatures.

In earlier studies, total levels of GA-like substances were measured using bioassay procedures. Van Bragt (1971a), using a dwarf pea bioassay, reported almost equal levels of gibberellins in sprouts per g FW after 12 weeks of bulb storage at either 5° or 20°C (chapter 1, Table 1.2a). In the present study too, at the end of 5° or 17°C bulb treatment sprouts had almost identical total GA levels per g FW (chapter 4). These total GA levels equaled the data of van Bragt, expressed as ng GA_3 equivalents per g FW. From these results, van Bragt (1971a) already suggested that there is no clear relation between the level of endogenous GAs at the end of cooled or noncooled bulb storage and floral stalk elongation at subsequent higher temperatures. In most of the other studies (Aung and De Hertogh, 1967; Aung and De Hertogh, 1968; Hanks and Rees, 1980a), the essential control of noncooled bulbs of the same age was not included in the GA analyses.

Apparently, the concentration of a particular GA or the total content of free GAs in bulb sprouts at the end of bulb storage, is not suited as parameter in a test for properly cold treated bulbs.

In the present study, in sprouts from both cooled and noncooled bulbs the presence of GA conjugates was demonstrated (chapter 3). They were not quantified and their possible relation to a cooling period remains to be investigated. In addition, the levels of free GAs in individual parts of bulb sprouts were not measured; hence, local differences within the sprouts, e.g. between the various internodes of the floral stalk, cannot be excluded.

Alternatively to an increase in the level of free GAs during cooled storage, GA precursors or inactive GAs may accumulate in noncooled bulbs due to a block in the conversion to active GAs (Metzger, 1990). For this reason, it might be considered that

GA_4 is the accumulating inactive precursor in noncooled bulbs, pointing to GA_1 being the active GA for cold-stimulated stalk elongation. However, also during cooled storage the level of GA_4 increased. The level of GA_1 was low for both bulb treatments.

Hypothesis 2: cold treatment and GA sensitivity

Inspired by the different responses of cooled and noncooled bulbs to applied GA_3, Hanks (1982) suggested a second hypothesis to explain the role of GAs in the cold requirement of tulip: a cooling period is needed to increase the sensitivity of the tulip sprout for GAs. An increase in GA sensitivity during the cold treatment was indeed observed in this study, as was shown by the experiments with isolated sprouts and applied GA_1, GA_4 and GA_9 (chapter 5). However, the sensitivity for GAs appeared to be dependent not only on bulb treatment but also on the starting date of incubation of the isolated sprouts. At early starting dates, sprouts from cooled bulbs were already sensitive for GAs and noncooled sprouts did not respond to applied GAs. At later starting dates the response of cooled sprouts to GAs further increased and sprouts from noncooled bulbs responded to GA application as well. Comparable changes in GA response during the storage season, have been mentioned for GA application to complete bulbs (Hanks and Rees, 1977; Moe *et al.*, 1978; Hanks and Rees, 1980b).

The results demonstrate that at least at later starts of incubation, GA sensitivity is not limiting for the development of noncooled sprouts *in vitro*, since sprouts from both cooled and noncooled bulbs showed a response to applied GA. Apparently, during bulb storage an increase in GA sensitivity occurs. Yet, GA sensitivity is not the only factor involved in the cold requirement of tulip.

GA biosynthesis after bulb storage

Earlier studies with tulip suggested a possible role for GA biosynthesis after the cold treatment, because application of GA biosynthesis inhibitors such as paclobutrazol or ancymidol at that time, could reduce sprout growth (Saniewski, 1989; Suh *et al.*, 1983; Shoub and De Hertogh, 1974; Hanks and Rees, 1977).

In this study, using paclobutrazol after bulb storage, an inhibitory effect of paclobutrazol on floral stalk elongation was confirmed and appeared to be dependent not only on the pretreatment of the bulbs, but also on the starting date of the incubation (chapter 5). At early starting dates of incubation, floral stalks showed

hardly any growth and paclobutrazol had no effect on the elongation of the floral stalk in both cooled and noncooled sprouts. At later starts, paclobutrazol reduced floral stalk elongation in both types of sprouts. The results indicated that at least at later starting dates, GA biosynthesis can occur in sprouts from cooled as well as from noncooled bulbs, thus providing GAs for floral stalk elongation.

Remarkable was the increasing performance of sprouts in the absence of exogenous GA, when incubated at later starting dates (chapter 5). Especially sprouts from noncooled bulbs elongated more when isolated and incubated at later starting dates. This corresponds with results obtained in earlier studies with intact bulbs (Saniewski, 1989), sprouts (van Bragt, 1971b), stalk explants (Kawa and Saniewski, 1990a) or leaf explants (Kawa and Saniewski, 1990b). These plants or explants elongated more when planted or isolated at later dates in the season.

Despite the increased floral stalk elongation of the noncooled control sprouts at later starts of incubation, paclobutrazol reduced the stalk lengths for consecutive incubations to comparable low values. Since paclobutrazol inhibits the oxidative reactions from *ent*-kaurene to *ent*-kaurenoic acid, the increasing performance of the isolated sprouts and the increasing effect of paclobutrazol, suggest an increase in the availability of precursors for the synthesis of GAs. This 'early' GA biosynthesis, enhanced by storage and low temperatures, might supply the sprout with the necessary precursors for GAs when the conditions are favourable for further GA biosynthesis.

Since the experiments with paclobutrazol demonstrated that GA biosynthesis after 'planting' is essential for growth, it is not surprising that the GA levels at the end of bulb storage, before planting, could not be related to floral stalk elongation after planting. Hence, the more relevant changes in GA levels were to be expected after planting the bulbs, at subsequent higher temperatures.

Cooled and noncooled bulbs were planted and GA levels were measured until 11 days after planting (chapter 4). After planting cooled bulbs, the sprouts started to grow and within the first 11 days the level of GA_4 in the floral stalks increased. In noncooled bulbs, sprout growth was negligible and the increase in the level of GA_4 did not occur. The level of GA_{24}, a precursor of GA_4, was higher 4 days after planting than the level in the total sprout immediately after storage, at planting. This indicated that production of GA_{24} had occurred, either in the floral stalk, or in the leaves or basal plate followed by transport to the floral stalk.

As sites of GA biosynthesis in higher plants have been mentioned: developing fruits or seeds, elongating shoot apical regions, roots and young leaves (Graebe and Ropers, 1978; Sponsel, 1987; Moore, 1989). In tulip, the results of Kawa-Miszczak *et al.* (1992) suggested the bulb roots as site of GA biosynthesis. Results of Aung (1971) with intact, rooted bulbs indicated that the basal plate might be a site of GA production. However, except for a decrease in the amount of GA_4 in the basal plates of planted, noncooled bulbs, no significant changes in GA levels were observed in these bulb organs neither during storage nor after planting the pretreated bulbs. The absence of increased GA levels during storage in the present study, might be explained by the non-rooting conditions, since Aung (1971) emphasized the effect of water. Yet, after planting and watering the pretreated bulbs, the general GA levels in the basal plates and roots showed no increase but decreased instead. It is possible that GA biosynthesis occurred and the GAs were converted to GA conjugates, which were not determined in the present study. Furthermore, GA precursors might have been synthesized in the basal plates or roots, followed by transport of these precursors to other parts, such as the floral stalk or flower.

The results in this study with paclobutrazol (chapter 5), demonstrate that at least later in the storage season, the sprout may be an important site of GA production. Since the sprout consists of various organs, the exact localization of GA biosynthesis within the sprout remains to be determined.

Nature of active gibberellin

Among the GAs present in tulip sprouts (Figure 7.1), GA_4 or GA_1 might be the active GAs, the others being precursors (GA_9, GA_{12} and GA_{24}), or an inactivated metabolite (GA_{34}). Comparing the endogenous levels, GA_4 appeared to be the most abundant GA in sprouts, basal plates, floral stalks and flowers. Further, in the first 11 days after planting an increase in the level of GA_4 was observed together with the start of floral stalk elongation. In contrast, GA_1 was present at much lower levels and these levels did not increase during the first 11 days after planting (chapter 4).

Comparing the effects of GA_1, GA_4 and GA_9 applied to isolated sprouts, all three of them could stimulate floral stalk elongation. For stimulating full flower development, GA_9 was the least effective GA (chapter 5). It is possible that the activity of GA_9 is due to its conversion to GA_4. Using [^3H]- and [$^2H_3$]-labelled GA_9 applied to isolated sprouts, it was shown that isolated sprouts indeed are able to convert GA_9 to GA_4 (chapter 6). However, GA_4, on its turn might have to be

converted to GA_1 before becoming biologically active. The involvement of GA_1 cannot be excluded based only on its low endogenous levels, since rapid turnover of GA_1 into its inactivated metabolite (GA_8) is possible (Figure 7.1). GA_8, however, was not detected as endogenous GA in any of the extracts (Rebers *et al.*, 1994, chapter 3). Additionally, after application of [^{3}H]GA_9 or [$^2H_3$]GA_9 to isolated sprouts, labelled GA_4 was detected but no evidence was found for the production of labelled GA_1 or GA_8. This suggests that the endogenous GA_1 is not produced from endogenous GA_4, but will originate from the early-13-hydroxylation pathway. Other GAs of this pathway, such as GA_{19} and GA_{20}, might have been present in amounts below detection.

In *Thlaspi arvense*, it was suggested that two pathways for GA metabolism function in the regulation of different processes. The non-hydroxylation route leading to GA_9 (GA_4 was not detected) may regulate thermoinduced stem growth, while the 13-hydroxylation pathway leading to GA_1 possibly controls processes that proceed in noninduced plants (Metzger, 1990)

Interactions of gibberellins with other hormones

Besides the involvement of GAs in floral stalk elongation, the role of other hormones has been implicated as well. Since auxin is well known for its effect on stem elongation, GA-induced growth has been explained by an effect of GA on auxin biosynthesis, with auxin being the real regulator of growth (Métraux, 1987). In tulip, several studies suggested the involvement of auxin in controlling the elongation of the internodes (Hanks and Rees, 1977; Saniewski and de Munk, 1981; Okubo and Uemoto 1985; 1986; Okubo *et al.*, 1986). Their results suggested that GAs have an effect on auxin biosynthesis, transport or sensitivity. Some possible interactions between GAs and auxin in regulating floral stalk elongation are outlined in Figure 7.2.

Removal of the flower bud at an early stage of growth, considerably reduced floral stalk elongation. This effect could be reversed by application of auxin (Hanks and Rees, 1977; Saniewski and de Munk, 1981; Okubo *et al.*, 1986; Saniewski, 1989), where GA_3 was ineffective (Hanks and Rees, 1977; Okubo *et al.*, 1986). These results demonstrated that the presence of the flower is essential, possibly as a site of auxin biosynthesis, providing auxins for floral stalk growth. Within the flower, especially the presence of the gynoecium appeared necessary, and poor development of the gynoecium was suggested to cause reduced stalk elongation, due to an inadequate supply of auxin (Hanks and Rees, 1977). Using isolated stalk explants,

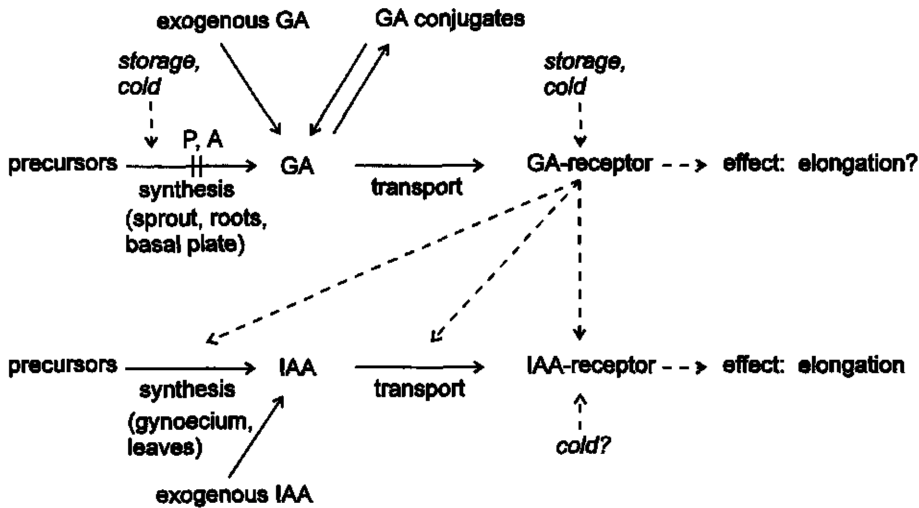

Figure 7.2 Possible interactions between GAs and auxin in regulating floral stalk elongation of tulip. P: paclobutrazol, A: ancymidol.

Kawa and Saniewski (1990a) showed that auxin could induce stalk elongation only when basipetal transport was possible. In addition to the flower, the leaves too have been suggested to produce and release auxin-like substances (Saniewski and de Munk, 1981).

Okubo *et al.* (1986), using bioassays, measured the amount of diffusable auxin from the flower bud. Application of the GA biosynthesis inhibitor ancymidol reduced both the elongation of the upper internode and the amount of diffusable auxin. Both effects were reversed by application of GA_3. These results indicated that GA stimulated the production and/or the transport of auxin, as was also suggested by Saniewski and de Munk (1981). In pea stems too it was shown that GA enhanced auxin biosynthesis (Métraux, 1987). The auxin-induced floral stalk elongation in tulip could be inhibited by application of the GA biosynthesis inhibitor paclobutrazol (Okubo et al., 1986; Saniewski, 1989), suggesting that in addition continued GA biosynthesis is required for the auxin-mediated stimulation of floral stalk growth.

Next to an effect on auxin biosynthesis, GA was suggested to have a stimulating effect on the tissue sensitivity to auxin (Saniewski and de Munk, 1981; Okubo *et al.*, 1986). Results of Rietveld *et al.* (1994) indicated that both applied GA and a cold bulb treatment enhance the sensitivity of floral stalk explants to auxin.

Although Hanks and Rees (1977) suggested two different mechanisms controlling

the elongation of the upper and the lower internodes, results of Okubo and Uemoto (1985; 1986), suggested that elongation of all internodes is controlled by interactions of auxins and gibberellins. The effect of paclobutrazol on all internodes (Saniewski, 1989; this study, unpublished results) supports the view that the elongation of all internodes is regulated by these interactions. Furthermore, results of Saniewski (1989) indicated that GA biosynthesis might occur in each internode.

Summarizing the preceding discussion, it is suggested that the elongation of the internodes is under direct control of auxin, but that auxin biosynthesis, transport and/or sensitivity are controlled by GA (Figure 7.2).

Other factors might also be involved in the development of the floral stalk. Light seemed to be required for sufficient elongation of the last internode, but not for flower development. Full flower development could be observed in dark-grown conditions (Okubo *et al.*, 1986).

The presence of leaves appeared necessary for floral stalk elongation and flower development especially in the early stage of growth (Saniewski and Kawa-Miszczak, 1992). When the leaves were removed, growth of all internodes was inhibited and the flower desiccated. The effect could be reversed by application of cytokinin to the flower bud, suggesting the leaves to provide cytokinins for the growing flower bud (Saniewski and de Munk, 1981). Rakhimbaev *et al.* (1978) reported that a low temperature increased the level of cytokinins in tulip bulbs.

The flower might be the main sink in the tulip plant, and flower bud abortion can be seen as a lack of substrate supply to the flower. The sink function of the flower can be strengthened by GA or cytokinin, and weakened by ethylene and ABA; ethylene is known to cause flower bud blasting (de Munk and Gijzenberg, 1977; Moe *et al.*, 1978). Methyl jasmonate is known for its strong stimulatory effect on leaf senescence in many plants, including tulip (Saniewski and Kawa-Miszczak, 1992).

The effects of these hormones on floral stalk elongation might be indirect: by influencing the development of the flower or the leaves, the availability of auxin is affected and as a result the elongation of the floral stalk.

It has been suggested that inhibitors present in the sprouts, leaves or scales are degraded or removed during the cooling period. The presence of abscisic acid (ABA) in tulip bulbs has been established, and the roots (Aung and De Hertogh, 1979) and basal plate (Aung and De Hertogh, 1979, Terry *et al.*, 1982) have been suggested as sites of ABA biosynthesis. Rakhimbaev *et al.*, (1978) reported that an ABA-like

growth inhibitor, measured in extracts of whole bulbs, decreased during a cold treatment. Terry *et al.* (1982) suggested that the lack of extension growth of the floral shoot in noncooled bulbs, might be related to its high ABA content. However, Franssen and Voskens (1992) reported that no significant changes in the ABA levels per g FW sprout occurred, neither during bulb storage at 5°C nor during noncooled storage at 17°C. With regard to the growth of especially isolated sprouts from noncooled bulbs *in vitro* (chapter 5), it is possible that dissecting the basal plate also removed a putative site of ABA biosynthesis, removing the inhibitor and hence improving sprout growth.

The elongation of floral stalk and leaves after planting pretreated bulbs, is due almost entirely to the elongation of cells produced early in development (Gilford and Rees, 1973). A cold-stimulated hormone action may lead to cell wall loosening and/or lowering of the osmotic cell potential. The growth of the flower stalk mainly depends on nutrients imported from the scales. Mobilization of reserves in the scales is important for floral stalk elongation, at least until the leaves are functional (Lambrechts, 1993). The poor floral stalk elongation and prevailing flower bud blasting of intact planted, noncooled bulbs contrasted with the increasing performance of isolated sprouts from noncooled bulbs *in vitro* (chapter 5). Possibly in intact planted, noncooled bulbs the transport of nutrients from the scales to the sprout is limited, as a consequence of dehydration during the preceding storage at high temperatures. In the isolated sprouts, the uptake of nutrients might have been facilitated by cutting away the scales and basal plate.

Conclusion: role of gibberellins

In conclusion, each of the two hypotheses alone cannot explain the role of GAs in the cold requirement of tulip. An increase in the amount of free GAs occurs, but is more pronounced in noncooled bulbs and no changes in the content of free GAs have been found that are directly related to the preparation of the rapid development at subsequent higher temperatures. Therefore, the GA content at the end of bulb storage, cannot be used as a marker in a test for properly cold-treated bulbs. The sensitivity to GAs increases in cooled sprouts, but also noncooled sprouts are responsive to applied GAs and GA sensitivity apparently is not limiting for the development of noncooled sprouts *in vitro*.

After cooled bulb storage, GA biosynthesis is essential for floral stalk elongation

to proceed, either in intact bulbs or in isolated sprouts. This leads to the hypothesis that a cold treatment stimulates GA biosynthesis at subsequent higher temperatures, then leading to an increase in GA levels. GA might stimulate the auxin biosynthesis in the flower bud and the leaves, and/or its transport and the sensitivity to auxin. The elongation of the floral stalk then is primarily controlled by auxin, transported basipetally, but the auxin system is regulated by GA. The response of isolated sprouts to GA_4, the increase in the level of GA_4 in the growing floral stalks of cooled bulbs and the inability of isolated sprouts to produce detectable amounts of GA_1 from applied GA_9, support the idea that the major responsible gibberellin is GA_4.

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Summary

Tulip bulbs (*Tulipa gesneriana* L.), with terminal buds containing a complete flower, require a period of low temperature to prepare the bud for floral stalk elongation and full flower development at subsequent higher temperatures. For the cultivar Apeldoorn, a dry-storage treatment of 12 weeks at 5°C prior to planting at 20°C, will lead to proper floral stalk elongation and full flower development. Shorter periods at 5°C usually result in slower shoot elongation and delayed flowering. Without any cold treatment, the growth of the shoot is strongly reduced and often flower abortion occurs. In these processes, the involvement of gibberellins (GAs) has been implicated, because application of GAs could partly replace the cold treatment. In addition, GA biosynthesis inhibitors could reduce the floral stalk elongation of cold-treated bulbs and this effect was reversed by simultaneous application of GA.

In horticultural practice, there is a need for a practical assay to test whether a particular bulb has received a proper cold treatment. The amount of GAs or of one particular GA, might provide a suitable parameter in a test for properly cold-treated bulbs.

In this study, the role of GAs in the cold requirement of tulip was investigated by analysing the GA levels in cooled and noncooled tulip bulbs, and by studying the effect and metabolism of applied GAs in combination with a GA biosynthesis inhibitor.

An inventory was made of GAs, in sprouts of cooled (12 weeks 5°C) and noncooled bulbs (12 weeks 17°C) (chapter 3). By combined gas chromatography-mass spectrometry (GC-MS) and GC-selected ion monitoring (SIM), GA_1, GA_4, GA_9,

GA_{12}, GA_{24}, GA_{34} and three GA-related compounds were detected. They all occurred in sprouts of both cooled and noncooled bulbs. Most of them were found in the conjugated form as well. Among these GAs, GA_4 and/or GA_1 might be the active forms, the others being precursors (GA_9, GA_{12}, and GA_{24}), or an inactivation product (GA_{34}).

Using GC-SIM and deuterated GAs as internal standards, the changes in endogenous GA levels were measured in sprouts and basal plates during cooled and noncooled bulb storage, as well as after planting these bulbs (chapter 4). GA_4 and GA_{24} were the major occurring gibberellins, with levels up to ca. 10 ng per sprout or basal plate. GA_1, GA_9 and GA_{34} were present in much lower amounts. The levels of GA_{12} and of the GA conjugates and GA-related compounds were not analysed.

During bulb storage, the level of GA_4 per sprout increased, especially in noncooled bulbs. After 12 weeks, these sprouts contained more GA_4 and also more GA_1 than cooled sprouts. However, sprouts in noncooled bulbs did hardly show any development after planting and it is unlikely that the increased level at the end of bulb storage is correlated with floral stalk elongation at subsequent higher temperatures. In the basal plates no significant changes occurred in the GA levels during storage. Therefore, the GA content in sprouts or basal plates at the end of bulb storage, cannot be used as marker in a test for properly cold-treated bulbs.

After planting cooled bulbs, the sprouts started to grow and within the first 11 days the level of GA_4 in the floral stalks increased. In planted noncooled bulbs, sprout growth was negligible and an increase in the level of GA_4 did not occur.

The biological activity of GA_1, GA_4 and GA_9, was tested on isolated sprouts, cultivated on a liquid medium *in vitro* (chapter 5). To compare the sensitivity to exogenous GAs, sprouts from both cooled and noncooled bulbs were used. The growth retardant paclobutrazol was used to study the role of GA biosynthesis. The growth of these isolated sprouts, the response to GAs and the effect of paclobutrazol, appeared to be dependent not only on the pretreatment of the bulbs, but also on the time in the season that the sprouts were isolated and incubated.

At early starting dates of incubation, floral stalks from both cooled and noncooled bulbs hardly showed any elongation in the absence of exogenous GA. Paclobutrazol had no effect on floral stalk elongation, and the response to GAs of sprouts from cooled bulbs was greater than the response of sprouts from noncooled bulbs. At later starts, considerable floral stalk elongation already occurred without GA application. Paclobutrazol inhibited this floral stalk elongation, and the growth of sprouts from

both cooled and noncooled bulbs was stimulated by GA application. The three tested GAs were not significantly different in stimulating floral stalk elongation. The effect of paclobutrazol was reversed by simultaneous application of GA. The results of these *in vitro* experiments demonstrated that, although depending on the time of the year, sprouts from both cooled and noncooled bulbs are responsive to exogenous GAs. Moreover, sprouts from both bulb treatments are capable of GA biosynthesis. The increasing performance of the isolated sprouts when incubated at later starting dates, and the increasing effect of paclobutrazol on these sprouts, suggested an increase in the availability of precursors for the synthesis of GAs. Apparently, low temperatures as well as bulb storage itself enhance GA biosynthesis and GA sensitivity, and consequently floral stalk elongation after planting when conditions are favourable for growth.

The isolated sprouts did not develop a full-grown flower without the addition of GA. GA_4 was more effective than GA_9 in stimulating this flower development. GA_1 could also stimulate flower development, but was no more effective than GA_4.

The activity of applied GA_9 might be due to its conversion to GA_4. GA_4 on its turn, might have to be converted to GA_1 before becoming biologically active. The metabolism of applied GA_9 was studied, with the purpose to investigate whether tulip sprouts are able to metabolize GA_9 to biologically active GA_4 or GA_1, and whether sprouts from cooled and noncooled bulbs show differences in GA metabolism (chapter 6). $[^3H]GA_9$ and $[^2H]GA_9$ were applied to isolated sprouts by injection into the floral stalk and the metabolites were analysed in the sprouts after 24 h. According to HPLC analyses, $[^3H]GA_9$ was converted to GA_4-like and GA_{34}-like compounds. The labelled metabolites of $[^2H]GA_9$ were identified by GC-SIM, which demonstrated the conversion of $[^2H]GA_9$ to $[^2H]GA_4$ and $[^2H]GA_{34}$. Sprouts from both cooled and noncooled bulbs were able to convert GA_9 to GA_4 and GA_{34} *in vitro*. No evidence was found for the production of labelled GA_1. In the presence of prohexadione (BX-112), known for its inhibiting effect on 2- and 3ß-hydroxylations of GAs, the formation of $[^2H]$ metabolites was less or absent.

In conclusion, there is no direct correlation between the cold-stimulated growth and a change in the endogenous GA status in sprouts or basal plates during cold bulb storage. Further, the sensitivity to GAs increases in cooled sprouts, but also noncooled sprouts are responsive to applied GAs, and GA sensitivity apparently is not limiting for the development of noncooled sprouts *in vitro*. After cooled bulb storage, GA biosynthesis is essential for floral stalk elongation to proceed. The increase in the level of GA_4 in the growing floral stalks of cooled bulbs, the response of isolated sprouts to

GA_4 and the inability of isolated sprouts to produce detectable amounts of GA_1 from applied GA_9, support the hypothesis that GA_4 is the major intrinsically active GA in the floral stalk elongation of tulip.

Samenvatting

Inleiding en probleemstelling

Eén van de belangrijkste factoren die de groei en ontwikkeling van tulpen (*Tulipa gesneriana* L.) beïnvloeden, is de temperatuur. Tulpebollen hebben na de aanleg van de bloem in de zomer, een winterse koudeperiode nodig om bij het daarop volgende voorjaar bij hogere temperaturen een goede groei van bloemsteel en bloem te verkrijgen. Voor de cultivar Apeldoorn is een periode van 12 weken bewaring bij 5°C (koelcel) optimaal: wanneer de bollen daarna geplant worden bij hogere temperaturen (17-20°C), worden goed groeiende planten verkregen met een hoge bloemkwaliteit. Een kortere of langere koudebehandeling leidt vaak tot afwijkingen in de groeisnelheid en de bloemontwikkeling. Zonder koudebehandeling is de spruitgroei sterk geremd en treedt meestal bloemverdroging op.

In de praktijk bestaat behoefte aan een toets, om na te gaan of aan de koudebehoefte van een bol is voldaan. Daarom wordt gezocht naar één of meerdere goed te bepalen chemische stoffen, die maatgevend zijn voor een op de juiste wijze gekoelde bol. Uit de literatuur zijn resultaten bekend die er op wijzen dat een groep van plantehormonen, de gibberellinen (GA's), betrokken zijn bij de koude-gestimuleerde groei van tulpen: toedienen van GA's kon de koudebehandeling vaak gedeeltelijk vervangen. Bovendien kon de stengelstrekking geremd worden met stoffen die de eigen produktie van GA's blokkeren. De hoeveelheid van deze GA's, of van één GA in het bijzonder, zou een geschikte parameter kunnen zijn in een toets voor op een juiste wijze gekoelde bollen.

Gibberellinen, een groep van plantehormonen

Gibberellinen zijn bekend om hun rol bij de kieming van zaden, de inductie van bloei en de lengtegroei van stengels en bladeren. De groep van gibberellinen bestaat uit meer dan 90 stoffen die chemisch nauw met elkaar verwant zijn. Zij kunnen in elkaar worden omgezet, waarbij actieve vormen ontstaan of actieve stoffen juist worden geïnactiveerd. Niet alle vertegenwoordigers komen in alle plantensoorten voor. Naast het voorkomen in de vrije vorm, kunnen gibberellinen ook gebonden aan bijvoorbeeld suikers voorkomen. Deze vorm is zelf niet biologisch actief, maar kan actief worden nadat het gibberelline is vrijgemaakt.

Dit onderzoek

Een eerste vraag was of de koudeperiode nodig is om de hoeveelheid gibberellinen in de bol, of van één GA in het bijzonder, te doen toenemen. Daartoe werd eerst vastgesteld welke gibberellinen in tulpen voorkomen. Vervolgens werd nagegaan hoe hun hoeveelheden veranderen tijdens de koudebehandeling, en tijdens het uitgroeien van de spruit na het planten. Ter vergelijking werd dit ook gedaan in ongekoelde bollen.

Het is ook mogelijk dat niet de hoeveelheden van de hormonen veranderen, maar de gevoeligheid van het weefsel voor hormonen. Dit vormde de tweede vraagstelling en werd onderzocht door gibberellinen van buitenaf toe te dienen en het effect op de stengelstrekking en de bloei te bestuderen. De reactie van zowel gekoelde als ongekoelde bollen werd weer bekeken. Hierbij werd ook gebruik gemaakt van een remstof die de eigen produktie van GA's blokkeert (paclobutrazol). Bovendien werd onderzocht of toegediende GA's in andere GA's worden omgezet.

Meten van gibberellinen

Om GA's te kunnen bepalen werden ze geëxtraheerd uit het plantemateriaal. Omdat de gezochte stoffen slechts in zeer kleine hoeveelheden voorkwamen (enkele delen per miljard), was een uitgebreide zuivering nodig. De methode die hiervoor gebruikt werd, is beschreven en toegelicht in hoofdstuk 2.

Met behulp van gaschromatografie (GC) en een daaraan gekoppelde massaspectrometer (MS), werden GA_1, GA_4, GA_9, GA_{12}, GA_{24} en GA_{34} aangetoond in extracten van tulpespruiten (hoofdstuk 3). Daarnaast werden ook drie gibberellineverwante stoffen gevonden. Alle gevonden GA's en de verwante stoffen kwamen voor

in spruiten van zowel gekoelde (12 weken 5°C) als ongekoelde bollen (12 weken 17°C). De meeste GA's werden ook in de gebonden vorm aangetroffen. In deze serie gibberellinen kunnen GA_4 en GA_1 het actieve GA zijn, de andere zijn voorlopers (GA_9, GA_{12} en GA_{24}), of een inactiveringsprodukt (GA_{34}).

Met behulp van gedeutereerde [^{2}H] GA's als interne standaarden, werden de veranderingen in de GA hoeveelheden gemeten in spruiten en bolschijven, voor en na een behandeling bij 5° of 17°C (hoofdstuk 4). Bovendien werden de GA hoeveelheden de eerste 11 dagen na het planten van de bollen gevolgd. GA_4 en GA_{24} kwamen in de grootste hoeveelheden voor, tot circa. 10 ng per spruit of bolschijf. De hoeveelheden van GA_1, GA_9 en GA_{34} waren veel kleiner. De hoeveelheden van GA_{12}, van de gebonden GA's en van de drie GA-verwante stoffen werden niet gemeten.

Gedurende de bolbehandeling nam de hoeveelheid GA_4 per spruit toe, vooral in ongekoelde bollen. Na 12 weken bevatten deze spruiten meer GA_4 en ook meer GA_1 dan gekoelde spruiten. Na het planten groeiden de spruiten van ongekoelde bollen echter nauwelijks, en het lijkt er dus niet op dat de verhoogde hoeveelheden na een bolbehandeling, de oorzaak zijn van stengelstrekking na het planten. In de bolschijven werden geen significante veranderingen in GA hoeveelheden gemeten. Het gibberelline-gehalte in spruiten of bolschijven aan het eind van de bolbewaring, is daarom niet geschikt als parameter in een test voor op de juiste wijze gekoelde bollen.

Nadat gekoelde bollen geplant waren, begonnen de spruiten uit te groeien en nam de hoeveelheid GA_4 in de bloemstengel toe. In de geplante ongekoelde bollen was de groei te verwaarlozen en nam de hoeveelheid GA_4 niet toe.

Toedienen van gibberellinen

De biologische werkzaamheid van GA_1, GA_4 en GA_9 werd getest op uitgeprepareerde spruiten die werden gekweekt op een vloeibaar medium in buizen (*in vitro*) (hoofdstuk 5). Om de gevoeligheid voor toegediende GA's te vergelijken, werden zowel spruiten uit gekoelde als uit ongekoelde bollen gebruikt. De groeiremmer paclobutrazol werd gebruikt om de rol van eigen GA produktie te bestuderen.

Bij deze experimenten bleek dat de stengelstrekking van de spruiten, het effect van de toegediende GA's en het effect van paclobutrazol, afhankelijk waren van de voorbehandeling van de bollen, maar ook van de tijd in het seizoen dat de spruiten waren uitgeprepareerd en op medium gezet. Wanneer de spruiten vroeg in het seizoen waren uitgeprepareerd, groeiden de bloemstengels van zowel gekoelde als ongekoelde spruiten, zonder toegediend GA nauwelijks. Toegediende GA's hadden meer effect op

spruiten uit deze gekoelde bollen dan op die uit ongekoelde bollen. Paclobutrazol had geen effect op de stengelstrekking. Wanneer de spruiten later in het seizoen waren uitgeprepareerd, groeiden de bloemstengels al aanzienlijk zonder dat GA was toegevoegd. Paclobutrazol remde deze stengelstrekking, wat weer werd opgeheven wanneer ook GA was toegediend. Het toevoegen van GA stimuleerde de groei van zowel spruiten uit gekoelde als ongekoelde bollen. De drie geteste GA's vertoonden onderling geen significante verschillen in hun effect op de stengelstrekking.

De resultaten van deze in vitro experimenten lieten zien dat, hoewel afhankelijk van de tijd in het seizoen, spruiten van zowel gekoelde als ongekoelde bollen gevoelig zijn voor toegediend GA. Bovendien kunnen spruiten van beide bolbehandelingen zelf GA's produceren. De toename in de groei van spruiten die later waren uitgeprepareerd en het toenemende effect van paclobutrazol op deze spruiten, suggereren een toename in de beschikbaarheid van stoffen voor de produktie van GA's. Deze toename wordt blijkbaar beïnvloed door zowel een koudebehandeling als de bolbewaring zelf.

De bloemontwikkeling van de uitgeprepareerde spruiten was niet volledig wanneer geen GA was toegediend: de bloemen kleurden niet rood en de meeste verdroogden. Wanneer GA was toegediend, werden meestal wel volledig ontwikkelde, rood gekleurde bloemen verkregen. Hierbij was GA_4 effectiever dan GA_9. Ook GA_1 kon de bloemontwikkeling stimuleren, maar had niet meer effect dan GA_4.

Het is mogelijk dat GA_9 pas actief is nadat het in GA_4 is omgezet, en GA_4 is misschien pas actief na omzetting in GA_1. De omzettingen van toegediend GA_9 werden bestudeerd, om te onderzoeken of uitgeprepareerde spruiten in staat zijn GA_9 om te zetten in het biologisch actieve GA_4 of GA_1. Daarbij werd ook onderzocht of spruiten uit gekoelde en ongekoelde bollen hierin verschillen (hoofdstuk 6). Getritiëerd [^{3}H] en gedeutereerd [^{2}H] GA_9 werden toegediend aan uitgeprepareerde spruiten door middel van injectie in het bloemstengeltje. Na een dag werden de omzettingsprodukten geanalyseerd. Volgens de analyse door middel van HPLC (hoge-prestatie vloeistof chromatografie), was het getritiëerde GA_9 omgezet in GA_4-achtige en GA_{34}-achtige stoffen. De gemerkte omzettingsprodukten van het gedeutereerde GA_9 konden geïdentificeerd worden met behulp van GC-MS. Gedeutereerd GA_4 en gedeutereerd GA_{34} werden gevonden en dit bevestigde de omzetting van toegediend GA_9 naar GA_4 en GA_{34}. Deze produkten werden gevonden in zowel spruiten uit gekoelde als spruiten uit ongekoelde bollen. Gemerkt GA_1 werd niet gevonden. In aanwezigheid van de remstof prohexadion (BX-112; remt hydroxyleringen van GA's), werden minder of geen gemerkte produkten gevormd.

Conclusies

Uit de resultaten kan geconcludeerd worden dat er geen direkte correlatie is tussen de koude-gestimuleerde groei en een verandering in de GA hoeveelheden in spruiten of bolschijven tijdens de koudebehandeling.

In vitro neemt de gevoeligheid voor GA's toe in spruiten van gekoelde bollen, maar ook spruiten uit ongekoelde bollen kunnen reageren op toegediende GA's en een vergelijkbare stengellengte opleveren. De GA gevoeligheid blijkt dan dus niet de beperkende factor voor de ontwikkeling van ongekoelde spruiten.

Na de koudebehandeling is GA produktie noodzakelijk voor de groei. De toename in de hoeveelheid GA_4 in de groeiende bloemstelen van geplante gekoelde bollen, het effect van GA_4 op uitgeprepareerde spruiten en het niet aantoonbaar zijn van GA_1 na toediening van GA_9, ondersteunen de hypothese dat GA_4 het voornaamste intrinsiek actieve GA is bij de bloemsteelstrekking van tulpen.

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Curriculum vitae

Mariken Rebers werd geboren op 1 november 1961 in de stad Groningen. In mei 1980 behaalde ze het VWO diploma aan het Eemland-College Noord in Amersfoort. In september van dat jaar begon ze met de studie Biologie aan de toenmalige Landbouwhogeschool in Wageningen. Het kandidaatsexamen werd in 1984 behaald. De doctoraalfase begon met een bijvak Hydrobiologie, wat werd uitgevoerd bij het Nederlands Instituut voor Onderzoek der Zee op Texel. Daarna volgden een hoofdvak Microbiologie en een hoofdvak Plantenfysiologie. Na een stage van 9 maanden bij het Institute of Biological Chemistry in Pullman, Washington (USA), werd in september 1988 het doctoraalexamen met lof behaald. Tot en met november van dat jaar bleef zij werkzaam in Pullman.

Van juni 1989 tot en met mei 1993 werd het promotie-onderzoek verricht bij de vakgroep Plantenfysiologie van de Landbouwuniversiteit te Wageningen. De resultaten van dat onderzoek zijn beschreven in dit proefschrift. Een korte werkperiode werd doorgebracht bij het Frontier Research Program van het RIKEN institute in Wako-shi, Japan.

Zij heeft een beurs gekregen van de Science Technology Agency in Japan, om vanaf februari 1995 twee jaar onderzoek te doen bij dit zelfde Frontier Research Program.