

VERNALIZATION AND GIBBERELLIN PHYSIOLOGY OF
WINTER CANOLA

KAREN P. ZANEWICH
(Bachelor of Science, University of Lethbridge, 1986)

A Thesis Submitted
in Partial Fulfillment for
Requirements for the Degree

MASTER OF SCIENCE
(SPECIAL CASE MASTER'S)

UNIVERSITY OF LETHBRIDGE
LETHBRIDGE, ALBERTA, CANADA

March 19, 1993

© Karen P. Zanewich, 1993

ABSTRACT

Winter canola (*Brassica napus* cv. Crystal) requires vernalization, exposure to chilling, to induce bolting and flowering. Since gibberellins (GAs) have been implicated in the regulation of stem elongation and reproductive development in numerous plants, the role of GAs in events induced by vernalization was investigated.

Three classical approaches for studying GA physiology were taken. Plant growth regulators were applied and showed that: (i) GA application induced stem elongation but not flowering in nonvernalized plants and (ii) plant growth retardants that block GA biosynthesis prevented elongation and flowering in vernalized plants. Endogenous GAs were extracted from vernalized and nonvernalized shoot tips, chromatographically purified and quantified by gas chromatography-selected ion monitoring. GA_{1,3,8,19} and GA₂₀ concentrations were higher in the vernalized shoots following vernalization. Feeds of [³H]GA₂₀ to vernalized and nonvernalized plants demonstrated higher rates of [³H]GA₁ formation after vernalization, suggesting increased metabolism to the biologically active form. Collectively, these studies indicate a regulatory role for GAs in the control of stem elongation in winter canola, but the role of GAs in flowering was less clear. Vernalization apparently induces stem elongation by increasing GA synthesis and particularly the biosynthesis of GA₁.

ACKNOWLEDGEMENTS

Ten years ago during my management career initiative, when I first met Stewart Rood in an introductory microbiology class, it never occurred to me that I would study botanical things, much less complete a Master's Thesis under his supervision. Shortly thereafter, however, following an intensive two week crash course offered during Botany 2000, 'Everything Everybody's Discovered About Plant Physiology', I decided to go green. Eventually, I too discovered that gibberellins (GAs) are a powerful resource in the universe and worthy of further attention. I am extremely grateful and thankful to Stewart for providing me with the advice, opportunity and facilities (in essence everything!) that enabled me to study an aspect of plant physiology that I find very fascinating. As a novice paddler in the GA wild waters, I am appreciative of invaluable river reading skills that were passed on as well as the occasional Eskimo rescue.

The list of thank you's continues with: John Bain for providing me with another vantage point from which to view the mysteries of botany (my pin-box camera is almost a format camera now!); Trevor Potter for being my capable GA stunt double, a tough sounding board and a good listener; Keith Topinka for playing 'Canola Pursuit' at a moment's notice, always finding more winter canola seed, and providing field canola plants that he "didn't need"; Professor RP Pharis for sharing his vast collection of GAs; David Pearce for the excellent technical advice, creative consultation and Calgary Express Courier service; John Mahoney for providing alternative, new wave scientific discussion as well as being my chief recreation consultant (was that the purple or blue wax?); Roger Mandel for his helpful experimental suggestions and loan of his humonsterous computer literature search; Joanne Golden for aiding in the ambush against greenhouse pests and acting as my watering shadow on short notice; Bruce McMullin for his creative troubleshooting approaches and mechanical expertise; and everyone else who

dared to set eyes on my winter canola plants, Thesis, or parts there of.

The challenge of the last year or so, the 'Thesis' thing that has dominated so much of the my time, is actually the result of many people and I am grateful to those people who allowed me to take the complete immersion approach. To my friends, thanks for hanging in there with me (honest, you can give me a call at home now). Finally, to my family, the 'original-model' and the 'new and improved version', thanks for being so patient, understanding, and supportive - for this I am eternally grateful.

TABLE OF CONTENTS

Title Page	i
Signature Page	ii
Abstract	iii
Acknowledgements	iv
Table of Contents	vi
List of Tables	vii
List of Figures	viii
Abbreviations	xii
Chapter 1 - Introduction	1
Chapter 2 - General Materials and Methods	25
Chapter 3 - Distribution of Endogenous Gibberellins in Vegetative and Reproductive Organs of <i>Brassica</i>	36
Chapter 4 - The Effects of Vernalization and Plant Growth Regulators on Growth and Flowering in Winter Canola	54
Chapter 5 - Influence of Vernalization on Endogenous Gibberellin Concentrations in Winter Canola	83
Chapter 6 - Metabolism of [³ H]GA ₁ and [³ H]GA ₂₀ in Winter Canola Following Vernalization	93
Conclusions	110
References	116
Appendix 1 - Gibberellin Structures	133
Appendix 2 - Plant Growth Regulator Structures	137

LIST OF TABLES

Table 2-1	Solvents used for SiO ₂ partition chromatography of gibberellins in <i>Brassica</i> extracts.	34
Table 2-2.	HPLC gradients used for purification and separation of gibberellins.	35
Table 3-1.	Endogenous gibberellin concentrations in leaves from 28-day old (vegetative) <i>Brassica napus</i> cv. Westar plants.	44
Table 3-2.	Methyl ester trimethylsilyl ether GAs identified by capillary gas chromatography-mass spectrometry selected ion monitoring from <i>Brassica napus</i> cv. Westar immature siliques.	50
Table 6-1.	Distribution of metabolites in vernalized and non-vernalized winter canola following feeds of [³ H]GA ₂₀ at 6, 12-, and 15-days post-vernalization.	105
Table 6-2	Distribution of metabolites in vernalized and non-vernalized winter canola following feeds of [³ H]GA ₁ at 6, 12-, and 15-days post-vernalization.	111

LIST OF FIGURES

Figure 1-1.	Proposed chromosomal and genetic relationship between several <i>Brassica</i> species and their hybrids.	4
Figure 1-2.	Skeletal structure of a C ₁₉ gibberellin.	14
Figure 1-3.	Proposed gibberellin biosynthetic pathway in <i>Brassica</i> from GA ₁₂ -aldehyde.	17
Figure 3-1.	Gibberellin concentrations in different organs of 28 day old (vegetative) <i>Brassica napus</i> cv. Westar plants.	43
Figure 3-2.	Gibberellin concentrations in different organs of 64 day old <i>Brassica napus</i> cv. Westar plants during early silique-filling.	46
Figure 3-3.	Gibberellin concentrations of <i>Brassica napus</i> cv. Westar siliques at different developmental stages.	48
Figure 3-4.	Gibberellin concentrations of immature and mature <i>Brassica napus</i> cv. Westar seeds and silique coats.	49
Figure 4-1.	Photograph of nonvernalized and vernalized <i>Brassica napus</i> cv. Crystal plants.	62
Figure 4-2a.	Final plant height of <i>Brassica napus</i> cv. Crystal plants exposed to different lengths of vernalization.	63
Figure 4-2b.	Percentage of flowering <i>Brassica napus</i> cv. Crystal plants exposed to varying lengths of vernalization.	63

Figure 4-2c.	Days post-vernalization taken to reach anthesis in <i>Brassica napus</i> cv. Crystal plants exposed to varying lengths of vernalization.	63
Figure 4-3a.	Height of nonvernalized <i>Brassica napus</i> cv. Crystal plants treated with several concentrations of GA ₃ .	66
Figure 4-3b.	Dose-response curve of nonvernalized <i>Brassica napus</i> cv. Crystal plants treated with GA ₃ .	66
Figure 4-4.	Response of 2-, 4-, and 6-week partially vernalized and nonvernalized <i>Brassica napus</i> cv. Crystal plants to exogenous GA ₃ .	67
Figure 4-5.	Photograph of nonvernalized <i>Brassica napus</i> cv. Crystal plants treated with 10 ⁻¹ M GA ₃ , GA ₁ , GA ₄ , GA ₅ and 0 M GA.	71
Figure 4-6.	Effect of 0, 10 ⁻³ , 10 ⁻² , and 10 ⁻¹ M GA ₁ , GA ₃ , GA ₄ , and GA ₅ on plant height in nonvernalized <i>Brassica napus</i> cv. Crystal plants.	72
Figure 4-7.	Photograph of vernalized <i>Brassica napus</i> cv. Crystal plants treated with 0, 10 ⁻⁵ M PP333 at 12 DPV and 10 ⁻⁵ M PP333 immediately following vernalization.	74
Figure 4-8.	Effect of 10 ⁻⁵ M paclobutrazol on plant height and flowering of vernalized <i>Brassica napus</i> cv. Crystal.	75
Figure 4-9.	Photograph of vernalized <i>Brassica napus</i> cv. Crystal plants treated with 0, 10 ⁻⁴ , 10 ⁻³ , 10 ⁻² , and 10 ⁻¹ M CGA 163'935.	76

Figure 4-10.	Effect of 0, 10 ⁻⁴ , 10 ⁻³ , 10 ⁻² , 10 ⁻¹ M CGA 163'935 on height and flowering of vernalized <i>Brassica napus</i> cv. Crystal.	77
Figure 4-11.	Foliar and growth forms of vernalized <i>Brassica napus</i> cv. Crystal plants treated with 10 ⁻¹ M CGA 163'935.	82
Figure 5-1.	Gas chromatography-mass spectrometry selected ion monitoring profiles of the molecular ions from methyl ester trimethylsilyl ether derivatives of endogenous GA ₁ and GA ₃ and [2H ₂] GA ₁ and GA ₃ .	87
Figure 5-2.	Height of vernalized and nonvernalized <i>Brassica napus</i> cv. Crystal plants.	91
Figure 5-3.	Concentrations of GA ₁₉ , GA ₂₀ , GA ₁ , GA ₃ , and GA ₈ in shoot tips from vernalized and nonvernalized <i>Brassica napus</i> cv. Crystal plants at 0, 8 and 18 days post-vernalization.	92
Figure 6-1.	Elution of [³ H]GA ₁ and metabolites from SiO ₂ partition columns loaded with extracts from vernalized <i>Brassica napus</i> cv. Crystal shoot tips.	100
Figure 6-2. (top)	Percentage [³ H]GAs recovered in the EtOAc fractions from SiO ₂ partition chromatography after a [³ H]GA ₁ feed to <i>Brassica napus</i> cv. Crystal plants 6-, 12-, and 15- days post vernalization.	101
Figure 6-2. (bottom)	Percentage [³ H]GAs recovered in the EtOAc fractions from SiO ₂ partition chromatography after [³ H]GA ₂₀ fed to <i>Brassica napus</i> cv. Crystal plants 6-, 12-, and 15- days post vernalization.	101

- Figure 6-3. Elution of [^3H]GA₂₀ and metabolites from C₁₈-reversed phase HPLC loaded with the EtOAc-soluble fractions after silica gel partition chromatography from vernalized *Brassica napus* cv. Crystal shoots. 106
- Figure 6-4. Elution of [^3H]GA₁ and metabolites from C₁₈-reversed phase HPLC loaded with the EtOAc-soluble fractions after silica gel partition chromatography from vernalized *Brassica napus* cv. Crystal shoots. 110

ABBREVIATIONS

ANOVA = analysis of variance
BSTFA = bis-(trimethyl-silyl)-trifluoroacetamide
CGA 163'935 = cimectacarb
cv. = cultivar
DAP = days after planting
DPV = days post-vernalization
ein = *elongated internode* mutant
EtOAc = ethyl acetate
EtOH = ethanol
h = hour
dw = dry weight
GA = gibberellin
GA_n = gibberellin n
GC-MS (SIM) = gas chromatography-mass spectrometry (selected ion monitoring)
HPLC = high performance liquid chromatography
KRI = Kovats retention index
LD = long day
LDPs = long day plants
MeOH = methanol
PAR = photosynthetically active radiation
PGR(s) = plant growth regulator(s)
PGR(s) = plant growth retardant(s)
PP333 = paclobutrazol
PVPP = polyvinylpyrrolidone
ros = *rosette* mutant
R_t = retention time
SD = short day
SiO₂ = silicic acid
TMCS = trimethylchlorosilane

CHAPTER 1

INTRODUCTION

The production of canola or oilseed rape (*Brassica* spp.) in Canada has steadily increased since its introduction in the 1940s. Breeding and development of new varieties with higher oil content and lower glucosinolate concentration has served to promote canola as an attractive field crop alternative. Canola production ballooned from 6,100 tonnes in 1967 to 4.3 million tonnes in 1991, making canola Canada's second most valuable crop, exceeded only by wheat (More, 1992).

Although spring-sown canola accounts for almost all of the canola presently grown in Canada, fall-sown 'winter canola' also shows promise as an oilseed crop. Since planting and seedling emergence occur during the fall, young plants are able to take advantage of winter moisture reserves during the subsequent spring. Furthermore, since seed maturation occurs early in the summer growing period, the time when many insects and pathogens are prevalent, winter canola is able to minimize exposure to many pests which would result in yield reduction. In Europe, winter canola is a popular and successful oilseed crop and consistently out-yields spring varieties by 20% or more (Bunting, 1984).

Although winter canola varieties are well-adapted to European climates, present cultivars are not adapted to the climatic condition of the Canadian prairies. To understand the lack of adaptation and to assist efforts in winter canola breeding, further investigations of the physiology of winter canola may be helpful. Additionally, these studies also have value to basic science, since the regulation of flowering remains one of the most prominent

mysteries in plant biology.

The studies described in this Thesis were used to investigate the phytohormone physiology of winter canola, *Brassica napus* cultivar Crystal. Studies focussed on gibberellin (GA) physiology and its involvement in the regulation of bolting and flowering responses that are induced by vernalization, the cold treatment that is required by winter canola and other winter annuals as well as biennials for the induction of reproductive development.

The Botany of Canola

Canola, or oilseed rape, plants are dicotyledonous and members of the Brassicaceae family, a diverse group that is characterized by the presence of mustard-oils or isothiocyanates, metabolic products of glucosinolates (Cronquist, 1988). Isothiocyanates are sulphur-containing organic substances that serve as antiherbivory compounds which are effective against a wide variety of browsing insects and animals (Harborne, 1988). The oilseed members of *Brassica* are not all a single species; rather, they include several primary species including, *Brassica nigra*, *B. oleracea* and *B. campestris* (synonym: *B. rapa*) and their allotetraploid hybrids, *B. carinata*, *B. juncea* and *B. napus*, which resulted from paired crosses (Figure 1-1) (U, 1935; Williams, 1985; Bunting, 1986). The resulting allotetraploid hybrids described above have also been referred to as amphidiploids; one set of chromosomes was derived from one species and the other set came from another species to form the hybrid (Strickberger, 1976).

Current hypotheses which are supported by restriction fragment length polymorphism (RFLP) analyses suggest that the genomes of diploid species of *Brassica* arose from a $2n = 12$ progenitor and have evolved in ascending chromosome number (Song *et al.*, 1990). Furthermore, results from RFLP studies have lead Song *et al.* (1988) to suggest that the cultivated *Brassica* diploid species evolved via two distinct pathways. *Brassica nigra* and related taxa arose from one pathway, whereas *B. campestris* and *B. oleracea* originated independently.

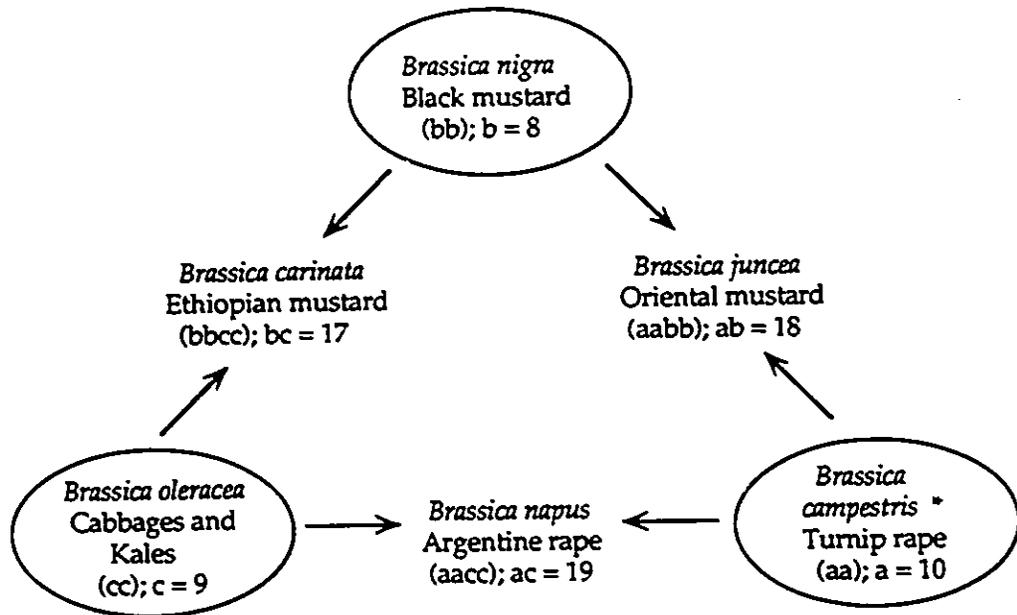


Figure 1-1. Proposed genetic and chromosomal relationships between several *Brassica* species and hybrids (adapted from Williams, 1985 and Bunting, 1986). Lower case letters indicate the nuclear genome such that a = 10 chromosomes, b = 8 chromosomes and c = 9 chromosomes. * Some authorities consider *B. rapa* to take precedence over *B. campestris*, but the names are synonymous.

According to Bunting (1986) some of the earliest reports indicate that a plant related to cabbage was first grown in Europe for production of rape oil during the sixteenth century. In addition to its merits as a crop plant, *Brassica* was and still is recognized as a pioneer plant for reclamation projects in the Netherlands and elsewhere (de Jong, 1981, cited by Bunting 1986). Although oilseed rape was first introduced into Canada prior to the 1940s, little was grown since there was no seed market (Kneen, 1992). Originally, it was primarily cultivated because it provided an excellent source of oil that was high in erucic acid and could be used as a marine lubricant during World War II. However, following the end of that war, the demand for lubricant oil declined and much of the oilseed was exported to Europe and Japan for crushing. Today, Japan continues to be a major importer of Canadian canola seed (More, 1992).

Two species of rape were initially introduced into Canada for the production of oil (Bunting, 1986). The introduction of *Brassica napus* seed into Canada seems almost accidental; a bag of seed found abandoned on a wharf in Argentina was subsequently shipped to Canada (Kneen, 1992). Consequently, the term 'Argentine rape' has been associated with *B. napus* in Canada. Similarly in 1936, a Canadian farmer of Polish descent provided the source of *B. campestris* seed and consequently the term 'Polish rape' was associated with this species. Initially, approximately 80% of spring-sown canola was *B. campestris*. However, with different breeding objectives and the development of early flowering *B. napus* cultivars, both species are now approximately equally grown in Canada (Bunting, 1986).

Breeding programs are currently emphasizing yield improvement, increased quality of oil and meal, increased disease resistance and specifically defined oil composition. The early oilseed rape varieties grown were limited

in their use as edible oils because of undesirably high contents of erucic acid and glucosinolates. Canada has been the leader in the development of new cultivars of 'double low'; oilseed rape varieties that are low in erucic acid and glucosinolate content (Bowman, 1984; Kimber, 1984). The name 'canola' was originally introduced by the Western Canadian Oilseed Crushers Association to designate only those varieties and cultivars of oilseed rape that possessed 5% or less erucic acid in the oil and 3 mg/g or less glucosinolate content in the meal (Kneen, 1992). However, in 1980 the trademark was transferred to the Canola Council of Canada and subsequently in 1986, the erucic acid content requirement in the oil was decreased to 2% or lower and the glucosinolate content was reduced to 30 micromoles or less per gram of meal (Kneen, 1992).

In Canada, canola oil is used largely as an edible oil and is often preferred in such products as shortenings, margarines, and salad and cooking oils (Paszowski, 1983). Canola oil is nutritionally desirable because it is low in saturated lipids and relatively high in mono-unsaturated lipids (Whitney *et al.*, 1990). The meal which is produced as a byproduct of oil extraction can be incorporated into the livestock feed for multigastric animals and is slowly gaining acceptance as a supplemental feed (Paszowski, 1983). Alternatively, the meal may also be used as a fertilizer, a use that is common in the citrus groves of China and Japan (Kneen, 1992).

Although spring-sown canola is primarily cultivated in Canada, winter canola can also be cultivated for the production of edible oil. Generally, spring varieties are sown in Alberta during the third week in March to mid-April and harvested in the fall, whereas winter varieties are planted from mid-August to mid-September and harvested in the late spring of the following year (K Topinka, personal communication).

Unlike its spring counterpart, winter canola is a cold-requiring plant that requires vernalization, an extended chilling period, before flowering and seed production. Typically, plants germinate, emerge and become established as seedlings in the autumn. As the seasonal temperatures fall, the plants stop growing so that during the winter the plant remains in a stunted rosette form, a morphology that minimizes chilling and/or freezing injury. Further, since the plants are close to the ground, snow cover can serve as effective insulation. In addition to these morphological characteristics, physiological mechanisms such as the accumulation of reducing sugars in the leaves provide winter hardiness for winter canola genotypes (Thompson and Hughes, 1986). The extended period of vernalization or chilling during the winter induces the transition from the vegetative to reproductive plant resulting in floral initiation and subsequent flowering.

Vernalization

In some previous publications, 'vernalization' has been used to describe accelerated flowering in response to various treatments including photoperiod. However, throughout this Thesis vernalization will be used to describe the promotion of flowering solely by exposure to low temperature.

Reports of flower promotion by exposure to a period of low temperature have been documented as early as the middle of the 19th century (Lang, 1965). The mechanisms of vernalization are unique, inasmuch as the perception and transduction of the environmental stimulus occurs during the chilling period, but the effect is expressed following vernalization (Metzger, 1988a). Klebs (1903, cited in Napp-Zinn 1987) equated vernalization with the occurrence of biochemical processes during chilling that permitted the

development of 'internal conditions' that subsequently resulted in flowering.

The term "jarovizacija" or its anglicized equivalent, vernalization, was originally coined by Trofim Denisovich Lysenko, a Russian geneticist (Evans, 1969). The English equivalent translated from Latin for the word vernalization is 'springization', suggesting that winter varieties are converted to the spring genotype by cold treatment (Salisbury and Ross, 1992).

The response(s) induced by vernalization involve two components, one genetic, the other environmental (Napp-Zinn, 1987). Numerous putative vernalization genes and alleles have been described for plants such as winter wheat, pea and *Arabidopsis*, however their role in the metabolism of nucleic acids, amino acids and plant hormones is ambiguous (Napp-Zinn, 1987). A common question arises: could the observed metabolic changes be involved in the regulation of flowering or are they solely the biochemical consequences of chilling?

Plant responses to vernalization are numerous and are often variety- or cultivar-dependent (Wiebe, 1990). The vernalization requirement may be facultative or quantitative, in which low temperature treatment results in hastened flowering. Plants with a quantitative vernalization response will eventually flower even with no chilling treatment. Conversely, absolute or qualitative vernalization requirements are those in which low temperature is absolutely required for flowering. Plants with a qualitative vernalization requirement will remain vegetative indefinitely without chilling treatment. Responses may also be immediate or delayed. Most biennials and some winter annuals including winter canola, have an absolute vernalization requirement and a delayed response, whereas a majority of winter cereal varieties, such as Petkis rye (*Secale cereale*) and winter wheat (*Triticum*

aestivum) have a facultative requirement (Lang, 1965).

The age(s) or stage(s) of development at which a plant is receptive or responsive to vernalization varies with plant species (Metzger, 1988a). In a review by Wiebe (1990) it was noted that many biennial plants with an absolute cold requirement must reach a specific developmental stage prior to becoming sensitive or susceptible to treatment. The point at which a plant becomes receptive to the stimulus defines the end of the juvenile phase and the switch to the adult or mature phase (Metzger, 1988a).

In addition to whole plant vernalization in which the shoot tip seems to play an integral role in the flower-promoting action of low temperatures (Lang, 1965), a variety of specific tissues including leaf petioles (*Lunaria*) (Wellensiek, 1961), root cuttings (*Lunaria*) (Wellensiek, 1962), imbibed seeds (*Brassica oleracea*) (Nakamura, 1961) and embryonic callus derived from immature embryos (*Triticum aestivum*) (Whelan and Schaalje, 1992) are responsive to vernalization. Further, the temperature at which vernalization occurs seems to be important for induction of a response. Some studies utilizing sub-zero temperature treatments that suspended growth and cell division have shown that some vernalization is possible at sub-freezing temperatures (Lang, 1965). However, this is probably the exception and the temperatures at which vernalization is most successful range from approximately 1°C to 12°C, depending upon the plant species (Hänsel, 1953; Reid *et al.*, 1991).

It has been suggested that actively metabolizing and dividing cells are generally required for successful vernalization (Wellensiek, 1964). Cellular studies using the cold requiring plant *Thlaspi* have indicated that increased mitotic activity rather than cell elongation is primarily responsible for

induced stem growth following vernalization (Metzger and Dusbabek, 1991). Although the mechanisms by which vernalization influences cell division are unknown, it has been suggested that acceleration of the cell cycle occurs in response to prolonged periods of cold treatment, thus forming new cells at a more rapid rate (Metzger and Dusbabek, 1991).

While the effects of vernalization are species- and often even variety-specific, several other factors are also known to affect vernalization. Generally, the effect of duration of low temperature on floral initiation is quantitative; the time required for the evocation of a flowering response is often positively correlated with the duration of the vernalizing period up to the point at which the optimal vernalization period is reached (King and Bacon, 1992; Purvis, 1961).

Observations made using localized chilling treatment of specific plant tissues suggest that the shoot tip is the principal site of temperature perception (Curtis and Chang, 1930; Metzger, 1988b). Other experiments have involved grafting parts from vernalized plants to nontreated plants (Lang, 1965). If the shoot tip with the apical meristem from a vernalized plant is attached to a nonvernalized plant, flowering eventually occurs. However, the grafting of the meristem region from a nonvernalized plant to a vernalized plant does not result in flowering, the meristematic tissue region remains vegetative. Hence, these results suggest that the substance or condition responsible for floral induction is formed in the shoot tip.

Flowering

As described by Lang (1952), flowering can be divided into four major

stages: (i) floral initiation, the differentiation of the flower primordia; (ii) floral organization, the development of the different floral parts; (iii) floral maturation, including meiotic cell division and growth of floral parts; and (iv) anthesis, the shedding of pollen and receptivity of the female structures. Photoperiodic, biennial, and winter annual plants are unable to flower unless they are exposed to specific environmental conditions such as long or short photoperiods or cold temperature. Exposure to one or a combination of these treatments results in the induction of floral initiation. Floral initiation in qualitative plants is probably dependent on the attainment of a specific metabolic condition which enables the morphogenic switch from the vegetative to the reproductive plant.

The transition of a plant from a vegetative to reproductive state is a complex phenomenon involving changes in metabolism and translocation of nutrients and other substances essential for the development of organs involved in sexual reproduction and the alternation of generations (Chailakhyan, 1968). Observations made primarily from photoperiodic grafting experimentation involving donor and recipient plants have supported the transmission of a specific substance(s) responsible for flower induction (Chailakhyan, 1968; Lang, 1965). Chailakhyan (1937, cited by Evans 1969) coined the term 'florigen' to represent the chemical substance, perhaps a hormone, as the stimulus responsible for flower initiation. Other hypothetical hormone-like compounds, such as anthesins (Chailakhyan, 1958, cited by Chailakhyan, 1975) have also been proposed to be required for flower development. In later forms, the florigen concept was revised to propose a florigen complex that is composed of gibberellins (GAs) and anthesins; GAs were proposed to regulate stem elongation and anthesins could regulate flower induction and formation (Chailakhyan, 1970).

Similar to the studies with photoperiodic plants, donor grafts from

vernalized plants of biennial species such as *Hyoscyamus* have successfully been attached to nontreated recipient plants resulting in flowering, suggesting that a substance was capable of being transmitted across the graft union and resulted in flower formation (Melchers, 1939, cited by Purvis 1961). Equivalent to florigen, the term 'vernalin' has been adopted to represent the hypothetical chemical substance which promotes flowering in cold-requiring plants. However, vernalin is strictly associated with the low temperature controlled stimulus. It has alternatively been suggested that rather than a transmissible substance, precursors of vernalin are present in both vernalized and nonvernalized plants, but in the latter there is a block to vernalin formation which is overcome during vernalization (Lang, 1965).

Two types of relationships between vernalin and florigen have been proposed (Lang, 1952). The first proposes that vernalin is a metabolic precursor which is converted into florigen. An alternate proposal suggests that vernalin is a catalyst causing the formation of florigen. Both alternatives remain speculative since little direct evidence of the possible chemical nature of vernalin has been established.

A group of compounds that have been likened to florigen and vernalin are the gibberellins (GAs). In the absence of vernalization, the cold requiring biennial *Hyoscyamus niger* can be induced to flower by the application of gibberellic acid (GA₃) (Lang, 1956). However GA₃ treatment does not substitute for vernalization in all cold-requiring or biennial plants. Thus far, neither vernalin nor florigen has been successfully isolated and the possible association between vernalin or florigen and GA remains uncertain.

Gibberellins

The gibberellins (GAs) are a group of phytohormones which are chemically defined by their diterpenoid structure. At present, about 90 gibberellins have been identified from numerous plant and fungal species (Mander, 1992; Takahashi *et al.*, 1986; DW Pearce, personal communication). GAs have been implicated in the regulation of both growth and developmental events. Studies using a range of dwarf mutants (Reid, 1990a) have confirmed the involvement of GAs in the regulation of stem elongation (Phinney, 1985). However, the role of GA in flower initiation and differentiation is less clear (Pharis and King, 1985; Zeevaart, 1983).

Gibberellins are synthesized via the mevalonic acid pathway from acetate units of acetyl CoA. The first true gibberellane ringed substance in the pathway is the aldehyde of GA₁₂, a 20-carbon compound from which other 20 or 19 carbon tetra- or penta-cyclic compounds are derived (Fig 1-2). Oxidation of C₂₀ GAs at C(20) result in the formation of the lactone bridge in the A ring which characterizes the C₁₉ GAs. The primary distinction between the different GAs is the attachment or substitution of different side groups, mainly hydroxyl groups, to the carbon skeleton. Commonly, the hydroxyl groups are attached to C(3) and/or C(13), but also may be positioned on C(1), C(2), C(11), C(12), C(15), C(18) or elsewhere (Fig. 1-2).

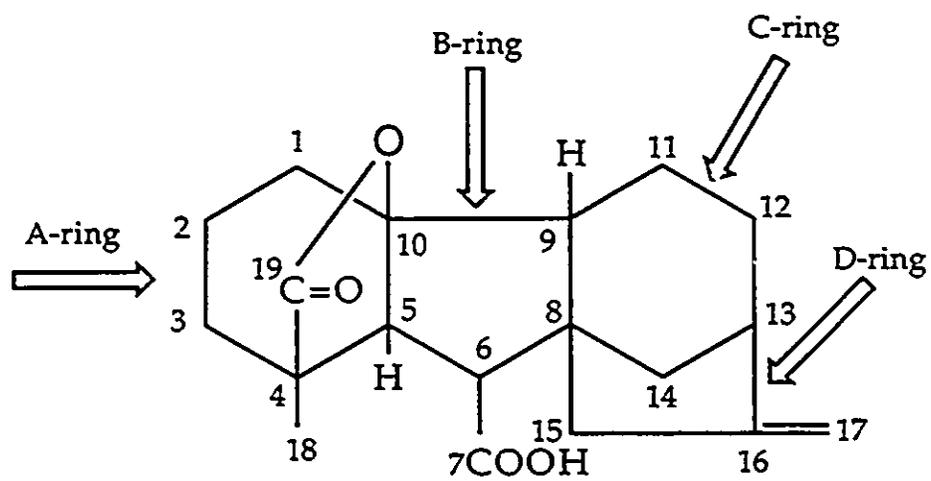


Figure 1-2. A C₁₉ gibberellin chemical skeleton showing the labelled rings and the numbered carbon atoms.

The structure of the biologically active GAs, those capable of evoking a physiological response, vary greatly (Pharis *et al.*, 1992). Generally, the C₁₉-GAs have a higher activity than the C₂₀-GAs (Hoad, 1983). Of the C₁₉-GAs, those which are characterized by 3 β -hydroxylation, 3 β ,13-dihydroxylation and 1,2-unsaturation tend to exhibit maximal biological activity (Hoad, 1983). Conversely, 2 β -hydroxylation results in decreased or complete loss of biological activity (Sponsel *et al.*, 1977).

The biosynthesis of GAs has been extensively reviewed (Graebe, 1987; Hedden *et al.*, 1983; Mander, 1992; Sponsel, 1987). Although early GA biosynthesis, those steps originating from mevalonic acid through *ent*-kaurene to GA₁₂-aldehyde, appears to be similar in most plants, the conversion of GA₁₂-aldehyde to subsequent GAs apparently varies with species (Graebe, 1987; Sponsel, 1987). Figure 1-3 illustrates the possible relationship between two GA biosynthetic pathways, the early 13-hydroxy and the late 13-hydroxy pathways in *Brassica* based on the native occurrence of GAs in *Brassica* (Hedden *et al.*, 1989) and the occurrence of similar pathways in other plants (Graebe, 1987).

GA₁₂-aldehyde probably serves as the precursor for all GAs. Subsequently, a series of hydroxylations and/or oxidations results in the formation of other GAs (Mander, 1992). The hydroxylation step at C(3) or C(13) is an important distinguishing feature that initially separates these two principal biosynthetic pathways (Fig. 1-3). However, the pathways may also converge to form a metabolic grid rather than distinct linear sequences (Sponsel, 1983). Rood *et al.*, (1987a) have suggested the presence of the early 13-OH pathway in *Brassica* following initial identification of prominent bioactive GAs. The occurrence of this pathway was supported by observing the conversion of [²H₂]GA₁₉ to [²H₂]GA₂₀ and [²H₂]GA₁ (Rood *et al.*, 1990a). Recent feeds of

[$^2\text{H}_2$]GA₉ to *Brassica* resulted in the formation of both the 13-OH and 13-non-OH GAs supporting not only the functioning of the 13-non-OH pathway in *Brassica*, but also the existence of an interconnecting metabolic web as shown in Figure 1-3 (SB Rood and P Hedden, unpublished).

The dominant biosynthetic pathway of many plants such as maize (Phinney and Spray, 1990) and probably *Brassica* (Rood *et al.*, 1989a, 1990a), is the early 13-hydroxylation pathway. Intermediate GAs of the early 13-OH pathway such as GA₅₃, GA₄₄, GA₁₉ and GA₂₀ are formed from GA₁₂-aldehyde and eventually give rise to GA₁, a biologically active GA shown to be responsible for stem elongation in numerous plants including maize (Phinney, 1985; Spray *et al.*, 1984), pea (Ingram *et al.*, 1984; Reid, 1990b) and probably *Brassica* (Rood *et al.*, 1987a). The conversion of GA₂₀ to GA₁ involving 3 β -hydroxylation is thought to be a pivotal metabolic conversion thereby allowing the regulation of GA₁ biosynthesis (Ingram *et al.*, 1984; Spray *et al.*, 1984). Subsequently, GA₁ can be 2 β -hydroxylated to form GA₈. Ultimately, the rate of formation of 2 β -hydroxylated catabolites could be one mechanism regulating the amount of GA₁ available for growth (Sponsel, 1983).

Non-13-OH GAs have also been identified in numerous plant species including *Brassica* (Hedden *et al.*, 1989), *Arabidopsis* (Talon *et al.*, 1990a), and *Thlaspi* (Metzger and Mardaus, 1986). Since the 13-non-OH GAs are abundant in reproductive organs in rice (Takahashi and Kobayashi, 1991) and maize (Murofushi *et al.*, 1991), this pathway might be more prominent during reproductive development. Alternatively, Graebe (1987) suggests that the 13-non-OH pathway may be 'accidental', arising from the metabolism of nonhydroxylated GAs by the same enzymes involved in the 13-OH pathway.

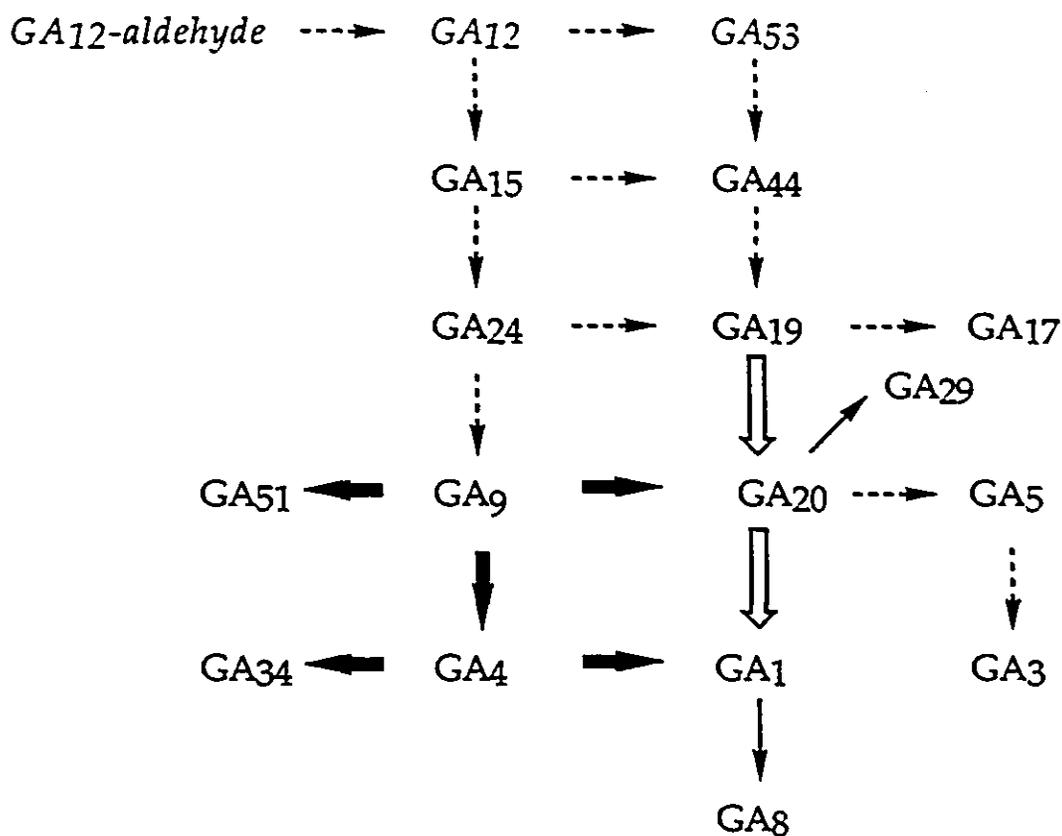


Figure 1-3. Proposed 13-OH and 13-non OH biosynthetic (metabolic) pathways from GA₁₂-aldehyde to C₁₉ GAs for *Brassica*. GA₁₂ and GA₅₃ (in italics) have not yet been identified in *Brassica* but have been found in other crucifers. (See Appendix 1 for GA structures.)

----> = proposed pathway based on occurrence of native GAs and similar pathways described in other plants (Graebe, 1987).

➡ = demonstrated pathway by metabolic feeds of [²H₂]GA₄ or [²H₂]GA₉ to the rapid cycling *Brassica rapa* genotype *ein* (SB Rood and P Hedden, unpublished).

⇨ = pathway demonstrated by metabolic feeds of [²H₂]GA₁₉ to the rapid cycling *Brassica rapa* genotype *ein* (Rood *et al.*, 1990a).

→ = proposed pathway based on metabolites identified by GC-MS following [³H]GA₂₀ feed to the rapid cycling *Brassica rapa* genotype *ein* (SB Rood, KP Zanewich, DW Pearce and RP Pharis, unpublished).

Several novel GAs have recently been identified in *Brassica*, however their biosynthetic relationship and role(s) in germination, growth and/or development remain undetermined. GA₈₅ was characterized from the cotyledons of young *Brassica* seedlings and demonstrated a bioactivity that was equal to or greater than that of GA₁ or GA₃, suggesting that this GA may be one of the primary bioactive GAs present in germinating canola seedlings (Sheng *et al.*, 1992a). Furthermore, just as some of the more active GAs have complementary inactive 2 β -hydroxyl forms, GA₈₅ has a similar inactive analogue, GA₈₉ (Sheng *et al.*, 1992b).

In addition to their free forms, GAs may also occur in bound, water-soluble forms which can be hydrolyzed to yield the free acid GAs (Schneider, 1983). These forms of GAs are referred to as the conjugated GAs with the GA being linked to a low molecular weight compound, most commonly glucose. GA conjugates are generally considered to be relatively biologically inactive but biological effectiveness may result following cleavage of the conjugate moiety (Schneider, 1983).

Flowering, Stem Elongation and Gibberellins

Evidence for the involvement of GAs in the regulation of flowering of angiosperms has been largely gathered from photoperiod and thermoinduction studies (Pharis and King, 1985; Zeevaart, 1983). Plants subjected to noninductive environmental conditions lack floral initiation, while those exposed to inductive conditions become capable of producing flower primordia and subsequently flowering. Direct or correlative and/or manipulative experimental approaches may be used to assess the relationship between GA content and flowering.

The direct approach relies primarily on the identification and quantification of endogenous GAs. Quantitative and/or qualitative changes in endogenous GA content would be expected prior to and during the transition from the vegetative to the reproductive condition (Zeevaart, 1983). There have been numerous attempts to establish such correlations between endogenous GA level and floral initiation and/or development.

Prior to the relatively recent development of GA quantification by combined gas chromatography-mass spectrometry (GC-MS), samples were quantified based on the ability of the extract to promote shoot elongation in GA bioassays. Harada (1962, cited in Zeevaart, 1983) assayed extracts from either thermoinduced or noninduced shoot tips of *Chrysanthemum morifolium* using an *Avena* mesocotyl assay and found that one particular zone (E) had a 10-fold increase in activity following thermoinduction. Changes in endogenous GA-like activity were also demonstrated in vernalized and nonvernalized winter rape using a bioassay with a dwarf variety of *Lathyrus*, although these changes were correlated with bolting or stem elongation rather than flowering (Margara, 1963). Experiments by Chailakhyan and Lozhnikova (1962) also noted increases in GA-like substances in the leaves of wheat, rape and rye following vernalization.

Preliminary results based on total ion response using gas chromatography-selected ion monitoring of extracts from cold-treated vegetative wheat seedlings, suggest that C₂₀ GAs are more abundant in the cold-treated seedlings while the C₁₉ GAs are more abundant in the nontreated seedlings (Lin and Stafford, 1987). However, these results may also be indicative of the role of GAs in the control of elongation since height was also profoundly affected by temperature.

Plant growth regulators (PGRs) are also useful tools for indirectly assessing physiological function associated with GAs (Grossmann, 1992). Certain PGRs block or inhibit GA biosynthesis, thereby reducing endogenous GA level. These PGRs also reduce plant growth and are referred to as plant growth retardants (PGRs). If GAs are required for flowering, the application of these PGRs before, during, and/or after the inductive treatment would also be expected to inhibit or retard flower initiation and development (Zeevaart, 1983).

Alternatively, genetic dwarf mutants that are deficient in endogenous GAs have also been used as tools to study the relationship between endogenous GA concentration and flowering capability (Reid, 1990a). Flowering is often altered or delayed in these mutants, substantiating the results of PGR studies. Thus, the effect of deliberate reduction of endogenous GA level on growth and developmental events can be evaluated in two manners: (i) artificially by the application of growth retardants that block GA biosynthesis, or (ii) with dwarf mutants that have reduced levels of endogenous GAs due to a genetically regulated lesion.

Metzger (1990a) noted that PGR-treated *Thlaspi arvense* still produced flower primordia, although, macroscopic flowers were not formed. This indicates that different phases of the flowering response may be differentially sensitive to GA level. In some long-day plants, plant growth retardants are completely effective in preventing flowering under inductive conditions, whereas other plants, stem elongation is retarded but flowering still occurs although it may be delayed (Pharis and King, 1985; Zeevaart, 1983). As a result of these variable and sometimes complex results, the research involving plant growth retardants should be interpreted cautiously.

Another manipulative approach in the study of GAs and flowering involves attempts to induce or stimulate flowering by the application of GA to the shoot tips of plants. Following gibberellic acid (GA₃) application, stem elongation generally occurs (Lang and Reinhard, 1961). Subsequently, depending on the type or kind of GA treatment and plant species, the degree of flowering varies from one that is similar to the response observed under optimal inductive conditions to a reduced response with delayed or incomplete flowering (Lang, 1957). Lang (1956) was able to induce stem elongation and flower formation in a non-cold-treated biennial, *Hyoscyamus niger*, solely with the application of GA₃. Since this initial observation by Lang, numerous cold-requiring plants have been treated with GA₃ (see Zeevaart, 1983). However, other responses have been varied, ranging from a complete flowering response to a complete lack of observable floral development.

Several explanations have been proposed to account for the lack of flower induction in certain plants after GA₃ application. First, Lang (1957) notes that with the variety of GAs that exist, there could be some species specificity with respect to inductive GAs. The failure to induce flowering could thus be the result of the use of an ineffective GA. Of the native GAs within the plant, one or a few could be responsible for flower initiation, while another(s) could regulate stem elongation (Metzger, 1990a). For example, in *Myosotis alpestris*, application of GA₇ caused both stem elongation and flower induction, whereas GA₃ resulted in only stem elongation (Michniewicz and Lang, 1962). Similarly, of various GAs tested, only GA₃₂, GA₅, 2,2-dimethyl GA₄, GA₃ and GA₇ were effective at inducing flower formation in a photoperiodic plant *Lolium temulentum* grown under noninductive conditions (Pharis *et al.*, 1987). Differential flowering responses to different GAs have prompted Evans and coworkers (1990) to suggest that GA structure

is related to florigenic activity in *Lolium temulentum*. Just as biological activity and specific GA structure are associated with the promotion of stem elongation, the florigenic capacity of GAs is also likely to be structurally defined. Furthermore, it is possible that structural specificity could vary for the control of elongation versus flowering.

An alternative explanation for the variable floral induction by exogenous GA₃ is that GA is probably not the sole factor that controls flowering in cold-requiring plants. GAs are likely to act in combination with other chemical and environmental factors (Napp-Zinn, 1984; Zeevaart, 1983). Lang (1957) observed a faster and more uniform flowering response after GA₃-application to plants that were also exposed to low temperatures that was otherwise non-inductive. It is likely that vernalization in cold-requiring plants shifts the balance between substances that promote or inhibit developmental processes in favor of the former, consequently promoting flowering (Lang, 1952). Vernalization is a complex process and is unlikely to influence only a single metabolic system; rather it probably affects several processes. GAs may thus only facilitate the expression of the vernalization response (Evans, 1971).

Floral initiation and the onset of stem elongation occur almost simultaneously in some flowering plants (Zeevaart, 1983). Pharis and King (1985) have noted that a major concern associated with prescribing GAs with a role in vernalization is one of discerning the effects on bolting (shoot elongation) versus floral initiation and development. As one experimental approach, Metzger (1990a) suggests that while GAs may be involved in the flowering process, genes responsible for the flowering process should be characterized to determine whether GA plays a role in the selective changes in gene expression that result in flowering. This molecular approach provides a worthy direction for future research focused on the elusive

substance or balance of substances associated with flowering.

Research Approach

These studies focus primarily on the GA physiology of canola cultivars of oilseed rape, *Brassica napus*. Preliminary investigations into the distribution of GAs within the canola plant were accomplished using the annual canola cultivar Westar. The use of the annual plant for these initial studies avoided the complexities of discriminating GA changes associated with development versus GA changes due to temperature condition alone. To assess the involvement of GAs in flowering and stem elongation associated with vernalization, three approaches were utilized with the winter annual cultivar Crystal:

1. The effects of several plant growth regulators on vernalized and nonvernalized *Brassica* plants were studied. Several concentrations of GA₁, GA₃, GA₄, and GA₅ were applied to determine whether exogenous GAs could substitute for vernalization and induce stem elongation and/or flowering in nonvernalized plants. Conversely, plant growth retardants that block GA biosynthesis, including a triazole and an acylcyclohexadione, were applied to vernalized plants to inhibit stem elongation and/or flowering in vernalized plants.
2. Endogenous GA content of shoot tips from vernalized and nonvernalized vegetative and reproductive winter canola *Brassica* plants was analyzed following chilling treatment.
3. The rate of metabolism was studied in winter canola *Brassica* plants

following vernalization to determine what metabolites were formed from feeds of [³H]GA₁ or [³H]GA₂₀ and if the rates of these metabolites were altered.

The combination of these research approaches comprises the classical sequence for investigating phytohormonal physiology. Collective results refer not only to the possible correlation between a phytohormone and a process but the experimental expansion to include manipulative studies can also investigate a causal or regulatory association between a phytohormone and the physiological process.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Plant Growth Conditions

Greenhouse-Grown Plants

Certified seeds of *Brassica napus* cvs. Crystal (winter annual) or Westar (spring annual) obtained from Mr. Keith Topinka, Canola Agronomist, Agriculture Canada Research Station, Lethbridge, Alberta, were sown in 11.5 (diameter) X 9.5 cm (height) pots filled with Terra-Lite 2000 Metro-Mix (W.R. Grace & Co. of Canada Ltd., Ajax, Ontario). Plants were watered to saturation daily and fertilized as required with a water-soluble 20-20-20 fertilizer with chelated trace elements (The Professional Gardener Co. Ltd., Calgary, Alberta). All plants were grown at $23^{\circ}\text{C} \pm 4^{\circ}$ (day and night) in the University of Lethbridge greenhouse (latitude 49.6°N) and provided with a 16 h photoperiod provided by natural sunlight supplemented with high pressure sodium vapor lights (Reflector PL90M (medium) N400, P.L. Light Systems Canada Inc.). The lights were positioned 1.4 m above the plants and provided $280 \mu\text{mol s}^{-1} \text{m}^{-2}$ photosynthetically active radiation (PAR) as measured with a Li-Cor quantum sensor (LI-190S-1; Lincoln, Nebraska). One week after seeding, plants were uniformly thinned to one seedling per pot.

Following four weeks of growth in greenhouse conditions, plants that were to be vernalized were transferred to an upright refrigerator maintained at $4^{\circ}\text{C} \pm 2^{\circ}$. A 10 h photoperiod was provided by cool white fluorescent lights delivering $50 \mu\text{mol s}^{-1} \text{m}^{-2}$ PAR. After 10 weeks at 4°C , plants were transferred to a cold room ($13^{\circ}\text{C} \pm 2^{\circ}$) for 72 h (light was from a 150W

incandescent bulb and cool white fluorescent tubes delivering $15 \mu\text{mol s}^{-1} \text{m}^{-2}$ PAR) and subsequently returned to the original greenhouse conditions. Plant height and developmental stage (Harper and Berkenkamp, 1975) were determined at weekly intervals (or more frequently as specified) throughout the experimental period.

Field Grown Plants

Brassica napus cv. Westar plants were grown in an irrigated field plot at the Agriculture Canada Research Station, Lethbridge (latitude 49.6°N) by canola agronomist Keith Topinka. Soil and fertility conditions and planting dates were typical for the region and details of those parameters are available from Alberta Agriculture records.

Plant Growth Regulator Applications

Gibberellins

Gibberellin (GA) solutions consisting of either GA₁, (from Professor RP Pharis, University of Calgary), gibberellic acid (GA₃) (119 3093; Eastman Kodak Co., Rochester, New York), GA₄ (Abbott Laboratories, North Chicago, Illinois) or GA₅ (Professor RP Pharis, University of Calgary) was dissolved in 50% (v:v) aqueous ethanol (EtOH) and serially diluted to appropriate concentrations of 1.0×10^{-1} , 1.0×10^{-2} , 1.0×10^{-3} M in 50 % EtOH. Applications of 5 μl each were made to the shoot tips of plants using a Hamilton syringe on four consecutive days starting 28 days after planting (DAP).

Paclobutrazol

Paclobutrazol (PP333; [(2RS, 3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-pentan-3-ol]), Imperial Chemical Industries, United Kingdom) was applied as a root drench (25 ml of a 10^{-5} M aqueous solution) to vernalized plants either immediately following 10 weeks of vernalization or 12 days post-vernalization (DPV). Dates of anthesis were recorded throughout the experimental period.

CGA 163'935

CGA 163'935 (proposed ISO name: cimectacarb; ethyl 4-cyclopropyl (hydroxy) methylene-3,5-dioxocyclohexane-carboxylate, Ciba-Geigy Ltd., Basle, Germany) at aqueous concentrations of 1.0×10^{-1} , 1.0×10^{-2} , 1.0×10^{-3} or 1.0×10^{-4} M was applied by foliar spray (until run-off; approximately 10 ml per plant) to plants which had been vernalized for 10 weeks. Dates of anthesis were recorded over the experimental period.

Endogenous Gibberellin Analysis

Tissue Collection

Tissue was harvested, quickly frozen in liquid nitrogen, and lyophilized for 72 h. All tissue was stored with desiccant at -20°C until analysis.

GA Extraction and Purification

Prior to extraction, all tissue was re-lyophilized during thawing for 24 h. Tissue was ground using a mortar and pestle in cold 80% aqueous methanol (MeOH; ACS531; BDH Inc., Edmonton, Alberta) and allowed to extract for at least 12 h at 4°C. Extracts were filtered under vacuum and phosphate buffer (0.1 M, pH 8) and 10 to 20 ng each of [17-²H₂]GA₁, [17-²H₂]GA₃, [17-²H₂]GA₄, [17-²H₂]GA₅, [17-²H₂]GA₈, [17-²H₂]GA₉, [17-²H₂]GA₁₉, and [17-²H₂]GA₂₀ (all greater than 99% enrichment; Professor LN Mander, Australian National University) were added as quantitative internal standards. The MeOH was removed *in vacuo* and the pH of the resulting aqueous extract was increased to 9 with 1.5 N KOH. Aqueous extracts were partitioned twice against water-saturated diethyl ether (B10094; BDH Inc.). The aqueous extract was acidified to pH 7 using 1.5 N HCl and a slurry was made using polyvinylpyrrolidone (PVPP; P-6755; Sigma, St. Louis, Missouri). After 30 minutes, the aqueous PVPP slurry was vacuum filtered. The aqueous filtrate was acidified to pH 3 with 1.5 N HCl and partitioned three times against water-saturated ethyl acetate (EtOAc; B10108; BDH Inc.). The EtOAc fraction was frozen and filtered to remove water. EtOAc was removed *in vacuo* and the fraction was taken to dryness. Dried residues were transferred to Whatman GF/D glass microfibre discs (09-874-48; Fisher Scientific, Edmonton, Alberta) using a 1:1 (v:v) solution of MeOH:EtOAc. Additionally, 250 Bq each of [1,2-³H]GA₁ and [1,2-³H]GA₄ (Amersham Corporation, Arlington Heights, Illinois) was added to the discs as chromatographic standards.

Samples were further purified using step-wise elution silica gel (SiO₂) partition chromatography (Durley *et al.*, 1972; Rood *et al.*, 1983). All elution solvents were pre-saturated with 5% formic acid (A118-4; Fisher Scientific). Five g of deactivated (20% H₂O by weight) SiO₂ (2766; SA Scientific

Adsorbents, Atlanta, Georgia) was slurried with a 95:5 hexane (B90210; BDH Inc.):EtOAc solution and poured into a glass column. After settling and low pressure compaction, the samples on the glass microfibre discs were positioned on the top of the column's stationary phase. The column was initially eluted with 30 ml of a 60:40 hexane:EtOAc solution followed by 35 ml of a 5:95 hexane:EtOAc solution (Table 2-1). Five ml fractions were collected and ^3H -distribution was determined by liquid scintillation counting of 0.3 ml aliquots.

Appropriate SiO_2 fractions were dried and then grouped together using MeOH, filtered using 0.45 μm filters (SJHV004NS; Millipore Waters Chromatography, Mississauga, Ontario), refiltered using C18 Sep Pak cartridges (51910; Millipore Waters Chromatography) and dried under N_2 at room temperature. The GAs were further purified and fractionated by reversed-phase high performance liquid chromatography (HPLC) using a $\mu\text{Bondapak C}_{18}$ column (3.9 x 300 mm; 27324; Millipore Waters Chromatography) attached downstream from a C18 Guard-Pak precolumn (88070; Millipore Waters Chromatography). A linear gradient of 10-73% MeOH with 1% acetic acid in the aqueous component at a flow rate of 1.7 ml min^{-1} was used (Table 2-2). Two ml fractions were collected at 1.2 min intervals. Aliquots from fractions 20-40 were counted to determine the distribution of ^3H -standards.

Analyses by GC-MS SIM

HPLC fractions were air-dried and then grouped and transferred into 1 ml Reacti-vials according to the retention times (Rts) of authentic standards and those reported by Koshioka *et al.* (1983). Samples were dried at room

temperature under N₂. Residues were methylated in 100 µl of ethereal diazomethane (Cohen, 1984) at room temperature for 60 min. Subsequently, samples were dried under N₂ and 100 µl of ethereal diazomethane was again added. Methylated samples were dried under N₂ and silylated with 50 µl of pyridine (27530; Pierce, Rockford, Illinois) and 50 µl BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) + 1% TMCS (trimethylchlorosilane) (38832, Pierce) at 90°C for 30 min. GA8 samples were silylated twice. All samples were dried under N₂ and allowed to cool to room temperature before analysis.

Samples were dissolved in hexane and 1 µl aliquots were introduced by cool on-column injection into a mega-bore precolumn. GAs were resolved using a Hewlett-Packard 5890 Series II GC containing a DB-5 (133-5012; J&W Scientific, Folsom, California) or DB5MS (122-512; J&W Scientific) 15 m X 0.25 mm fused silica column with a 0.25 µm film of polymethyl (5% phenyl) siloxane. Head pressure of the carrier gas (He) was 265 kPa and the resulting flow rate was 1.4 ml min⁻¹. At injection, the column head pressure was 28 kPa and the temperature was 60°C. Following a 30 second delay at 60°C, the following temperature program was used: a rapid temperature ramp of 25°C min⁻¹ to 200°C, a slow rate temperature ramp of 5°C min⁻¹ to 270°C and a final rapid temperature ramp of 20°C min⁻¹ to 300°C. The transfer line from the gas chromatograph to a Hewlett Packard 5970 mass selective detector was maintained at 300°C. Selected ion monitoring programs were used.

The amounts of endogenous GA₁, GA₃, GA₄, GA₅, GA₈, GA₉, GA₁₉ and GA₂₀, were calculated from the peak area ratios of 506/508, 504/506, 418/420, 416/418, 594/596, 298/300, 434/436 and 418/420, respectively, using a modified version of the equation for isotopic dilution analysis described by Fujioka *et al.* (1986):

$$\text{ng GA} = S/100 [(BCE / (D-FC)) - A]$$

where S = ng of standard added to extract, B = % labelled molecules in the internal standard, C = measured intensity of M⁺ of unlabelled GA, E = a factor calculated from the relative intensities of ions in the M⁺ cluster of unlabelled GA and the amounts of partly labelled GA relative to fully-labelled GA in the internal standard, D = measured intensity of M⁺ of completely labelled GA, F = the intensity of the ion of m/z equivalent to M⁺ of the fully-labelled GA in the M⁺ cluster of the unlabeled GA relative to the intensity of M⁺, C = measured intensity of the M⁺ of unlabelled GA, and A = % of unlabelled molecules in the internal standard. When labelled standards were not available, the relative levels of GAs were estimated based on the ease of detection (abundances) of the M⁺ ion, recognizing that fragmentation patterns were different for each GA.

GA identifications were based on Kovats Retention Indices calculated from a coinjected hydrocarbon standard from 'parafilm' (Gaskin *et al.*, 1971) and from the comparison of the abundances of eight monitored ions with KRI and ion abundances of authentic standards or from published reports.

Metabolism of [³H]GA₁ and [³H]GA₂₀ in Vernalized and Nonvernalized Plants

Application of [³H]GAs

Metabolism of [³H]GAs were examined in two separate experiments. In one experiment, 2.1 KBq of either [1,2-³H]GA₁ (from Amersham; specific activity: about 1.2 TBq per mmol) or [2,3-³H]GA₂₀ (from Professor RP Pharis,

University of Calgary [see Murofushi *et al.*, 1977]; about 62 GBq per mmol) in 95% aqueous EtOH was applied by Hamilton syringe to the shoot tip of vernalized and nonvernalized *Brassica napus* cv. Crystal plants 5 DPV (no observable stem elongation) or 11 DPV (some stem elongation). In a second experiment, 2.1 KBq of either [1,2-³H]GA₁ or [2,3-³H]GA₂₀ was applied to the shoot tips of plants 14 DPV (stem elongation). Following an incubation period of 24 h, plant apices were harvested, quickly frozen in liquid nitrogen and lyophilized. All tissue was stored with desiccant at -20°C until analysis.

Analysis of Metabolic Products

Tissue was ground in cold 80% aqueous MeOH and extracted for at least 12 h. Following vacuum filtering, extracts were taken to complete dryness *in vacuo*. Dried residues were transferred onto glass microfibre discs using 1:1 MeOH:EtOAc and a final transfer with a minimum volume of 50% aqueous MeOH. Silicic acid partition chromatography was carried out as previously described with minor modifications in solvent volumes and the inclusion of an additional step (Table 2-1). Initially, 50 ml of 60:40 hexane:EtOAc was passed through the column, followed by 50 ml of 5:95 hexane:EtOAc and finally 40 ml of MeOH. Five ml fractions were collected and aliquots were removed to determine the distribution and ratio of 'free' acidic [³H]GAs to putative conjugated [³H]GAs. Acidic GAs elute in the EtOAc fractions while GA conjugates elute in the methanolic fractions. Recovery of GAs in either the EtOAc or MeOH fractions was expressed as a percent of the total recovered radioactivity.

Appropriate fractions were grouped together for each of the free and conjugated GA regions. After drying under N₂, aliquots of each sample were

analyzed by reversed-phase C₁₈ HPLC (as previously described) to determine the relative distributions of the [³H]GA precursor ³H-metabolites. Tentative identification of ³H-metabolites was based on comparison of chromatographic Rts of authentic [³H]GA standards and Rts described by Koshioka *et al.* (1983). Further, previous identification of [²H]GA metabolites has already been made following similar feeds to *Brassica* (Rood *et al.*, 1990a). Metabolite recovery was expressed as percent of total recovered ³H.

Table 2-1. Solvents used for purification of gibberellins by step-wise silica gel partition chromatography.

Solvent (Hexane:EtOAc)	Volume (ml)	Fractions Collected
<i>Endogenous GA Analysis</i>		
(60:40)	30	6
(5:95)	35	7
<i>Metabolite Analysis</i>		
(60:40)	50	10
(5:95)	50	10
100% MeOH	40	8

Table 2-2. Solvent gradients for separation of gibberellins on C₁₈ μ Bondapak reversed-phase high pressure liquid chromatography.

Solvent	Time (min)	Flow Rate (ml min ⁻¹)	Volume (ml fraction ⁻¹)
10% MeOH	10	1.7	2
10% -> 73% MeOH	38	1.7	2
73% ->100%	15	1.7	2

CHAPTER 3

Distribution of Endogenous Gibberellins in Vegetative and Reproductive Organs of *Brassica*

Abstract

Since there is physiological diversity between different plant organs, studies were conducted to investigate the distribution of endogenous gibberellins (GAs) in *Brassica* (canola or oilseed rape). GA₁ and its biosynthetic precursors, GA₂₀ and GA₁₉, were extracted, chromatographically purified, and quantified by gas-chromatography-selected ion monitoring (GC-SIM), using [²H₂]GAs as internal standards. In young (vegetative) *Brassica napus* cv. Westar plants, GA concentrations were lowest in the roots, increased acropetally along the shoot axis and were highest in the shoot tips. GA concentrations were high but variable in leaves. GA₁ concentrations also increased acropetally along the plant axis in reproductive plants. During early silique filling, GA₁ concentrations were highest in siliques and progressively lower in flowers, inflorescence stalks (peduncles plus pedicels), stems, leaves, and roots. Concentrations of GA₁₉ and GA₂₀ showed similar patterns of distribution except in leaves, in which concentrations were higher, but variable. Immature siliques were qualitatively rich in endogenous GAs and GA₁, GA₃, GA₄, GA₈, GA₉, GA₁₇, GA₁₉, GA₂₀, GA₂₄, GA₂₉, GA₃₄, GA₅₁ and GA₅₃ were identified by GC-SIM. In whole siliques, GA₁₉, GA₂₀, GA₁ and GA₈ concentrations declined during maturation due to declining levels in the maturing seeds; their concentrations in the silique coats remained relatively constant and low. These studies demonstrate that GAs are differentially distributed in *Brassica* with a general pattern of acropetally increasing concentration in shoots and high concentration in actively growing and developing organs.

Introduction

Gibberellins (GAs) are involved in the regulation of many aspects of shoot growth and development in *Brassica* (Rood *et al.*, 1990b; Zanewich *et al.*, 1990) and other crop plants (Pharis and King, 1985; Phinney, 1985). The role of endogenous GAs in the control of shoot elongation has been well documented for many plants (Phinney, 1985) and reduced stature is a phenotypic consequence in GA-deficient or GA-insensitive *Brassica* mutants (Rood *et al.*, 1989b; Rood *et al.*, 1990b; Zanewich *et al.*, 1991). Additionally, reproductive development is retarded and anthesis is delayed or does not occur in GA-deficient *Brassica* dwarfs (Rood *et al.*, 1989b; Zanewich *et al.*, 1990; Zanewich *et al.*, 1991). Consistent with this, the application of triazole plant growth retardants that block GA biosynthesis can prevent or inhibit *Brassica* flowering in addition to retarding shoot elongation (Rood *et al.*, 1989a).

Studies of the physiological role of GAs in plant growth and development frequently involve qualitative and/or quantitative analyses of endogenous GAs. Major portions of shoots, whole shoots, or even whole plants are often extracted for GA determinations. However, such procedures will obscure any tissue- or organ-specific GA distribution and will dilute GAs from GA-rich organs with relatively GA-deficient, metabolically less-active tissues. Such dilution could prevent meaningful analyses of GA level or concentration.

Differences in GA distribution in several plants including pea (Smith *et al.*, 1992), maize (Murofushi *et al.*, 1991), rice (Takahashi, 1990), *Silene* (campion) (Talon and Zeevaart, 1990b) and oat (Kaufman *et al.*, 1976) have been recognized. To investigate the distribution of GAs within *Brassica* plants, studies were performed to determine the GA distribution in vegetative and reproductive organs of *Brassica*. Since GA₁ is probably a principal bioactive

GA in *Brassica* (Hedden *et al.*, 1989; Rood *et al.*, 1987a; Rood *et al.*, 1989b), the studies focused on analyses of GA₁ and two of its biosynthetic precursors, GA₂₀ and GA₁₉. Additionally, the major endogenous GAs in the GA-rich immature siliques (including seeds) were investigated.

Methods and Materials

Plant Materials and Growth

Seeds of *Brassica napus* cv. Westar were planted in 15 cm pots filled with Metro mix (W.R. Grace & Co., Ajax, Ontario). Seedlings were subsequently thinned to one healthy plant per container. Plants were grown in the University of Lethbridge greenhouse under the conditions described in Chapter 2.

Twenty eight-day old vegetative Westar plants were dissected into the following parts: 1) shoot tips (containing the apical meristem, small leaf primordia and subtending stem to provide approximately 5 mm long segments), 2) medial stems (stems between the shoot tip and cotyledons), 3) hypocotyls, 4) roots (primary and some secondary roots), 5) young leaves (those leaves nearest the shoot tip which had not yet fully expanded and had short petioles), 6) expanded leaves (those leaves with large blades and completely elongated petioles, and 7) old leaves (those leaves nearest the base of the plant which were senescing).

Another group of Westar plants was harvested nine weeks after planting and separated into: immature siliques, flowers (about one day post-anthesis), inflorescence stalks (peduncles plus pedicels), stems, leaves (all non-senescent leaves), and roots. Material for each replicate was collected from five plants and dry weights from 1.3 g (for flowers) to 39 g (for roots).

Siliques at various developmental stages were collected over two 45 day periods beginning July 31, 1989 and July 18, 1990 from Westar plants grown in an irrigated field plot at the Agriculture Canada Research Station, Lethbridge.

Criteria used for judging silique developmental stage were length, degree of filling, and color. Immature siliques (stage 1) were green, 1-3 cm long and had no seeds or tiny, moist, green seeds. In some instances the stigmas were still present. Stage 2 siliques were green and elongating and had small green 'watery' seeds. Siliques which were still green but with full seeds were categorized as stage 3. Stage 4 siliques had coats which had started to yellow or ripen, and hard, greenish-brown seeds. Mature siliques (stage 5) were dry, yellow, greater than 5 cm long, appeared full, and contained hardened black seeds. The dry weights for immature silique samples ranged from 2 to 10 g and for seeds from 0.89 to 1.8 g.

Endogenous GA Analyses

To quantify endogenous GA levels, 10 to 20 ng each of [17,17-²H₂] GA₁₉, [17,17-²H₂] GA₂₀, [17,17-²H₂]GA₁, and [17,17-²H₂]GA₈ standards (all greater than 99% enrichment) from Professor L.N. Mander, Australian National University, were added during extraction. Endogenous GAs were extracted and purified by step-elution silicic acid (SiO₂) partition chromatography followed by reversed-phase C₁₈ high-performance liquid chromatography (HPLC) as described in Chapter 2. Derivatized samples (Chapter 2) were analyzed by GC-MS-SIM.

For the identification of GAs from immature siliques, 2 g samples from silique harvest 1 were purified by SiO₂ and C₁₈ HPLC. HPLC fraction groupings were derivatized and analysed by GC-SIM, probing appropriate fractions for specific GAs as indicated in Table 3 in addition to GA_{5,7,12,13,25,27,36,44,70,77,85} and 2β-OH GA₅₃, relying on Kovats Retention Indices (Gaskin *et al.*, 1971) and ion abundances of authentic standards or from published reports. For identification,

eight ions were normally monitored but only five characteristic ions are included in Table 2. Since fragmentation patterns are different for each GA, estimates for quantitative comparisons were based on the ease of GA detection (abundances of the molecular ion or another ion of high intensity from) from 1/3 aliquots of each of the samples. Autotunes indicated similar instrument sensitivity before and after analyses.

Results and Discussion

GA Distribution in Vegetative Seedlings

GA₁ was detected in extracts from all parts of the seedling axis, including roots, in which GA concentrations were consistently low (Fig. 3-1). Shoot tips, regions of extensive cell proliferation, had the highest GA₁ concentrations, while stem concentrations were somewhat lower. Thus, within the plant axis, GA₁ concentration increased acropetally. A similar axial distribution of GA₁ has been recently reported for *Pisum* (Smith *et al.*, 1992).

GA₁ concentrations in leaves were generally high but variable (Table 3-1). Leaves that had already undergone expansion typically had the highest GA₁ concentrations, young actively growing leaves had the lowest concentrations and older senescing leaves had intermediate GA₁ concentrations. The leaves did not exhibit acropetally increasing concentrations of GA₁ as had been demonstrated in pea (Smith *et al.*, 1992). In contrast to pea, the young uppermost *Brassica* leaves had relatively low GA₁ concentrations.

Two immediate metabolic precursors of GA₁, GA₂₀ and GA₁₉, were also most concentrated in shoot apical regions and progressively less concentrated in stems and roots (Fig. 3-1). Although GA₂₀ and GA₁₉ concentrations were both consistently lower than GA₁, GA₁₉ concentrations were greater than GA₂₀ concentrations in the plant axis. However, unlike the high concentrations of GA₂₀ that have been reported in the upper leaves of pea (Smith *et al.*, 1992), concentrations of GA₂₀ in canola leaves were lower than those of GA₁ (Table 3-1). Additionally, GA₁₉ concentrations were also low in the leaves. Previous observation of lower GA concentrations in canola or oilseed rape shoots (Hedden *et al.*, 1989; Rood *et al.*, 1989a) probably resulted

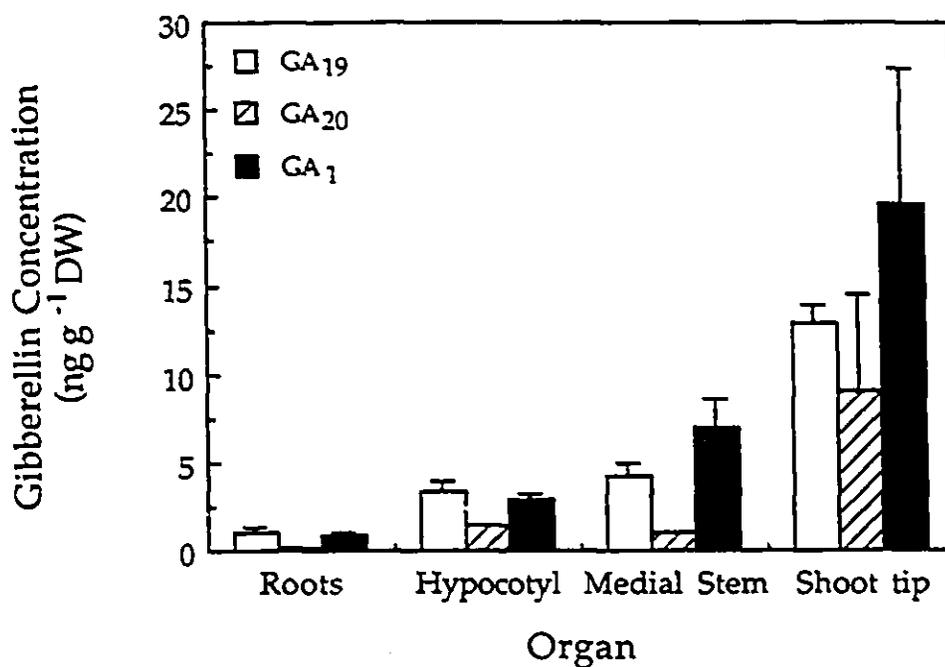


Figure 3-1. Gibberellin concentration (ng g^{-1} dry weight) in different organs of 28 day old (vegetative) *Brassica napus* (canola cv. Westar) plants. Values represent the means + s.e. of two replicates.

Table 3-1. Endogenous gibberellin (GA) concentration in leaves from 28 day old (vegetative) *Brassica napus* cv. Westar plants (means \pm standard errors are included).

Leaves	GA Concentration (ng g ⁻¹ DW)		
	GA19	GA20	GA1
Young	3.8 \pm 0.1	5.6 \pm 3.0	13.1 \pm 6.2
Expanding	1.8	6.7 \pm 2.0	34.6 \pm 23.3
Old (senescing)	3.0 \pm 1.3	3.9 \pm 0.2	23.5 \pm 5.4

from analyses of large shoot samples that contained substantial amounts of metabolically less-active, mature tissues combined with more vigorous younger tissues.

GA Distribution in Reproductive Plants

The pattern of distribution of GA₁₉, GA₂₀, and GA₁ in reproductive plants was similar to that observed in the vegetative seedlings. Concentrations of all three GAs were highest in the apical tissues that had differentiated into the reproductive organs (Fig. 3-2). Among the reproductive structures, concentrations of the three GAs tended to be highest in immature siliques (including seeds), intermediate in the flowers, and lower in the inflorescence stalks (peduncles and pedicels). The stems generally contained lower concentrations of GAs than the reproductive organs, and the lowest GA concentrations occurred in the roots. GA concentrations in the leaves were also variable in the reproductive plants. GA₁ concentration in the leaves was lower than in other shoot parts (Fig. 3-2).

Endogenous GAs in Siliques and Seeds

In the reproductive plants, GA concentrations were highest in the immature siliques (Fig. 3-2). To investigate changes of GA concentrations in developing siliques, weekly harvests were conducted to obtain siliques at five developmental stages. During this sequence of development, GA₁₉, GA₂₀ and particularly GA₁ concentrations progressively declined with age (Fig. 3-3). Consequently, the concentrations of these free GAs, and particularly free GA₁, were very low in the mature siliques. García-Martínez *et al.* (1987) also found

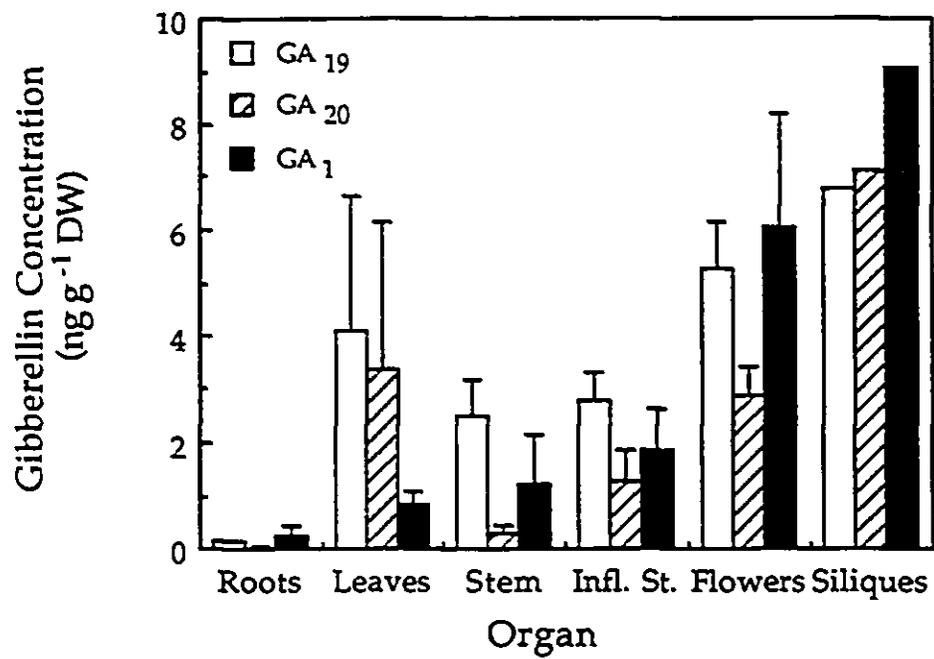


Figure 3-2. Gibberellin concentrations (ng g^{-1} dry weight) in different organs of 64 day old *Brassica napus* (canola cv. Westar) plants during early silique-filling. 'Infl. St.' indicates the inflorescence stalk that includes peduncles plus pedicels. Values represent the means of five replicates + s.e., except for siliques.

qualitative and quantitative differences in GAs in pea pods and seeds at different developmental stages.

To differentiate between the endogenous GA contribution of the seeds and the silique coats, seeds were dissected from immature (stage 1) and mature (stage 5) siliques. Concentrations of GA₁₉, GA₂₀, GA₁ and GA₈ and were consistently low in the silique coats regardless of harvest timing (Fig. 3-4). In contrast, the immature seeds had GA concentrations which were significantly higher (ANOVA, $df = 3/9$, $F = 4.594$, $p = 0.0325$) than those in the mature seeds. This is consistent with numerous reports which indicate that the levels of GAs present in immature seeds of various plants are generally high (Takahashi *et al.*, 1986, Sponsel, 1987). Further, García-Martínez *et al.* (1991) have noted that GA concentrations are substantially higher in ovules than in pods of pea.

The immature seeds contained higher concentrations of all measured GAs relative to the mature seeds. GA₁₉, GA₂₀, GA₁ and GA₈ concentrations were, respectively, 18.2-, 9.9-, 13.6-, and 2.6-fold higher in immature than mature seeds. The mature seeds contained more GA₂₀ than the other GAs, an observation that corresponds to recent findings with the *Brassica napus* cultivar Parkland (HC Imeson, KP Zanewich and SB Rood, unpublished).

Since GA₁₉, GA₂₀ and GA₁ concentrations were high in immature siliques, the occurrence of other endogenous GAs in that plant part was subsequently investigated. GC-SIM analyses were focused to probe for specific GAs that are native to *Brassica* (Hedden *et al.*, 1989; Rood *et al.*, 1987a) or to other crucifers, *Arabidopsis* (Talon *et al.*, 1990a) and *Thlaspi* (Metzger and Mardaus, 1986), as well as other GAs that are metabolically associated. Thirteen GAs were detected (Table 3-2) including ten that had been previously identified from stems and apices (Rood *et al.*, 1987a) or whole shoots (Hedden *et al.*,

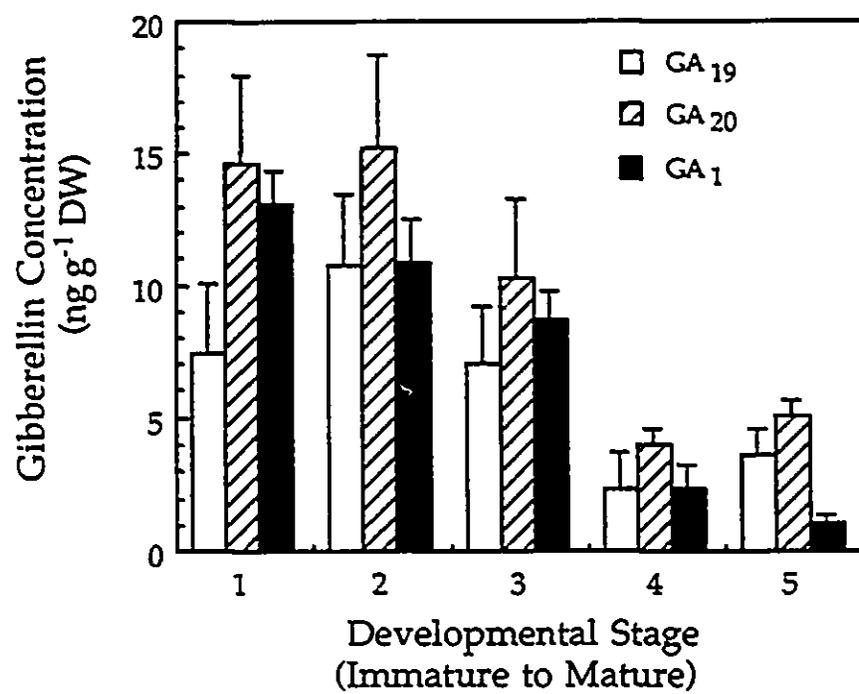


Figure 3-3. Gibberellin concentrations (ng g^{-1} dry weight) of *Brassica napus* (canola cv. Westar) siliques at different developmental stages. Values represent means of four replicates + s.e. for tissue harvested from plants grown in field tests at Lethbridge in 1989 and 1990.

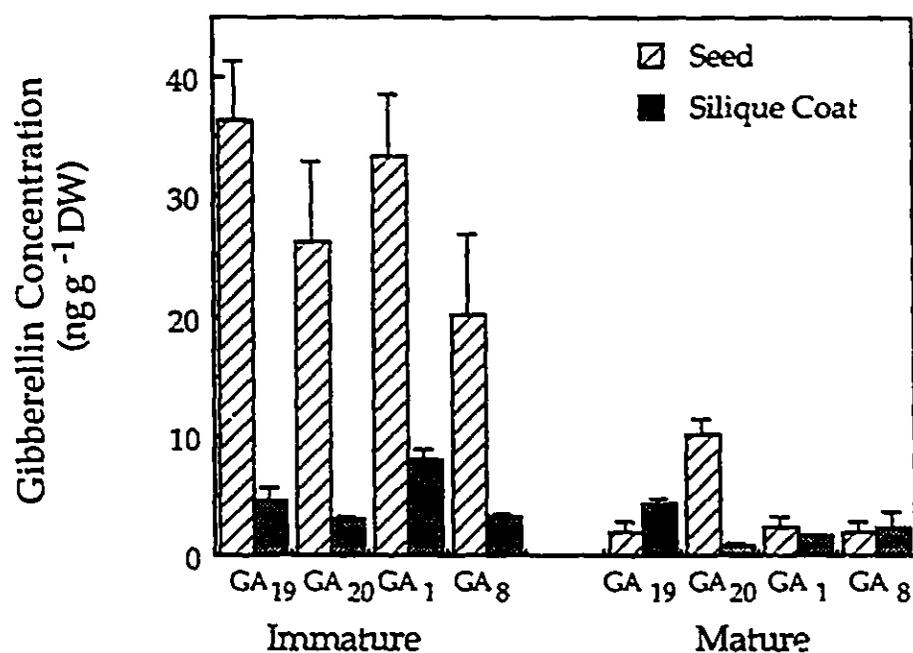


Figure 3-4. Gibberellin concentration (ng g^{-1} dry weight) of immature (stage 1) and mature (stage 5) *Brassica napus* (canola cv. Westar) seeds and silique coats. Values represent the means of two to five replicates + s.e. from plants grown in field tests at Lethbridge in 1990.

Table 3-2. MeTMSi gibberellins (GAs) identified by capillary gas chromatography-selected ion monitoring (GC-SIM) from *Brassica napus* cv. Westar immature siliques.

GA	HPLC fraction	KRI ^a	Ion <i>m/z</i> and (relative abundance)				
GA ₁	21-24	2709	506(100)	491(8)	448(18)	416(8)	390(6)
GA ₃	21-24	2739	504(100)	489(6)	445(9)	431(7)	347(8)
GA ₄	35-37	2564	418(9)	390(6)	386(19)	289 (66)	284(100)
GA ₈	10-14	2831	594(100)	579(7)	565(3)	553(5)	535(12)
GA ₉ ^b	38-40	2390	298(100)	270(61)	243(36)	227(30)	226(36)
GA ₁₇	31-34	2625	492(73)	460(34)	432(33)	401(21)	373(21)
GA ₁₉	31-34	2650	462(9)	447(7)	434(100)	431(15)	402(43)
GA ₂₀	28-30	2534	418(100)	403(14)	375(47)	359(20)	301(25)
GA ₂₄ ^b	38-40	2513	314(78)	286(60)	285(71)	226(100)	225(93)
GA ₂₉	15-18	2711	506(100)	491(14)	477(3)	447(3)	389(11)
GA ₃₄	31-34	2699	506(100)	459(7)	431(6)	416(6)	288(19)
GA ₅₁	35-37	2568	403(4)	386(28)	343(13)	328(42)	284(100)
GA ₅₃	38-40	2538	448(47)	419(5)	389(17)	251(41)	235(31)

^a Kovats Retention Index. Samples were analyzed on a DB-5 capillary column.

^b MeGA

1989). The three additional GAs, GA₄, GA₉ and GA₅₃ are members of GA biosynthetic pathways previously proposed for *Brassica* (Hedden *et al.*, 1989; Rood *et al.*, 1987a). GAs which were investigated but not detected included GA_{5,7,12,13,25,27,36,44,70,77,85} and 2 β -OH GA₅₃.

Based on the ease of detection by GC-SIM, GA₄, GA₉, GA₂₄, and GA₅₁ were apparently most abundant, GA₁, GA₃, GA₂₀, and GA₃₄ were intermediate, and GA₈, GA₁₇, GA₁₉, GA₂₉ and GA₅₃ were least abundant in the immature siliques. It must be noted that these estimates are very imprecise since the different fragmentation patterns of different GAs result in different relationships between ion abundance and GA abundance.

Of particular interest is the presence of GAs characteristic of both the early 13-OH and non-13-OH GA biosynthetic pathways. Based on the ease of detection and hence apparent abundance of GA₄, GA₉, GA₂₄, GA₃₄ and GA₅₁, it would appear that the non-13-OH GA biosynthetic pathway could be the dominant pathway in the immature siliques.

In apparent contrast to vegetative shoots (Hedden *et al.*, 1989), GA₄, and its precursor, GA₉, are abundant in immature siliques. GA₄ has been previously shown to be abundant in rice anthers (Kobayashi *et al.*, 1988) and maize tassels (Murofushi *et al.*, 1991) prompting the proposal that it may be involved in reproductive development. Further, Takahashi (1990) has presented evidence for the organ specificity of 13-OH versus non-13-OH GAs in rice. Further studies of the abundance and role of 13-OH versus non-13-OH GAs in vegetative and reproductive organs and tissues of *Brassica* are warranted.

The observation that GAs are concentrated differentially in organs of both vegetative and reproductive *Brassica* plants is consistent with results from pea

(Smith *et al.*, 1992), *Silene* (Talon and Zeevaart, 1990b), rice (Kobayashi *et al.*, 1988), and *Avena* (Kaufman *et al.*, 1986). In these other plants, concentrations of GAs were also found to be high in shoot tips providing a consistent pattern of high GA concentration in these centers of growth and developmental activity. Further, the studies provide evidence of acropetally increasing concentration of GAs, with highest levels in shoot tips or reproductive organs. The observation of lowest concentrations of GAs in *Brassica* roots suggests the pattern of increasing concentration upwards along the plant axis.

The observed moderately high concentrations of GAs in stems is relevant to the observation that this organ is highly GA-responsive. Stem elongation is a normal consequence of GA application. Elongation may also occur through the action of the single gene mutation seen in the *Brassica* mutant, *elongated internode*, which has accelerated GA₁ biosynthesis and in some conditions elevated GA₁ levels in the stems (Rood *et al.*, 1990a). Conversely, depressed stem elongation is a primary characteristic of the GA-deficient mutant *rosette* (Rood *et al.*, 1989b). Similarly, the application of plant growth retardants that block GA biosynthesis particularly inhibit stem elongation in *Brassica* (Hedden *et al.*, 1989; Rood *et al.*, 1989a). Thus, it is probably physiologically relevant that endogenous GA concentration is reasonably high in the GA-responsive organ, the stem.

In summary, the present study demonstrates greater than ten-fold differences in GA concentration amongst different *Brassica* organs. Increased concentrations of endogenous GAs upwards along the plant axis were observed in both seedlings and reproductive plants, with highest concentrations in the reproductive plants observed in immature seeds. Further, complete siliques were qualitatively as well as quantitatively rich in endogenous GAs. These observations emphasize the need to consider differential distribution of

endogenous GAs in plants for an accurate understanding of GA physiology.

CHAPTER 4

THE EFFECTS OF VERNALIZATION AND PLANT GROWTH
REGULATORS ON SHOOT ELONGATION AND FLOWERING IN WINTER
CANOLA

Abstract

Winter canola (oilseed rape) has a qualitative requirement for cold-treatment (vernalization) for the induction of stem elongation and flowering. The length of vernalization at 4°C required to induce bolting and flowering in *Brassica napus* cv. Crystal was 8 to 10 weeks. Gibberellic acid (GA₃) was applied to the shoot tip of 2-, 4-, 6-week partially-vernalized canola plants to determine whether GA₃-treatment could substitute for part of the vernalization requirement. Stem elongation was slightly promoted by partial vernalization but flowering was not observed in any of the partially-vernalized plants. Partially-vernalized plants were more responsive to exogenous GA₃ indicating that partial vernalization increases the sensitivity of the plants to GA.

To compare the efficacy of different GAs in promoting stem elongation and flowering, GA₁, GA₃, GA₄ or GA₅ at concentrations ranging from 10⁻³ to 10⁻¹ M were applied to the shoot tips of 28 day-old nonvernalized plants. All of the different GA concentrations promoted stem elongation and the GAs were ranked by declining ability to promote stem elongation as: GA₃ > GA₁ > GA₄ > GA₅. None of the GA treatments induced flower development.

Two plant growth retardants (PGRs), paclobutrazol and CGA163'935, were applied as a root drench or foliar spray, respectively, to 10 week (fully)

vernalized plants in an attempt to prevent stem elongation and/or flowering. Both PGRs inhibited stem elongation, but flowering still occurred although it was delayed. Collectively, these results suggest that GAs are involved in the regulation of stem elongation in winter canola, but the role in flowering is less direct. Further, the studies demonstrated that elongation and flowering can be separated in winter canola indicating partially independent regulation of these two processes.

Introduction

Gibberellins (GAs) have been implicated in the regulation of two major physiological processes in various plants: stem elongation (Graebe, 1987; Phinney, 1985) and flowering (Pharis and King, 1985; Zeevaart, 1983). Some dwarf plants, such as the single gene *Brassica* mutant *rosette* (*ros*), are partially deficient in endogenous GAs and respond to exogenous GAs by promoted elongation (bolting) which is concentration-dependent (Rood *et al.*, 1989b; Zanewich *et al.*, 1991). Flowering may be delayed or absent in this GA-deficient mutant and is subsequently induced or promoted by exogenous GA₃ (Rood *et al.*, 1989b). The application of plant growth retardants (PGRs) that block GA biosynthesis can prevent both stem elongation and flowering in *Brassica* (Rood *et al.*, 1987b; Rood *et al.*, 1989a), producing plants that phenotypically resemble *ros*.

Without vernalization, winter canola is somewhat similar to *ros* in that it remains as a vegetative rosette phenotype. Winter canola has a qualitative vernalization (chilling) requirement after which the plant switches from the vegetative to the reproductive phase. The thermoinductive treatment induces elongation and flowering in winter canola.

Although flower initiation is ultimately controlled genetically, (Lang, 1965; Metzger, 1990a), numerous studies have focussed on the environmental stimuli which are capable of inducing reproductive development (Lang, 1965; Pharis and King, 1985; Zeevaart, 1983). Many plants are photoperiodic; they are developmentally responsive to inductive or noninductive day lengths. Some annual *Brassica* species grown under noninductive short days (SD) can be induced to bolt and flower after the exogenous application of GAs (Lang, 1965). Similarly, some cold-requiring biennial *Brassica* varieties grown

under warm long days were induced to elongate and flower following exogenous gibberellic acid (GA₃) application (Chailakhyan, 1957; Lang, 1957). Furthermore, Mandel *et al.* (1992) were able to induce flowering in several cold-requiring cultivars of canola with exogenous GA and photoperiod manipulation.

The growth requirements of winter canola make this plant an attractive tool for studying the putative role of GAs in vernalization-associated events such as bolting and flowering. Accordingly, studies were conducted to investigate the effect of vernalization period, exogenous GA application and PGR application on stem elongation and flowering in a *Brassica napus* winter canola cultivar.

Materials and Methods

Growth conditions for *Brassica napus* cv. Crystal seedlings during the first four weeks after planting were as described in Chapter 2. Plant heights and growth stages (Harper and Berkenkamp, 1975) were recorded for all experiments.

Vernalization Length

Following four weeks of growth in greenhouse conditions, plants were partially- and fully-vernalized (refer to Chapter 2) for 1, 2, 3, 4, 5, 6, 7, or 8 weeks. Plants were grown from October, 1991 to May, 1992 and plant height, growth stage and dates of anthesis were recorded.

GA Applications

In a preliminary experiment that was started in June, 1992 and completed in September, 1992, 5 μ l of either a 1.0, 1.0×10^{-1} , 1.0×10^{-2} , or 1.0×10^{-3} M GA₃ in 50% ethanol (EtOH) was applied by syringe to the shoot tips of 4 week-old nonvernalized *B. napus* cv. Crystal plants. In a separate experiment that took place from November, 1992 until February, 1993, 5 μ l of 1.0×10^{-1} , 1.0×10^{-2} , 1.0×10^{-3} M GA₁, GA₃, GA₄, and GA₅ solutions in 50% EtOH were applied by syringe to the shoot tips of nonvernalized *B. napus* cv. Crystal plants four weeks after planting. Additionally, 4 week-old plants were partially vernalized for either 2, 4, or 6 weeks or vernalized for 8 and 10 weeks and then treated with 5 μ l of 1.0×10^{-3} M GA₃ in 50% EtOH. These plants were seeded during September, 1992 and harvested during February, 1993.

Application of Plant Growth Retardants

Following 10 weeks of vernalization, either 25 ml of 10^{-5} M paclobutrazol (PP333) or approximately 10 ml of CGA 163'935 (proposed compound name: cimectacarb) ranging in concentrations from 10^{-4} to 10^{-1} M was applied by root drench or foliar spray (Kerber *et al.*, 1989) respectively. Plants treated with PP333 were grown from January, 1992 through June, 1992 and CGA 163'935 treated plants were grown from September, 1992 until March, 1993. Dates of anthesis, plant height and other altered phenotypic characteristics were recorded.

Results and Discussion

Length of Vernalization and Bolting and Flowering Response

Although some plants have a facultative cold-temperature requirement whereby temperature hastens developmental events, winter canola has an absolute cold-requirement (vernalization) which is necessary for the transition from a vegetative to reproductive plant (Fig. 4-1). However, specific vernalization requirements vary between species (Chouard, 1960; Lang, 1965; Wiebe, 1990) and even cultivars (Myers *et al.*, 1982).

In winter canola, short periods of vernalization, such as 1 to 3 weeks were insufficient for the induction of stem elongation or flowering (Fig. 4-2a, b). Intermediate vernalization periods such as 4 to 6 weeks had slight effects on elongation and incomplete flowering resulted (Fig. 4-2a and b). The greatest elongation response and highest percentage of flowering was observed in those plants vernalized for longer periods of chilling, 7 and 8 weeks (Fig. 4-2 a and b). Regression analysis indicated that plant height was significantly correlated with the length of vernalization (regression coefficient (r) = 0.486; $p < 0.01$ [Steel and Torrie, 1960]). The r^2 value of 0.236 indicated that approximately 24% of the observed variation in plant height could be attributed to the length of vernalization. At some 'maximal' vernalization period, it is likely that additional vernalization would have no further effects on growth and development.

Bolting and flowering responses were correlatively related to the length of chilling exposure. The length of time required for flowering to occur following vernalization was directly related to the length of vernalization; the longer the period of vernalization, the shorter the time required for

anthesis (Fig 4-2c). The time required for the vernalized plants to reach anthesis was also highly correlated with the length of vernalization ($r = 0.659$; $p < 0.01$). Thus, approximately 43% of the observed variation in flowering could be attributed to the length of vernalization. However, this value may be under-represented, since it only accounts for plants which had successfully flowered.

Other *Brassica napus*, *B. campestris* and *B. oleracea* cultivars (Heide, 1970; Hodgson, 1978) and crucifers including *Thlaspi* (Metzger, 1985) have shown similar responses to varying lengths of vernalization. In addition to the length of vernalization, other factors such as the age of the plant at vernalization (Netzer *et al.*, 1986) and the vernalization temperature can also affect both bolting and flowering responses (Heide, 1970; Metzger, 1985; Tommey and Evans, 1991).

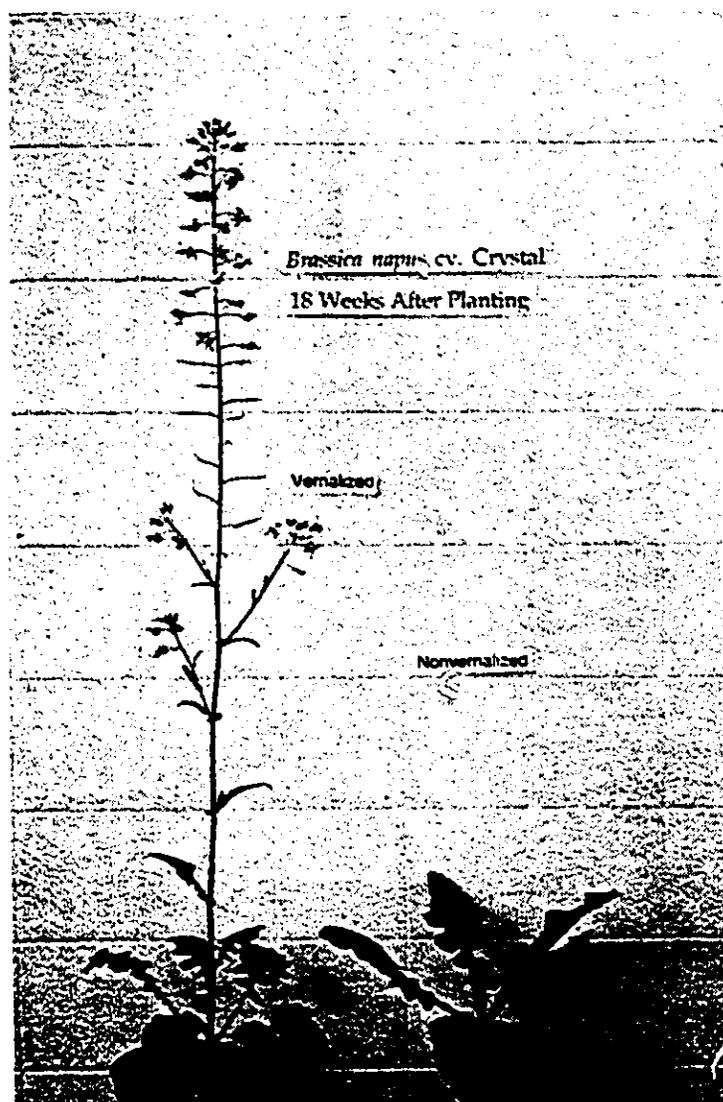


Figure 4-1. Nonvernalized (right) and 8-week vernalized (left) *Brassica napus* cv. Crystal plants, 126 days after planting (42 days post-vernalization).

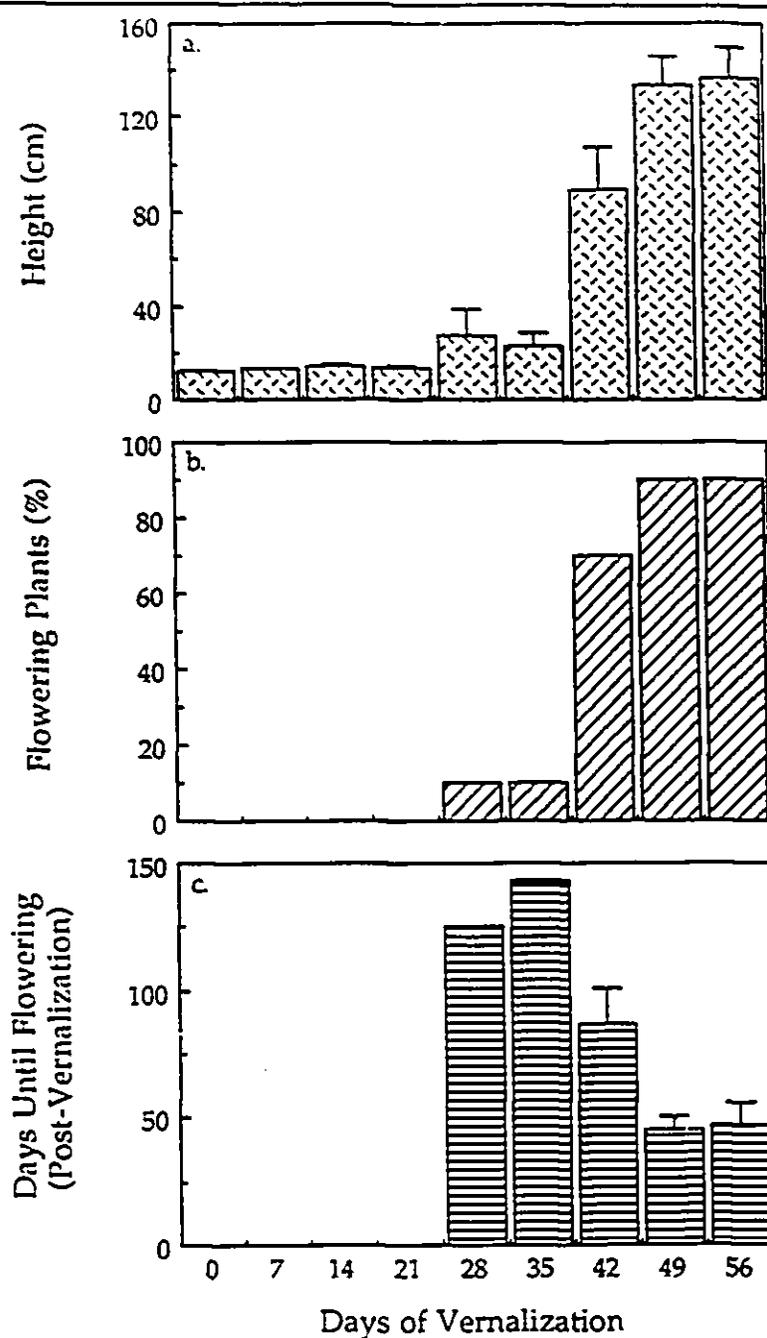


Figure 4-2. Plant elongation (a), percentage of flowering plants (b) and days post-vernalization to reach anthesis (c) in *Brassica napus* cv. Crystal plants exposed to lengths of vernalization at 4°C ranging from 0 to 56 days. Values represent the means + s.e. of ten plants.

Effect of GA Application to Nonvernalized Winter Canola

Application of GA₃ in amounts ranging from 1.73 µg to 1.73 mg to the shoot tips of nonvernalized winter canola resulted in promotion of stem elongation only (Fig. 4-3a); flowering was not induced. The elongation response was linearly related to the log of the dose applied ($r^2 = 0.99$; $p < 0.01$) (Fig. 4-3b). The influence of exogenous GA₃ on stem elongation is well documented for numerous plants (Phinney, 1985; Wittwer and Bukovac, 1958; Zeevaart, 1983). In some cold-requiring plants, GA₃ application has been successfully used as a substitute for cold treatment (Lang and Reinhard, 1961; Wittwer and Bukovac, 1957) while in other qualitative cold-requiring plants, GA application failed to result in earlier flowering (Chakravarti, 1958; Zeevaart, 1968).

GA₃ was applied to partially-vernalized winter canola plants to determine whether some of the vernalization requirement could be substituted by GA-treatment. No significant differences in height were observed between 2-, 4-, and 6-week partially vernalized plants and nonvernalized plants; all remained relatively short (Fig. 4-4). Partially vernalized plants treated with GA₃ were capable of significant (e.g. 6-week partially vernalized plants, 152 DAP, ANOVA, $df = 3/25$, $F = 7.73$, $p < 0.001$) stem elongation but did not flower (Fig. 4-4). Further, a quicker and larger elongation response following GA treatment was seen in the partially vernalized plants relative to the nonvernalized plants suggesting that these plants had a greater sensitivity to exogenous GA.

Metzger (1985) also indicated a similar enhanced elongation response to GA₃ in *Thlaspi* plants vernalized at different temperatures, prompting the suggestion that thermoinduced stem growth may occur in two separate

processes: initiation and regulation of growth. In another system involving the GA-induced initiation and control of hydrolytic enzyme activity, sensitivity to exogenous GA₃ was increased by pretreating deembryonated seeds with low temperatures prior to GA₃ treatment (Singh and Paleg, 1984).

Thus, thermoinduction might serve as a stimulus that increases the sensitivity of certain tissue to endogenous and exogenous GA concentration (Trewavas, 1982). Sensitivity might involve the presence or absence of specific receptor proteins which in turn regulate a response (Trewavas and Jones, 1981, cited in Trewavas, 1982). More likely though, a combination of interacting factors such as sensitivity (which may be perceived as a molecular response(s)) and metabolic mediation of endogenous GA status may be collectively influenced by environmental stimuli such as low temperature.

Since flowering was not observed in either non- or partially-vernalized GA₃-treated canola plants, GA₃ was unable to substitute for the floral induction component of vernalization. Flowering in the 10-week vernalized plants occurred at similar times in both the GA- and non-treated plants; there was no reduction in the time required for anthesis in the GA₃-treated plants (data not shown). Conversely, Suge (1986) was able to demonstrate a reduction in the time required for anthesis in both vernalized and nonvernalized radish plants treated with GA₃. In kohlrabi (*Brassica oleracea*), GA₃ treatment following an incomplete cold treatment also resulted in marked stem elongation but no acceleration of flowering compared to the controls (Van Marrewijk, 1976). In a biennial cold-requiring plant such as rutabaga (*Brassica napus*) or a facultative plant such as winter wheat, GA₃ pretreatment prior to the onset of partial vernalization leads to flower induction (Ali and Machado, 1982; Weibel, 1960). Other facultative cold-requiring plants treated with GA₃ prior to complete vernalization had

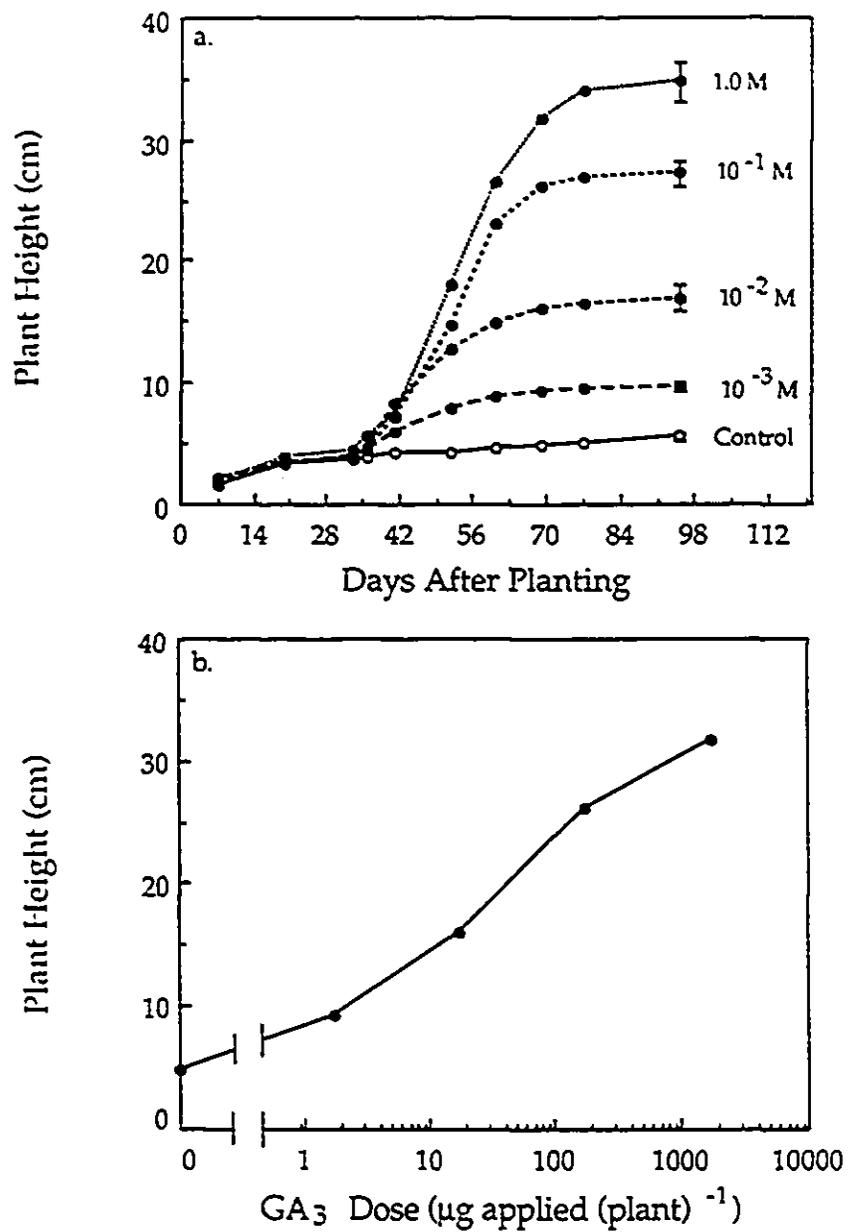


Figure 4-3. (a) Height of nonvernalized *Brassica napus* cv. Crystal plants treated with several concentrations of GA₃, 28 days after planting. Values represent the means \pm s.e. of ten plants. (b) Dose-response curve of Crystal nonvernalized plants treated with GA₃.

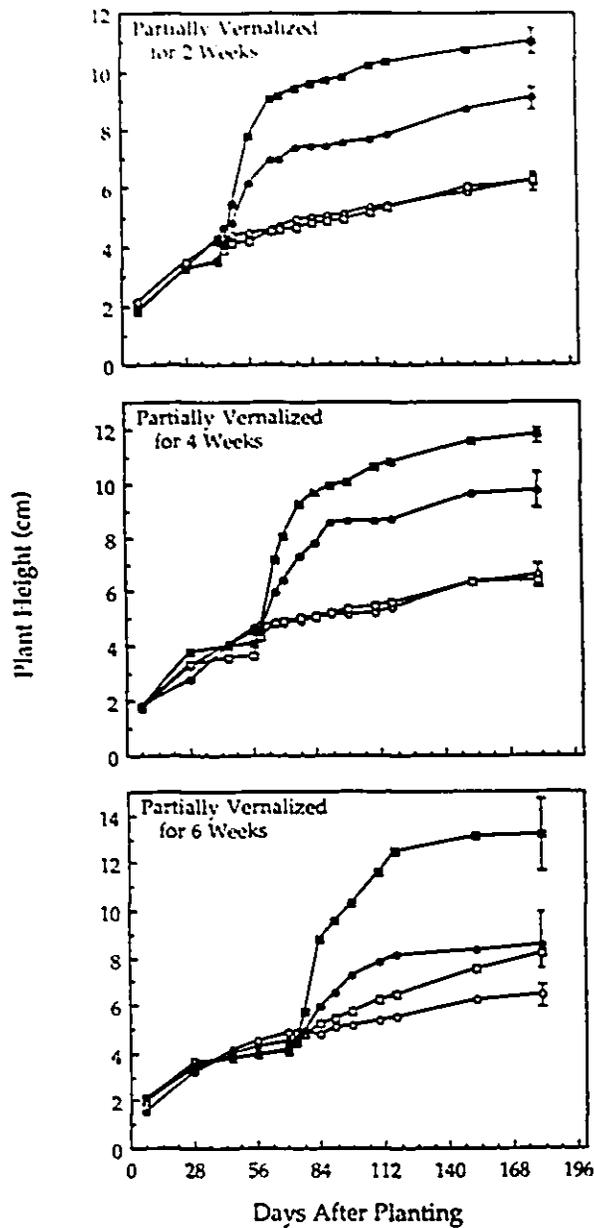


Figure 4-4. Response of 2-, 4-, and 6-week partially-vernallized and nonvernalized *Brassica napus* cv. Crystal plants to exogenous GA₃. No plants from any of the treatments flowered. Legend: O = Nonvernalized; □ = Partially-vernallized; ● = Nonvernalized + GA₃; ■ = Partially-vernallized + GA₃. Values represent the means \pm s.e. of ten plants.

accelerated flowering (Leshem and Steiner, 1968). Thus, the timing of GA₃ application may be crucial for flower induction.

The florigenic (flower inducing) activity of other GAs applied to plants grown under noninductive photoperiodic or temperature conditions has been observed (Evans *et al.*, 1990; Mandel *et al.*, 1992; Michniewicz and Lang, 1962; Ogawa *et al.*, 1990). Although GA₃ is very effective at inducing stem elongation, perhaps it is less florigenic in winter canola. The response of nonvernalized winter canola plants to other GAs including, GA₁, GA₃, GA₄ and GA₅ at various concentrations is shown in Figure 4-5 and 4-6. While all of the applied GAs promoted stem elongation, the efficacy varied. The GAs from highest to lowest efficacy were as follows: GA₃ > GA₁ ≥ GA₄ > GA₅ (Figs. 4-5 and 4-6). This relationship probably reflects the relative biological activities of each of the GAs (Hoad, 1983). GAs characterized by a free 7-carboxyl, the 19,10-γ-lactone function and a 3β-hydroxyl are considered biologically potent GAs (Mander, 1992). Since GA₅ lacks a hydroxyl on the β-position of C(3), this may account for the reduced stem elongation (see Appendix 1). Similar differences in the biological responses to exogenous GAs were also reported for the crucifer *Thlaspi* (Metzger, 1990b).

None of the nonvernalized *Brassica* plants treated with GA flowered under a 16 h photoperiod. Mandel *et al.* (1992) observed that nonvernalized winter canola grown under a 11 h SD photoperiod was not induced to flower, regardless of the type of GA applied. Conversely, nonvernalized plants grown under a 16 h photoperiod treated with GA₅ or 2,2 di-Me GA₄ were induced to flower. Suge and Rappaport (1968) also demonstrated that GA₃ application to nonvernalized radish plants grown under SDs was ineffective at inducing elongation or flowering, however, plants grown under 16 h photoperiods had enhanced bolting and flowering responses. Collectively,

these and other studies suggest that photoperiod and vernalization interactions may be important for flower induction.

Analogous to the differences in elongation responses induced by different GAs, differences in florigenic capabilities have also been noted in winter annuals. Mandel *et al.* (1992) observed greatest flowering promotion using the synthetic GA analogue, 2,2 di-Me GA₄, although GA₅ was also effective at inducing flowering. In two other cold-requiring plants, *Myosotis* and *Centaureium*, GA₇ and GA₁, and GA₃, GA₁, GA₄, GA₅ and GA₉, respectively, resulted in flowering (Michniewicz and Lang, 1962). Enhanced flower development was also observed in winter strains of *Arabidopsis* following application of GAs (Napp-Zinn, 1963, cited by Napp-Zinn, 1969). It has been suggested that in addition to differences in uptake, transport, and metabolism, GA structure may also affect the florigenic capacity of different GAs (Evans *et al.*, 1990).

The structural requirements for stem elongation may not be the same as those for flower induction. For example, GAs with florigenic capabilities in *Lolium* are characterized by the presence of a double bond in the A-ring, a free carboxyl group, and hydroxylation at C(12), C(13) and C(15), whereas those GAs promoting elongation generally possessed a free carboxyl group and hydroxylation at C(3) (Evans *et al.*, 1990).

Environmental conditions such as photoperiod may directly or indirectly influence the flowering responses of some cold-requiring plants (Mandel *et al.*, 1992; Suge, 1984; Suge and Takahashi, 1982). Photoperiod appears to be an essential component in the vernalization triggered cascade of events associated with flowering (Napp-Zinn, 1984). Additionally, the quantity of light also influences GA concentrations which may in turn affect flowering

responses (TI Potter and SB Rood, unpublished). Thus, some inherent uncontrolled parameters in the present greenhouse experiments may account for some of the deviations in flowering responses from those observed in the growth chamber experiments of Mandel *et al.* (1992).

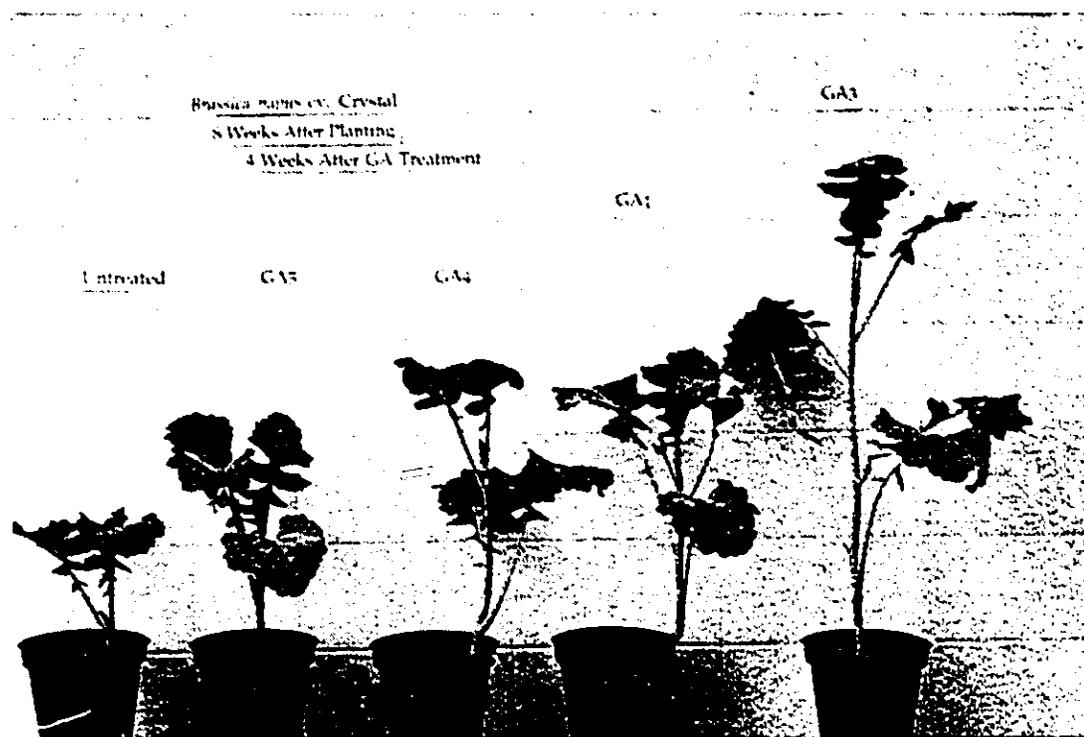


Figure 4-5. Nonvernalized *Brassica napus* cv. Crystal plants, 30 days following GA application (from left to right): untreated, 10^{-1} M GA5, GA4, GA1, and GA3.

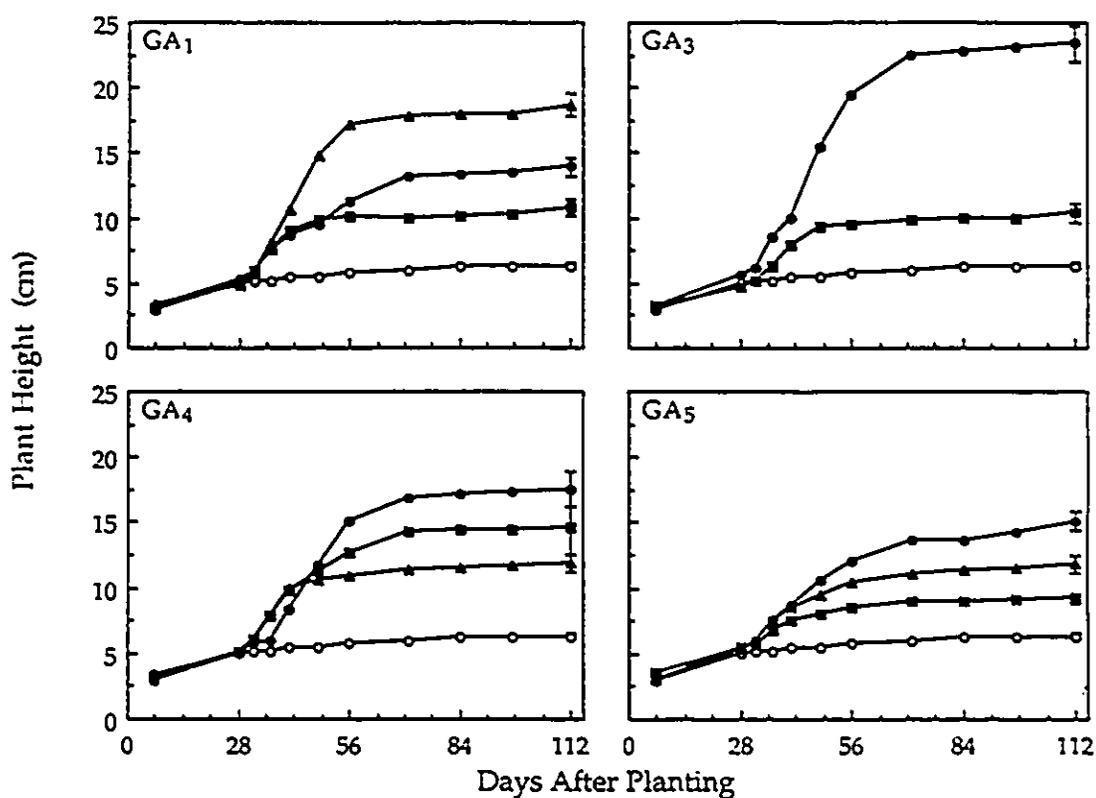


Figure 4-6. Effect of 0 (O), 10⁻³ (■), 10⁻² (▲), and 10⁻¹ (●) M GA₁, GA₃, GA₄, and GA₅ on plant height in nonvernalized *Brassica napus* cv. Crystal plants, 70 days after GA application. None of the plants flowered. Values represent the means \pm s.e. of ten plants.

Effect of Plant Growth Retardants on Growth and Development in Vernalized Winter Canola

Plant growth retardants (PGRs) are synthetic plant growth regulators (PGRs) which are primarily used to prevent lodging and increase harvestable yield of field crops (Amrein *et al.*, 1989; Jung and Rademacher, 1983). Recently, triazole type PGRs have also been used to increase cold hardiness in winter cultivars by decreasing plant size (Morrison and Andrews, 1992). PGRs also serve as useful research tools for studying GA physiology, allowing the GA biosynthetic pathway to be blocked at certain points of interconversion (Kamiya *et al.*, 1992)

The two PGRs used in this study, paclobutrazol (PP333) and CGA 163'935, were effective in inhibiting stem elongation in vernalized winter canola (Figs. 4-7 and 4-9). These PGRs probably inhibit stem elongation by preventing the biosynthesis of gibberellins. PP333 is a triazole that apparently targets early GA biosynthesis by blocking the enzyme kaurene oxidase that catalyzes the oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Rademacher *et al.*, 1984). Hedden *et al.* (1989) have shown that endogenous GA levels in *Brassica* plants were reduced following triazole application. Acylcyclohexadiones are a relatively new group of PGRs and include CGA 163'935 or cimectacarb. CGA 163'935 acts later in the GA pathway by interacting with the 2-oxoglutarate binding site of dioxygenases, cofactors of some biosynthetic GA enzymes including 3 β -hydroxylase which catalyzes the formation of GA₁ from GA₂₀ (Rademacher *et al.*, 1992). Barley plants treated with CGA 163'935 had reduced levels of endogenous GA₁, while levels of the immediate biosynthetic precursor, GA₂₀, were elevated (Adams *et al.*, 1991). Similarly in rice, endogenous GA₁ levels decreased following application of prohexadione, ar. acylcyclohexadione, while levels of precursors such as GA₁₉

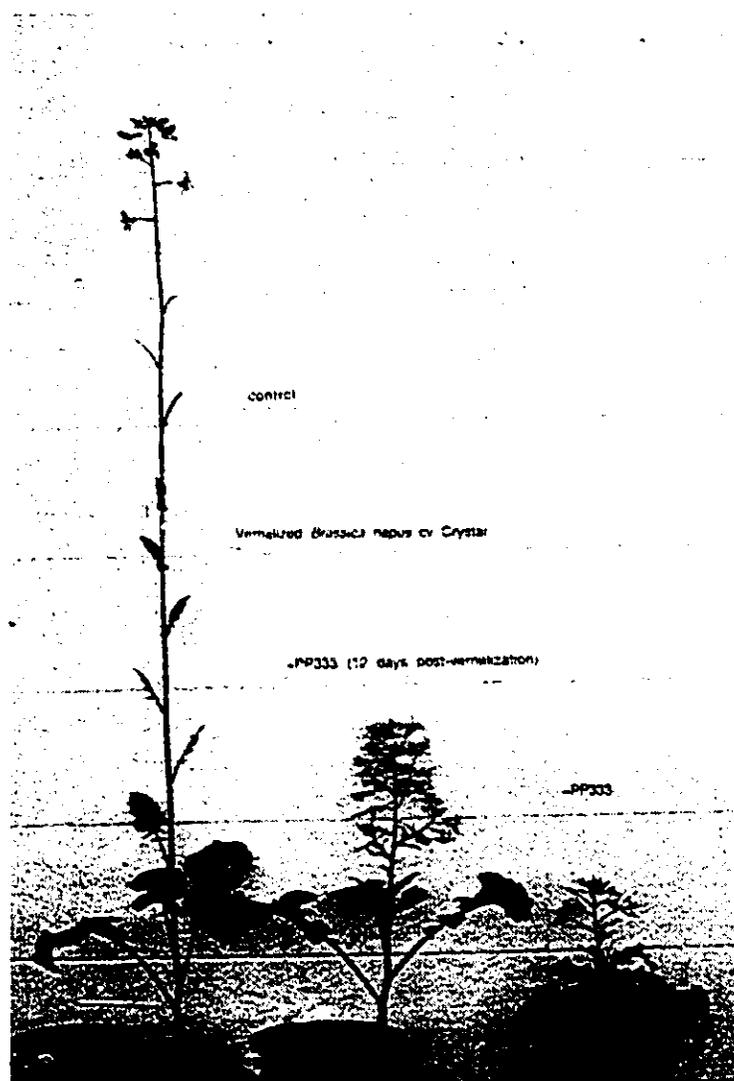


Figure 4-7. Ten week vernalized *Brassica napus* cv. Crystal untreated plants (left), PP333 treated 12 DPV (center) and PP333 treated immediately following vernalization (right), 28 days following PP333 root drench.

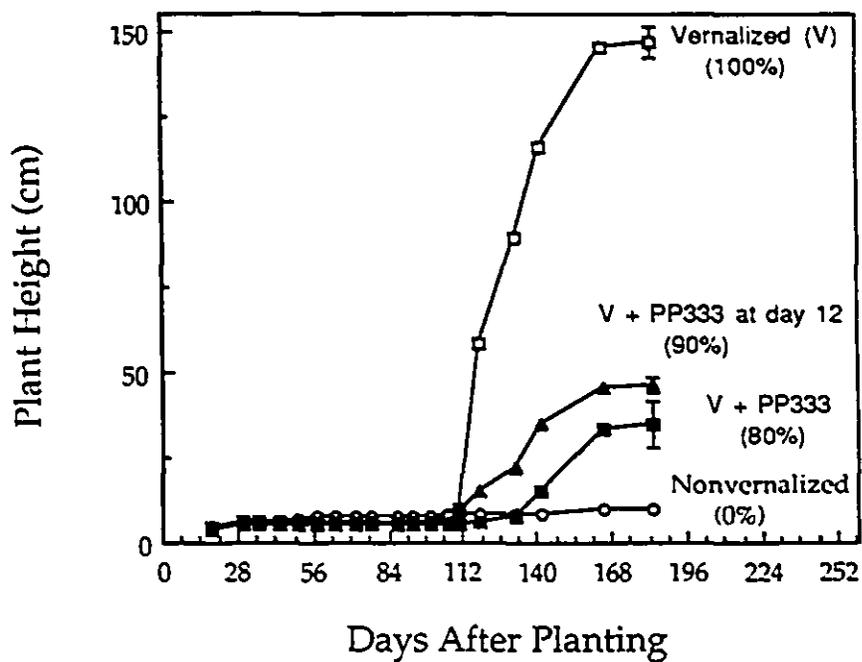


Figure 4-8. Effect of 10^{-5} M paclobutrazol on plant height of 70 day vernalized *Brassica napus* cv. Crystal, 182 days after planting. The percentage of flowering planting 84 days post vernalization is shown on the right in parenthesis. The values represent the means \pm s.e. of ten plants.

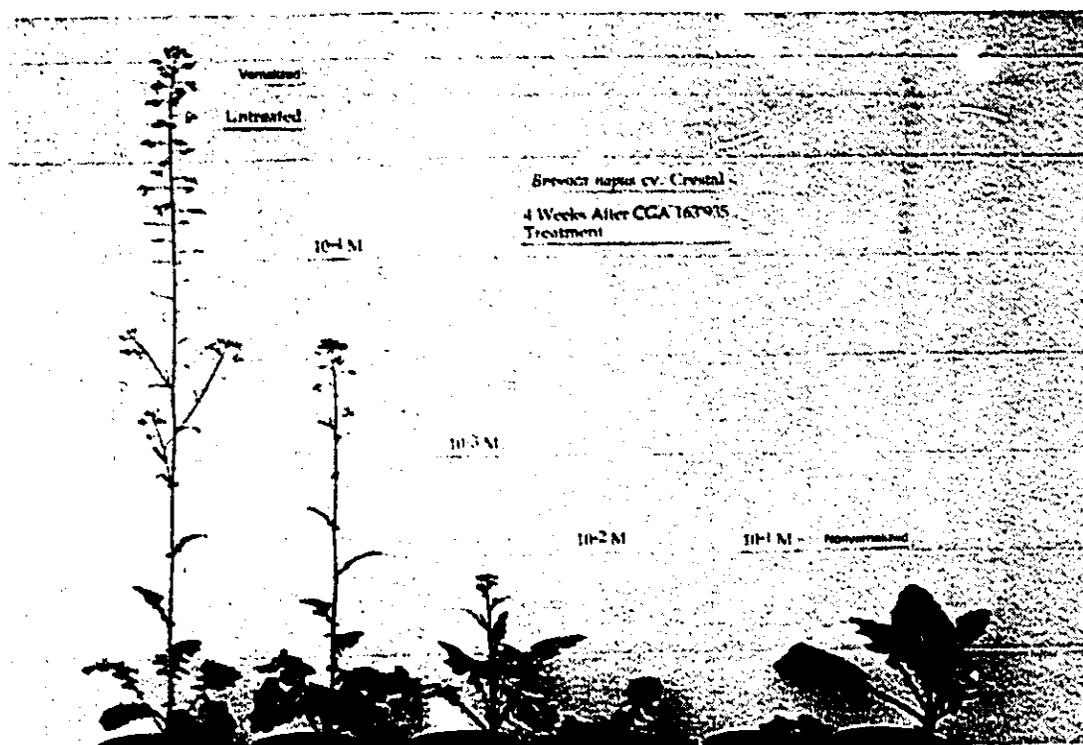


Figure 4-9. Seventy day vernalized *Brassica napus* cv. Crystal plants (from left to right) treated with 0, 10⁻⁴, 10⁻³, 10⁻², 10⁻¹ M CGA 163'935, 28 days following foliar application. The winter canola plant on the extreme right is nonvernalized and untreated.

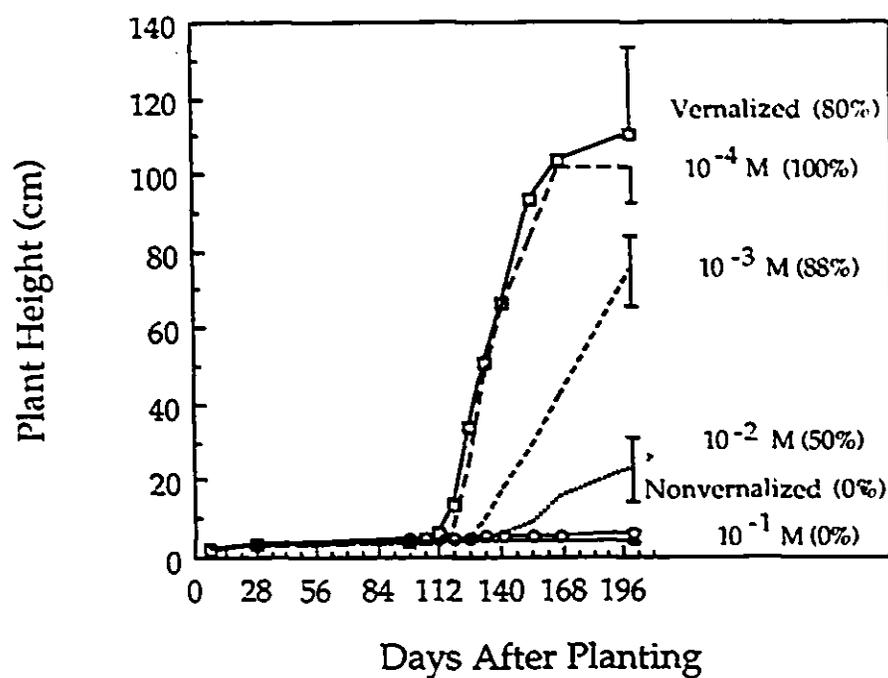


Figure 4-10. Effect of 0, 10⁻⁴, 10⁻³, 10⁻², 10⁻¹ M CGA 163'935 on plant height of 70 day vernalized *Brassica napus* cv. Crystal, 168 days after planting. Values represent the means \pm s.e. of nine plants. The percentage of flowering planting 70 days post vernalization is shown on the right in parenthesis.

and GA₂₀ increased or were unaffected (Nakayama *et al.*, 1992).

Stem elongation was inhibited in vernalized winter canola by the two PGRs and the degree of inhibition was dependent on PGR concentration and timing of application (Figs. 4-8 and 4-10). Application of PP333 immediately following vernalization resulted in the greatest retardation of plant growth (Fig. 4-7 and 4-8). Although the plants treated 12-days post-vernalization (DPV) had not begun to elongate prior to PP333 application, the observed elongation following PGR application was probably a result of residual GA biosynthesis that had occurred prior to PP333 application. In general PP333 was more potent than CGA 163'935 and able to effectively inhibit stem elongation at lower PGR concentration.

Elongation responses were also dependent on PGR concentration. At high concentrations of CGA 163'935, stem elongation was completely prevented and vernalized plants remained in a rosette-like plant form, while lower concentrations reduced elongation (Fig. 4-9 and 4-10). These results support the hypothesis that GAs are involved in the control of shoot elongation in winter canola.

Although flowering still occurred in the PGR-treated plants, it was delayed (Figs 4-7 through 4-10). Vernalized winter canola plants required 19 days until anthesis following vernalization while PP333 treated vernalized plants required 28 days to reach anthesis. All of the paclobutrazol treated plants had formed flower buds, but only 80-90% had flowered 12 weeks post-vernalization. At least 85% of vernalized plants treated with 10^{-4} , 10^{-3} , and 10^{-2} M CGA 163'935 also produced floral buds, however anthesis in these plants was delayed and related to the concentration of CGA 163'935. Untreated vernalized winter canola plants required 29 DPV to reach anthesis,

while plants treated with 10^{-4} , 10^{-3} , and 10^{-2} M CGA 163'935 required 39-, 66-, and 73-DPV to reach anthesis, respectively. None of the 10^{-1} M CGA 163'935 treated or nonvernalized plants initiated flower buds and consequently did not flower.

GAs have also been implicated in leaf morphogenesis in some plants, especially the development of foliar form (Robbins, 1957; Rogler and Hackett, 1975; Zanewich *et al.*, 1990). This was supported by observations with both the PP333 and CGA 163'935 treatments that resulted in the lack of leaf expansion and production of different leaf shapes. At a concentration of 10^{-1} M CGA163'935, exposed leaf tissue appeared necrotic 2 days post application. Subsequent leaves were brittle, crumpled and unexpanded forming a small, tight whorl around the shoot tip (Fig. 4-11). Other treatments using lower concentrations showed reduced leaf expansion but to a lesser degree.

Collectively, the results of application of PGRs to winter canola support previously published studies which indicate a regulatory role for GAs in the control of stem elongation in numerous *Brassica* species (Lang, 1957; Metzger, 1985; Suge 1982, 1984; Wittwer and Bukovac, 1957). Stem elongation was promoted in nonvernalized and partially vernalized winter canola plants by the application of several GAs including GA₁, GA₃, GA₄, and GA₅. Furthermore, partial vernalization amplified the effect of GA application, suggesting that vernalization may involve a change to phytohormone sensitivity, or perception and/or reception.

While exogenous GAs induced some stem elongation in nonvernalized winter canola, PGRs which acted as GA biosynthetic blocks reduced stem elongation in vernalized winter canola. These treatments separated bolting from flowering in winter canola. It is likely that exogenous GA would be able

to restore the elongation response in PGR treated vernalized plants as has been demonstrated in PGR-treated cowpeas (Martínez-García and García-Martínez, 1992) and *Brassica* (D.W. Pearce, personal communication). Given that stem elongation was inhibited by both early and late GA biosynthetic blocking agents, the 13-OH GA pathway is apparently important for elongation. As has been demonstrated in maize (Phinney, 1985) and proposed by Rood *et al.* (1987a) for *Brassica*, GA₁ appears to be an important bioeffector for stem elongation in winter canola.

Collectively, the present results suggest that effects of GA on flowering in winter canola are apparently indirect and complex; specific GAs and GA concentration probably influence flower differentiation and development in vernalized winter canola in combination with other limiting factors. Growth retardant treated plants with reduced endogenous GA levels were still capable of initiating flowers, although anthesis was delayed. This is similar to the growth and developmental status of the *Brassica* dwarf mutant, *rosette*, which has genetically reduced endogenous GA levels and has delayed flowering (Rood *et al.*, 1989b; Zanewich *et al.*, 1990). GA application to *rosette* or to non-induced cold-requiring cold plants is able to either accelerate or induce flowering (Ali and Machado, 1982; Lang, 1957; Rood *et al.*, 1989b; Van Marrewijk, 1976). The specific GA requirements of winter canola for flowering are probably complex and remain undetermined. Plants are probably receptive to GA treatments at specific or critical time periods during growth and development. After or prior to this receptive period, exogenous GAs may only elicit elongation responses. Furthermore, the PGRs used in this study are neither specific nor complete blocks, and as such could result in the production of minute quantities of GA which may be capable of inducing flowering.

These results suggest an indirect role for GA in the vernalization process, perhaps as Zeevaart (1983) proposed, a role associated with floral expression rather than floral induction. The vernalization process apparently also enables the plant to become more sensitive (or competent) to exogenous or endogenous GA. Though studies focusing on the quantification of endogenous GAs in the plant post-vernalization may provide further insights into the GA physiology of winter canola during bolting and flowering, other interactions between environmental factors such as photoperiod may also influence GA status within the plant. Furthermore, in addition to environmental conditions such as vernalization, numerous molecular mechanisms may be interacting to collectively regulate flowering (Martinez-Zapater and Somerville, 1990).

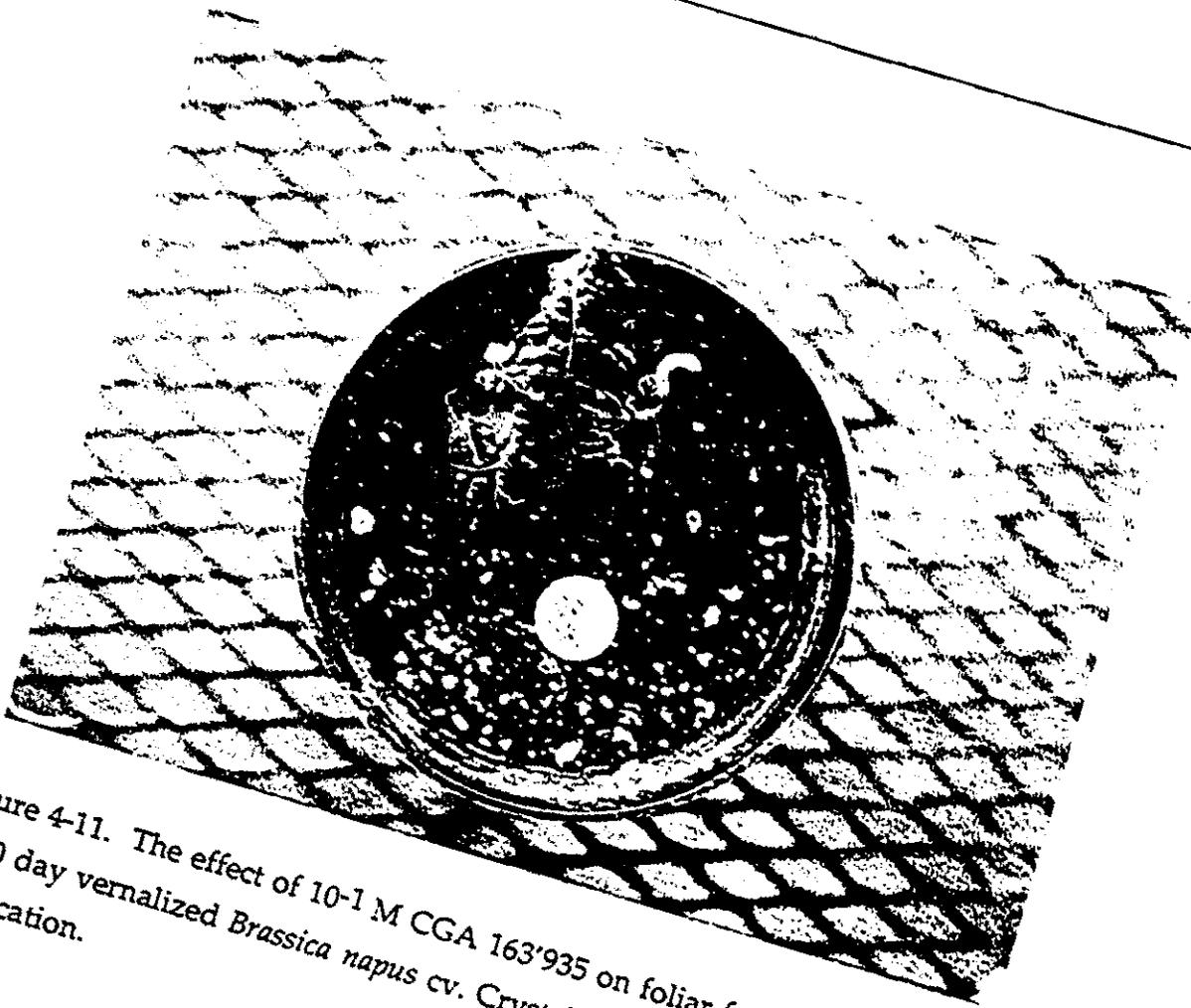


Figure 4-11. The effect of 10⁻¹ M CGA 163'935 on foliar form and plant height of 70 day vernalized *Brassica napus* cv. Crystal plants, 28 days following foliar application.

CHAPTER 5**INFLUENCE OF VERNALIZATION ON ENDOGENOUS GIBBERELLIN
CONCENTRATIONS IN WINTER CANOLA****Abstract**

Winter canola is an oilseed crop requiring chilling treatment (vernalization) for the induction of stem elongation and flowering. Shoot tips and upper stem tissue were harvested from vernalized and nonvernalized *Brassica napus* cv. Crystal plants at 0-, 8-, and 18-days after vernalization treatment at 4°C. Gibberellins (GAs) were extracted, purified using step-wise silicic acid partition chromatography and C₁₈ reversed-phase HPLC and quantified by gas chromatography-mass spectrometry, selected ion monitoring (GC-MS, SIM) using [²H₂]GA_{1,3,4,5,8,9,19}, and 20 as quantitative internal standards. All deuterated standards were recovered, but only 13-OH endogenous GAs were sufficiently abundant for confident quantification. Concentrations of these endogenous GAs were consistently higher in the vernalized plants at all harvest times following vernalization, with the greatest increase observed 8 days after vernalization. At this harvest, concentrations of GA_{1,3,8,19,20} were 15.8, 9.6, 8.1, 8.3, and 22-fold higher, respectively, in vernalized stem tissue. The elevated concentration of all GAs suggest promotion of GA biosynthesis prior to GA₁₉ formation. The greatest increases in GA concentrations, including GA₁ and GA₃, coincided with the onset of stem elongation and floral differentiation, suggesting that these GAs are involved in the regulation of these responses.

Introduction

Like other winter annuals, winter canola, a type of oilseed rape, requires vernalization (chilling) to induce the transition from the vegetative to a reproductive phase. Typically following vernalization, these plants undergo rapid stem elongation and then flowering. Since gibberellins (GAs) have been implicated in the regulation of both stem elongation and flowering (Pharis and King, 1985; Phinney, 1985; Zeevaart, 1983), it seems likely to expect that GAs might be involved in events associated with the vernalization response (Chailakhyan and Lozhnikova, 1962).

The investigation of the role of GAs in cold requiring plants may be examined using several approaches. Numerous studies have attempted to induce stem elongation and/or flowering by exogenous GA treatment of non- or partially vernalized plants (Lang, 1957; Lang and Reinhard, 1961). Unfortunately, results have been mixed. Some cold-requiring biennial and winter annual *Brassica* varieties have been induced to elongate and flower following exogenous GA₃ treatment and long-day (LD) photoperiod while those exposed to a short-day (SD) photoperiod and GA₃ treatment elongated but were incapable of flowering (Chailakhyan, 1957; Lang, 1965; Mandel *et al.*, 1992; Suge and Takahashi, 1982; Zeevaart, 1983). Conversely, other *Brassica* types failed to flower without at least partial vernalization.

While exogenous GA studies investigate the physiological consequence of increased GA levels, the complementary condition of reduced GA level can also be studied. GA-deficient dwarf mutants represent a group of plants that are unable to fully elongate and often have delayed or absent flowering because of reduced concentrations of endogenous GAs (Reid, 1990a). The phenotype of GA deficient mutants partially resembles nonvernalized winter annuals. This suggests that the failure of non-vernalized plants to bolt and flower could be due to an environmentally linked GA deficiency.

This hypothesis logically leads to a second approach in the study of gibberellin physiology and vernalization - the quantification of GA concentrations. Comparisons of endogenous GA status in vernalized and nonvernalized plants should determine correlations between the vernalization response and GA concentrations.

Although preliminary studies have attempted to examine the endogenous GA content of vernalized and nonvernalized plants using either bioassays or gas chromatography-mass spectrometry (GC-MS), results have been variable (Joseph *et al.*, 1983; Lin and Stafford, 1987; Michniewicz *et al.*, 1981; Suge, 1970;). Consequently, the present study investigated GA physiology and vernalization by determining the endogenous GA contents of vernalized and nonvernalized winter canola plants by GC-SIM using [²H₂]GA internal standards that enabled confident quantification and statistical analysis. This enabled the investigation of the prior hypothesis that (i) nonvernalized plants are relatively GA-deficient, and that (ii) vernalization enables bolting and flowering through the enrichment of GA content.

Methods and Materials

Plant Material and Growth

Brassica napus cv. Crystal seeds were planted June, 1992 and grown and vernalized as described in Chapter 2. Samples of plant tissue ranging in dry weight from 0.3 - 5.5 g (5 - 7 shoot tips and upper stem segments of approximately 1.5 cm in length) were collected during October, 1992, 0-, 8-, and 18-days post vernalization (DPV), flash frozen in liquid nitrogen and lyophilized for 72 h. Only vernalized plants at 18-DPV had macroscopically visible flower primordia and had begun to elongate.

Endogenous GA Content

Four replicates were independently extracted, purified and analyzed for endogenous GA content as described in Chapter 2. Following overnight extraction, 20 ng of [17-²H₂]GA₁, [17-²H₂]GA₃, [17-²H₂]GA₄, [17-²H₂]GA₈, [17-²H₂]GA₉, [17-²H₂]GA₁₉, [17-²H₂]GA₂₀ were added as internal standards which permitted quantitative analyses by gas chromatography-mass spectrometry (GC-MS) with selected ion monitoring (SIM) (Fig. 5-1). Additionally, 20 ng of [17-²H₂]GA₅ was added to a single replicate. GA quantities were calculated by comparing the molecular ions of the endogenous GAs to the internal standards and correcting for mutual ion contributions (see Chapter 2). Quantities were reported as concentrations (ng (g DW)⁻¹).

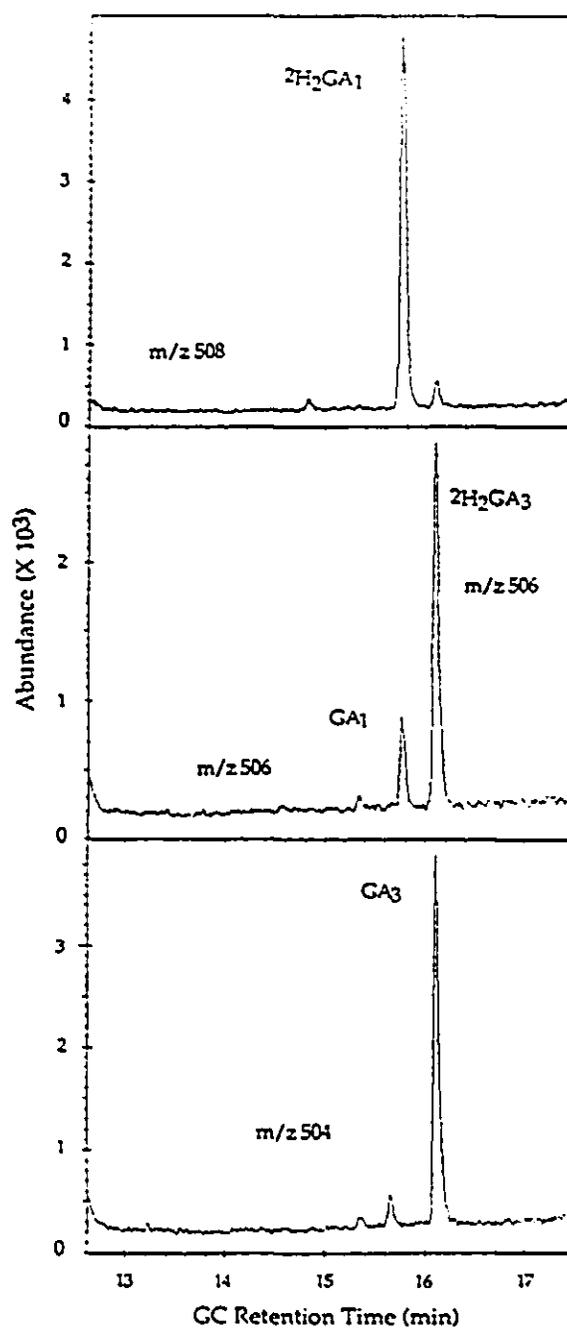


Figure 5-1. Gas chromatography-mass spectrometry selected ion monitoring (GC-MS SIM) profiles of the molecular ions from methyl ester trimethylsilyl ether derivatives of endogenous GA₁ and GA₃ and internal standards of [2H₂] GA₁ and [2H₂] GA₃.

Results and Discussion

Following vernalization, the vernalized and nonvernalized winter canola plants displayed distinctive phenotypes. Significant differences (e.g. height at 116 DAP, ANOVA, $df = 1/38$, $F = 15.5$, $p < 0.003$) in height were apparent after day 28 until the conclusion of the study (Fig. 5-2). Nonvernalized plants remained as short vegetative rosettes while all vernalized plants had started to elongate and form flower primordia by 18 DPV.

The observed phenotypic differences between vernalized and nonvernalized winter canola plants suggest that GA, known to be involved in the regulation of stem elongation and reproductive development (Pharis and King, 1985; Phinney, 1985; Zeevaart, 1983), could be associated with vernalization. Quantification of endogenous GAs further substantiate the proposed relationship between endogenous GA concentration and vernalization.

During the initial extraction, 13-OH and 13-non-OH [$^2\text{H}_2$]GAs were added as internal standards. While all [$^2\text{H}_2$]GA standards were recovered during GC-MS analysis, only endogenous GAs from the early 13-OH pathway were detected from the purified HPLC fractions. Traces of GA₄ may have been present, but peaks were minimal preventing confident quantification. The ranking of endogenous GA concentrations in the canola plants in decreasing order was GA₃ > GA₁₉ > GA₁ = GA₈ > GA₂₀. Relative abundances of these 13-OH GAs did not differ between vernalized and nonvernalized plants.

Differences in most of the measured GA concentrations were statistically significant (e.g. GA₁ at 8 DPV, ANOVA, $df = 1/2$, $F = 37.36$, $p = 0.025$) at all harvests (Fig. 5-3). Concentrations of GA₁ and GA₃ were significantly (e.g. GA₃ at 8 DPV, ANOVA, $df = 1/2$, $F = 38.36$, $p = 0.025$) higher in vernalized stem tissue after the plants had recovered from vernalization and started to elongate and initiate floral primordia (8 and 18-DPV) with increases being 3.1-

/2.3- (for GA₁/ GA₃), 15.8-/9.6-, and 5.2/4.6-fold higher at 0-, 8- and 18-DPV, respectively. Similarly, GA₈ concentrations were also statistically higher (e.g. at 8 DPV, ANOVA, df = 1/6, F = 114.16, p < 0.005) in vernalized plants over the entire sampling period. Although precursors of the biologically potent GA₁, GA₁₉ and GA₂₀, were both significantly increased (e.g. GA₁₉ at 8 DPV, ANOVA, df = 1/6, F = 24.55, p = 0.003) in vernalized canola, concentrations tended to decline over time. This may suggest that these GAs were being converted to GA₁, whose concentrations gradually increased over time. However metabolic feeds are required to confirm this speculative proposal. Additionally, the possible relationship between GA turnover rates is currently unknown (Metzger, 1985). The observation that all of the 13-OH GAs studied were elevated following vernalization suggests that a thermoinduced biosynthetic step probably occurs prior to GA₁₉. Future studies should include analyses of other GAs including GA₄₄, GA₅₃ and GA₁₂, as well as the kaurenoids to consider possible changes in these precursors. That study might reveal the biosynthetic interconversion(s) that is altered following vernalization.

In the annual canola cultivar Westar, concentrations of endogenous GA-like substances are relatively low during the vegetative phases of growth but increase following floral initiation and again during silique filling (Rood *et al.*, 1989a). However, since stem elongation and floral initiation occur at the same time, it is difficult to determine whether increases in endogenous GAs are associated with stem elongation and/or flower induction. In some thermoinduced plants, increases in endogenous GA-like substances have been observed but were proposed to be associated with stem elongation rather than flower induction (Margara, 1963; Zeevaart, 1983)

Increased concentrations of GA-like substances have been observed in other cold-requiring plants following vernalization. Results gathered from bioassays of numerous plants including *Brassica napus* (Margara, 1963), cabbage - *Brassica campestris* (Suge and Takahashi, 1982), chicory (Joseph *et*

al., 1983), chrysanthemum (Harada, 1962, cited in Zeevaart, 1983), radish (Michniewicz *et al.*, 1981; Suge, 1970; Suge and Rappaport, 1968), *Thlaspi* (Metzger, 1985), and winter wheat (Chailakhyan and Loznikova, 1962; Reda *et al.*, 1978) have indicated the presence of unique GA-like fractions or increases in the activity of certain fractions with GA-like activity from vernalized tissue.

Numerous environmental factors including photoperiod and temperature are capable of influencing stem elongation and flowering in plants (Lang, 1965; Zeevaart, 1983). Rapid stem elongation has been observed in some rosette long-day plant (LDPs) which have been grown under SDs and then transferred to LD conditions (Zeevaart, 1983). Since the response of winter canola grown under several photoperiods has been variable (Mandel *et al.*, 1992), it is not likely that a single factor is responsible for flower induction. Rather, several factors probably collectively affect the molecular mechanisms of stem control and/or flowering.

Combinations of appropriate exogenous GAs and photoperiod are able to induce both stem elongation and flowering in nonvernalized winter canola and plant growth retardants are able to prevent or inhibit stem elongation in vernalized winter canola (Mandel *et al.*, 1992; KP Zanewich, DW Pearce and SB Rood, unpublished). These observations coupled with the present observation of increased GA concentrations in vernalized canola indicate that the induced stem growth in winter canola is at least partly regulated by elevated endogenous GA.

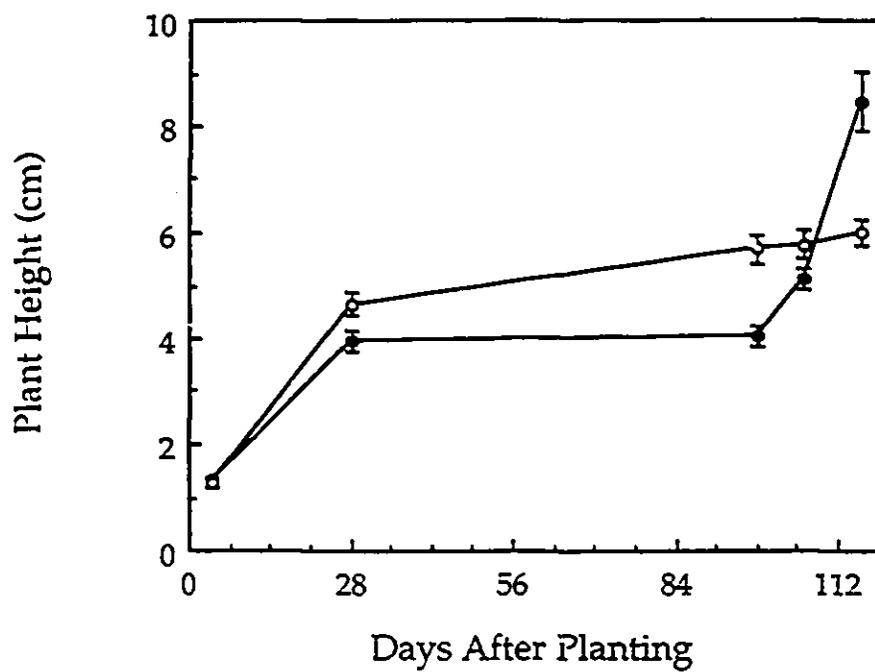


Figure 5-2. Height of *Brassica napus* cv. Crystal vernalized (●) and nonvernalized (○) plants. Plants were vernalized for 70 days from day 28 until day 98.

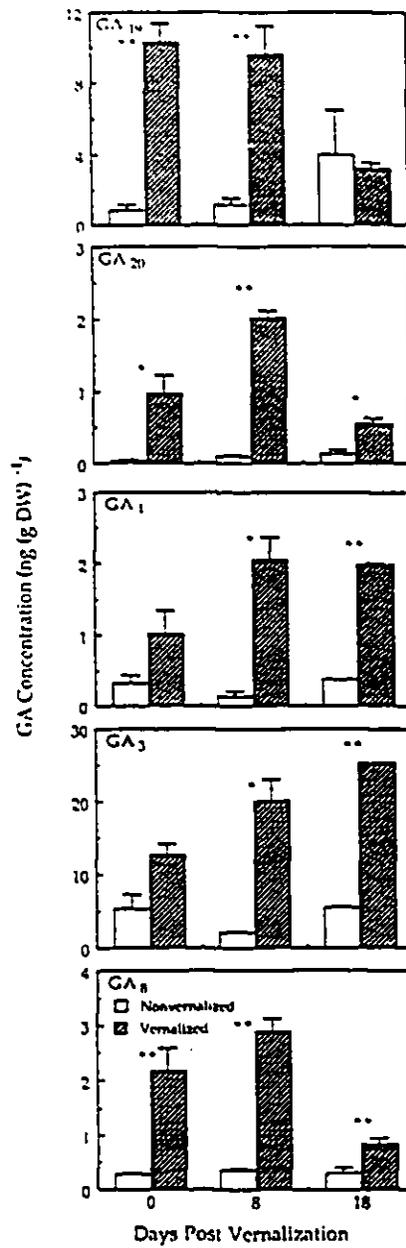


Figure 5-3. The concentrations of GA₁₉, GA₂₀, GA₁, GA₃, and GA₈ in shoot tips from vernalized and nonvernalized *Brassica napus* cv. Crystal plants at 0, 8 and 18 days post-vernialization. The *, ** indicates that values are significantly different between the vernalized and nonvernalized treatments (ANOVA; *-p < 0.05; **-p < 0.01). Note the different y-axis scales for the GAs.

CHAPTER 6

METABOLISM OF [³H]GA₁ AND [³H]GA₂₀ IN VERNALIZED AND
NONVERNALIZED WINTER CANOLA

Abstract

Winter canola is a winter annual that requires vernalization (chilling) for the induction of stem elongation and flowering. The metabolism of [³H]GA₂₀ or [³H]GA₁ applied to the shoot tips of *Brassica napus* cv. Crystal was compared 24 hours following application in nonvernalized and 10 week vernalized plants at 6-, 12- and 15- days post-vernalization (DPV). Extracts from the stem apices were purified by sequential step-wise elution silica gel partition chromatography followed by C₁₈ reversed-phase high pressure liquid chromatography. Vernalized plants contained significantly higher amounts of ³H recovered in the EtOAc-soluble fractions following [³H]GA₁ or [³H]GA₂₀ feeds. Conversely, nonvernalized plants had significantly higher amounts of ³H eluted with MeOH, a fraction that probably contained [³H]GA glucosyl conjugates. Of the recovered EtOAc-soluble radioactivity, approximately 46% and 56% were associated with the exogenous precursors, [³H]GA₁ and [³H]GA₂₀, respectively. Additional peaks of radioactivity corresponded to the Rts of [³H]GA₁ and [³H]GA₂₉ following the [³H]GA₂₀ feed and [³H]GA₈ following the [³H]GA₁ feed. Although there were no qualitative differences in [³H]GA₁ or [³H]GA₂₀ metabolism between vernalized and nonvernalized plants, vernalized plants generally had decreased rates of EtOAc-insoluble ³H metabolite formation. Furthermore, progressive increases in the formation of [³H]GA₁ from [³H]GA₂₀ were detected in vernalized plants at 6-, 12- and 15-DPV, an interval that coincided with the onset of stem elongation and initial floral development in

vernalized plants. This suggests that the latter stages of GA₁ biosynthesis and particularly the 3 β -hydroxylation of GA₂₀ to GA₁ and GA conjugate formation are correlated with stem elongation. However, since the metabolic differences were relatively small, other metabolic conversions prior to the biosynthesis of GA₂₀ are also likely to be responsible for the control of growth and developmental responses following vernalization in winter canola.

Introduction

Prior to GA₁₂-aldehyde formation, GA biosynthesis appears to be similar in most plants (Graebe, 1987; Sponsel, 1987). However, the conversion from GA₁₂-aldehyde to subsequent GAs varies across plants and even across tissues (Graebe, 1987; Sponsel, 1987). The endogenous gibberellins (GAs) characterized from *Brassica* indicate that both the early 13-OH and 13-non (or late)-OH pathways may be present (Hedden *et al.*, 1989; Rood *et al.*, 1987a). In elongating *Brassica* shoots, GA₁ and two of its precursors, GA₁₉ and GA₂₀, were abundant suggesting that the early 13-OH biosynthetic pathway is the dominant pathway during elongation (Rood *et al.*, 1987a). These results also support similar findings by Phinney (1985) and others that indicates that GA₁ is the principal bioeffector involved in the regulation of shoot elongation.

Metabolic feeds to the rapid-cycling single gene mutant of *Brassica*, *elongated internode (ein)*, have demonstrated the conversion of [²H₂]GA₁₉ to [²H₂]GA₂₀ and [²H₂]GA₁ (Rood *et al.*, 1990a). Furthermore, [³H]GA₂₀ feeds to *ein* result in the apparent formation of [³H]GA₁, [³H]GA₂₉ and [³H]GA₈ (Rood *et al.*, 1990a). These results support the occurrence of the early 13-OH GA pathway in *Brassica*.

Vernalization, or chilling, of winter canola is essential for the induction of stem elongation and flowering. Furthermore, endogenous GA concentrations have been found to be increased in vernalized canola shoot tips (Chapter 5). The purpose of the present investigation was to determine whether the observed increased endogenous GA concentrations in vernalized winter canola shoot tips were correlated with enhanced GA biosynthesis. Given the pivotal role of GA₁ in stem elongation, metabolic studies focussed

of the metabolism of [^3H]GA₁ and [^3H]GA₂₀, GA₂₀ being the immediate precursor of GA₁. Studies of GA biosynthesis may contribute to the understanding of GA physiology by further investigating the correlation between shoot elongation and endogenous GA physiology.

Methods and Materials

Plant Growth Conditions

Brassica napus cv. Crystal plants were grown and vernalized in the greenhouse and a refrigerated chamber, respectively, at the University of Lethbridge in two separate experiments from March, 1991 to June, 1991, and March, 1992 to June, 1992, with conditions already described in Chapter 2.

Application of Radioactive GAs

In one experiment, 2.1 KBq of either [1,2-³H]GA₁ (specific activity: about 1.2 TBq per millimole) or [2,3-³H]GA₂₀ (about 62 GBq per mmol) was applied by Hamilton syringe to the shoot tips of 10 week vernalized and nonvernalized *Brassica napus* cv. Crystal plants 5 days post-vernalization (DPV) (no observable stem elongation) or 11 DPV (some stem elongation and floral development). In a second experiment, 2.1 KBq of either [1,2-³H]GA₁ or [2,3-³H]GA₂₀ was applied to the shoot tips of plants 14 days post-vernalization (some stem elongation and flower buds present). Following 24 h of metabolism, plant shoot tips were harvested, representing 6-, 12- and 15- DPV, respectively. The first experiment that consisted of the 6- and 12-DPV plant tissue and had 5 replicates, while the second experiment (15-DPV) consisted of only 2 replicates.

Analysis of [³H]GA Metabolites

Plant tissue was ground and extracted in cold 80% aqueous methanol

(Chapter 2). Dried samples were loaded onto glass fiber discs that were placed on a 5 g SiO₂ partition column for sequential step-wise elution with the following solvents: 50 ml of 60:40 hexane:EtOAc, 50 ml of 5:95 hexane:EtOAc which eluted polar GAs such as [³H]GA₇, [³H]GA₈, [³H]GA₂₀, and [³H]GA₂₉ and 40 ml of MeOH which eluted ³H-metabolites that were insoluble in the above solvent steps (putative [³H]GA conjugates) (see Chapter 2). Fractions were combined into EtOAc- and MeOH-soluble groups and recovery of [³H]GAs was determined as per Chapter 2. Subsequently, samples were separated on HPLC and quantitative and qualitative [³H]GA metabolite assessments were performed (refer to Chapter 2).

Results and Discussion

Metabolite Distribution Following Silica Gel Separation

Following the [^3H]GA₁ and [^3H]GA₂₀ metabolic feeds to vernalized and nonvernalized winter canola plants, extracts from the apical shoot tips harvested at 6-, 12- and 15- DPV were separated by sequential step-wise silicic acid partitioning. Chromatographic separation of the [^3H]GA-precursor and acidic [^3H]GA metabolites (free [^3H]GAs) from the more polar EtOAc-insoluble ^3H -metabolites was achieved by manipulating the polarity of the elution solvent (Fig. 6-1). No compounds eluted in the relatively nonpolar 60% hexane solvent, free [^3H]GAs eluted in the 95% EtOAc, and EtOAc-insoluble ^3H -metabolites were eluted with MeOH. The increased polarity of the EtOAc-insoluble metabolites suggests the presence of [^3H]GA-conjugates and those probably represented glucosyl conjugates (Schneider, 1983).

The distributions of ^3H in extracts from both the [^3H]GA₁ and [^3H]GA₂₀ feeds are shown in Fig. 6-2 top and bottom, respectively. There were significant differences (3-factor ANOVA: vernalized vs nonvernalized effect, $df = 1/18$, $F = 5.02$, $p = 0.03$) in the amounts of recovered EtOAc-soluble [^3H]GAs between vernalized and nonvernalized winter canola shoots. Following 24 h of metabolism after [^3H]GA₂₀ application, nonvernalized plant extracts contained 69%, 66%, and 69% EtOAc-soluble ^3H at 6-, 12- and 15-DPV, respectively, while vernalized plant extracts had 75%, 71% and 75%.

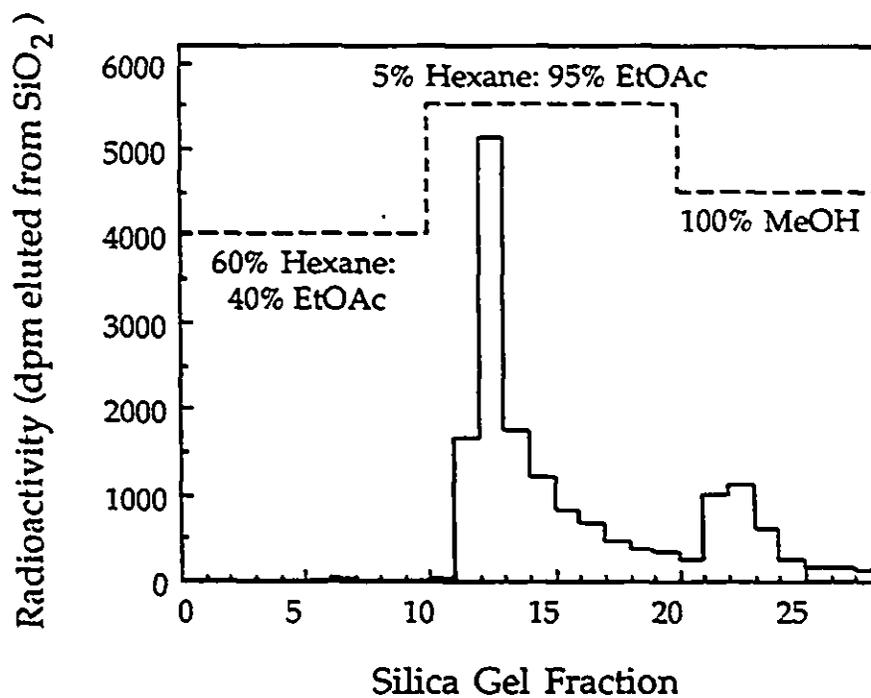


Figure 6-1. Elution of radioactivity from step-elution SiO₂ partition columns loaded with extracts from vernalized *Brassica napus* cv. Crystal plants fed [³H]GA₁.

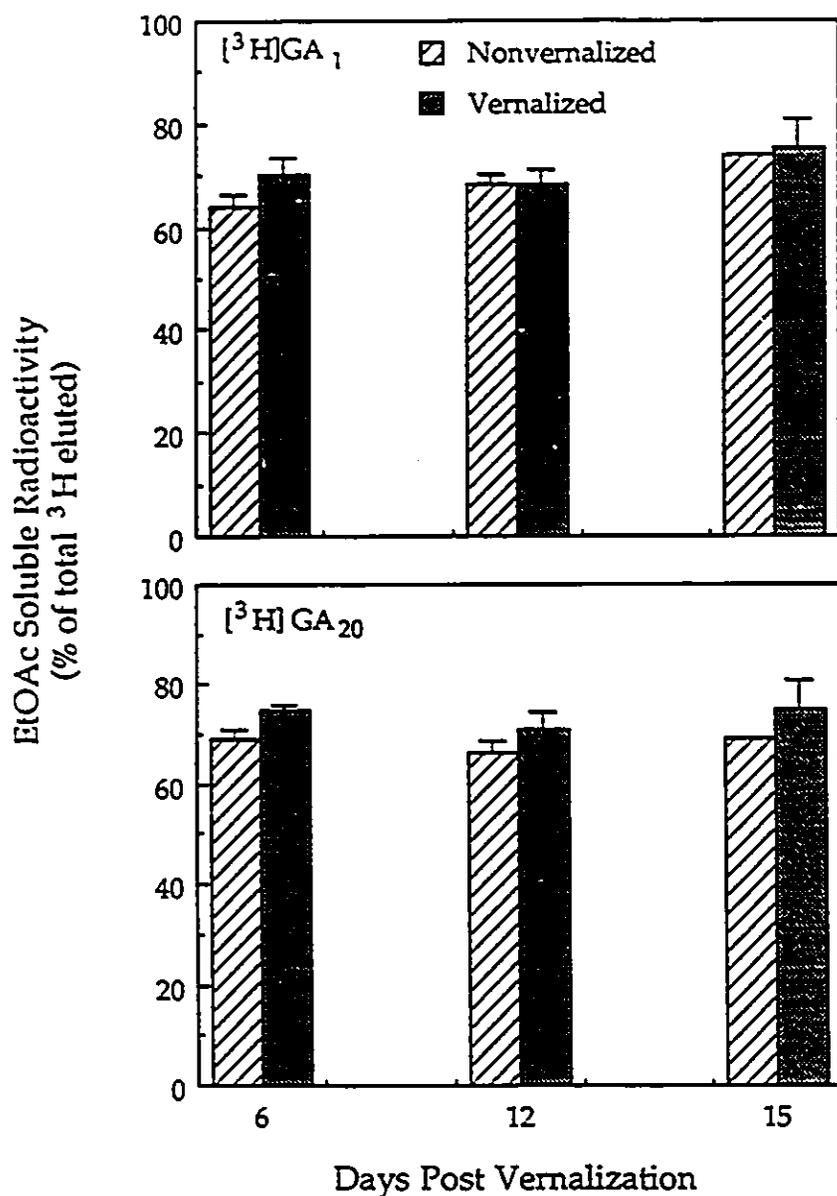


Figure 6-2. Percentage of total radioactivity eluted with EtOAc from step-elution SiO_2 partition column loaded with extracts from vernalized and nonvernalized *Brassica napus* cv. plants fed $[^3\text{H}]\text{GA}_1$ (top) and $[^3\text{H}]\text{GA}_{20}$ (bottom) at 6-, 12-, and 15-days post-vernalization. Values are means \pm s.e. of 5 (6-, 12-DPV) and 2 (15-DPV) replicates.

Appreciable amounts of EtOAc-insoluble radioactivity eluted in the MeOH from both vernalized and nonvernalized extracts. Since the relative amounts of EtOAc-soluble ^3H -metabolites were significantly different and a complementary relationship exists between the amounts of EtOAc-soluble and EtOAc-insoluble ^3H -metabolites, there was also a significant difference in the amounts of EtOAc-insoluble ^3H -metabolites formed in vernalized compared to nonvernalized winter canola (data not shown, but can be calculated as 100% - values in Fig. 6-2). Thus vernalized plants apparently had significantly reduced rates of ^3H -conjugate formation.

Metabolite Distribution Following [^3H]GA₂₀ or [^3H]GA₁ Feeds

A number of metabolites are commonly produced from GA₂₀, including GA₁, epi-GA₁, GA₃, GA₅, GA₈, and GA₂₉ and their glucosyl ester or ether counterparts (Graebe, 1987; MacMillan, 1990). Based on HPLC elution, the principal ^3H -metabolite formed after [^3H]GA₂₀ feeds to either vernalized or nonvernalized winter canola plants was [^3H]GA₁ (Fig. 6-3). In vernalized winter canola plants [^3H]GA₂₀ turnover was increased to produce significantly more [^3H]GA₁ (Table 6-1). At 6-DPV vernalized plants had increased rates of formation of [^3H]GA₁ and the apparent rate of 3 β -hydroxylation of [^3H]GA₂₀ to [^3H]GA₁ differed significantly between vernalized and nonvernalized plants at 12-DPV (ANOVA, $df = 1/18$, $F = 9.424$, $p = 0.015$). Vernalized plants also tended to have increased conversion rates at 15-DPV (Table 6-1). The lack of statistical significance at this latter sampling date was probably due to the limited sample size of only 2 plants.

The conversion rate from [^3H]GA₂₀ to [^3H]GA₁ was 6.7% to 39.4% higher in the vernalized compared to nonvernalized samples. Thus, the rate of 3 β -

hydroxylation was increased in vernalized in winter canola plants. This increase was determined following feeds of relatively high specific activity [^3H]GAs and hence, induced effects resulting from exogenous GA application should have been minimal. The positioning of ^3H -label in the A-ring of the gibberellin structure also leads to ^3H losses during metabolic conversions, although the underestimation of the actual amount of metabolites produced should be proportional between vernalized and nonvernalized plants. However, the difference between vernalized and nonvernalized plants would likely be even greater if GA pool sizes were considered, since vernalized plants have higher endogenous GA₂₀ concentrations. The total turnover from endogenous GA₂₀ to GA₁ would be the combined product of available GA₂₀ level and metabolic rate. Thus, the difference in 3β -hydroxylation rate in the vernalized compared to nonvernalized plants would probably be substantially greater than the 35% increase observed in this metabolic study.

Smaller peaks accounting for approximately 6% of the total recovered EtOAc-soluble [^3H]GAs in either the vernalized or nonvernalized plant tissue, corresponded to the Rt of [^3H]GA₂₉, another logical metabolite produced from [^3H]GA₂₀ (Fig. 6-3). There were no significant differences in the apparent rate of formation of [^3H]GA₂₉ in vernalized compared to nonvernalized plants (Table 6-1).

Generally, vernalized plants had reduced formation rates of all EtOAc-insoluble ^3H -metabolites. There were significant differences in the amounts of EtOAc-insoluble ^3H -metabolites produced following [^3H]GA₂₀ feeds at 6-DPV (Table 6-1; e.g. GA₁-conjugate at 6-DPV, ANOVA, $df = 1/8$, $F = 5.717$, $p = 0.044$). The largest ^3H -EtOAc insoluble metabolite peak was near the Rt of authentic [^3H]GA₂₀, the intermediate peak was near [^3H]GA₁, and smallest peak was near [^3H]GA₂₉. These peaks probably represent GA glucosyl

conjugates which elute from HPLC slightly before or coincidentally with their corresponding free GAs (Koshioka *et al.*, 1983). Positive identification of these metabolites could be ascertained using liquid chromatography-mass spectrometry (Murofushi *et al.*, 1992) or less definitively using chemical hydrolysis or enzymatic cleavage (Rood *et al.*, 1983).

The nonvernalized plants generally had lower levels of the precursor [^3H]GA₂₀ and more [^3H]GA₂₀ and [^3H]GA₁ conjugates. However, the physiological role of GA conjugates is not well understood. It has been suggested that conjugation could be a means of GA regulation in which GAs are stored or transported in relatively inactive conjugate forms which can later be cleaved (reversible conjugation) thereby releasing the biologically active free GA forms (Rood *et al.*, 1983; Schneider, 1983; Schneider *et al.*, 1992). The present results suggest that conjugation might play a minor regulatory role in the control of free GA level and subsequent induction of elongation following vernalization. Vernalized plants that are induced to bolt have reduced rates of GA-conjugate formation, whereas nonvernalized plants that remain in a rosette apparently have more rapid rates of conjugation.

Table 6-1. Distribution of principal metabolites from [³H]GA₂₀ feeds to vernalized and nonvernalized *Brassica napus* cv. Crystal plants at day 6-, 12-, and 15-days post-vernalization. EtOAc- and MeOH-soluble silica gel fractions were eluted on reversed-phase C₁₈ HPLC column. The eluant was MeOH:H₂O with 1% acetic acid, 10 min at 10% MeOH, 38 min of a linear gradient to 73% MeOH and 5 min of a linear gradient from 73 to 100% MeOH at a flow of 1.7 ml/min. Values are means \pm s.e. from 5 (6-, 12-DPV) or 2 (15-DPV) replicates.

Days After Vernalization	<u>% of Total Recovered Radioactivity</u>					
	EtOAc Fraction			MeOH Fraction		
	GA ₂₀	GA ₁	GA ₂₉	GA ₂₀	GA ₁	GA ₂₉
<i>Nonvernalized</i>						
6	37.1 \pm 0.8*	27.0 \pm 1.2	5.0 \pm 0.5	12.4 \pm 0.7*	14.5 \pm 0.8*	4.0 \pm 0.5
12	40.1 \pm 1.1	19.8 \pm 0.9*	4.2 \pm 0.3	17.1 \pm 1.3	15.2 \pm 0.7	3.6 \pm 0.3
15	57.4 \pm 4.1	11.4 \pm 5.1	2.2 \pm 0.7	18.6 \pm 2.4	11.1 \pm 3.7	2.0 \pm 0.3
<i>Vernalized</i>						
6	42.2 \pm 0.6*	28.8 \pm 0.8	4.7 \pm 0.3	9.6 \pm 0.4*	11.7 \pm 0.8*	3.3 \pm 0.6
12	41.1 \pm 1.2	26.0 \pm 1.3*	5.3 \pm 0.5	13.2 \pm 1.3	11.6 \pm 1.8	2.9 \pm 0.3
15	55.9 \pm 0.4	15.9 \pm 1.4	2.8 \pm 0.4	14.0 \pm 4.3	10.3 \pm 4.4	1.1 \pm 0.6

* significant difference between nonvernalized and vernalized treatments (ANOVA and/or t-tests).

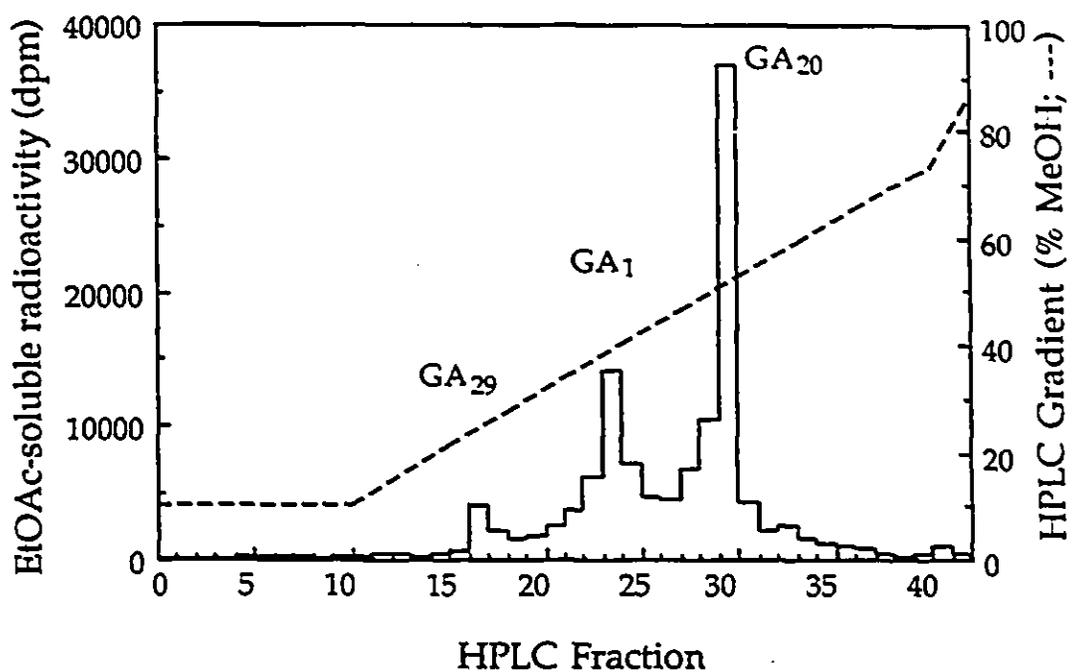


Figure 6-3. Elution of radioactivity from reversed-phase C18 HPLC column loaded with EtOAc-soluble fractions following a feed of [^3H]GA₂₀ to vernalized *Brassica napus* cv. Crystal plants 12-days post-vernalization.

The sole ^3H -labelled EtOAc-soluble metabolite indicated by C_{18} reversed-phase HPLC from either nonvernalized or vernalized winter canola plants fed ^3H GA₁ was ^3H GA₈ (Figure 6-4). However, there were no significant differences in the rates of the 2 β -hydroxylation conversion of the ^3H GA₁ to ^3H GA₈ in vernalized compared to nonvernalized plants (Table 6-2). Two peaks of ^3H -metabolites were detected from the EtOAc-insoluble SiO_2 fractions. The larger peaks had an R_t that was near the R_t of authentic ^3H GA₁, while the other peak had a R_t that was similar to that of authentic ^3H GA₈, suggesting that these two metabolites were glucosyl conjugates of ^3H GA₁ and ^3H GA₈, respectively. There were no significant differences in the proportion of putative GA-conjugates formed in either the vernalized or nonvernalized plants (Table 6-2). These results suggest that the rate of 2 β -hydroxylation is not substantially influenced by vernalization, nor is the apparent rate of GA₁ or GA₈ conjugation.

Some of the endogenous GAs present in *Brassica* have been identified by Rood *et al.* (1987a) and Hedden *et al.* (1989). Based on the occurrence of these GAs, metabolic pathways such as the early 13-OH and 13-non-OH pathways have been proposed to exist in *Brassica*. Collectively, the ^3H GA₁ and ^3H GA₂₀ feeds support previous metabolic studies and indicate that the latter portion of the the early 13-OH GA biosynthetic pathway is present in *Brassica* (Rood *et al.*, 1990a). ^3H GA₂₀ can be 2 β -hydroxylated to form ^3H GA₂₉, but is apparently more abundantly 3 β -hydroxylated to form ^3H GA₁. Subsequently, ^3H GA₁ is likely 2 β -hydroxylated to form ^3H GA₈. GA conjugates of all of the above metabolites are probably also formed. In other plant systems additional GA₂₀ metabolites exist, particularly GA₃ via a GA₅ intermediate (MacMillan, 1990). However, the formation of $^2\text{H}_2$ GA₃ from $^2\text{H}_2$ GA₂₀ has not been confirmed in *Brassica* (SB Rood and DW

Pearce, personal communication).

This study demonstrates increased [^3H]GA₁ biosynthesis and reduced ^3H -conjugate formation from [^3H]GA₂₀ in winter canola plants following vernalization. There were no significant differences in conversion rates of [^3H]GA₁ to [^3H]GA₈. In another cold-requiring crucifer, *Thlaspi arvense*, feeds of [^{14}C]GA₁₂-aldehyde showed little difference in metabolism but, [^{14}C]ent-kaurenoic acid (KA) feeds produced significant differences in metabolism suggesting that conversion of KA to GA₁₂-aldehyde may be an important regulatory step in thermoinduction (Hazebroek and Metzger, 1990). Enhanced metabolism during early GA biosynthesis has also been observed in cold-responsive *Pisum sativum* cultivars using [^{14}C]ent-kaurene (Moore and Moore, 1991).

The increase in GA₁ biosynthesis and reduced rate of conjugation in the vernalized winter canola plants coincided with the onset of bolting and flower bud initiation; small buds were evident in plants at both of the latter study dates. Furthermore, endogenous GA₁ concentrations in winter canola have also been observed to increase following vernalization (Chapter 5). These results coupled with the observations of promotion of stem elongation following application of GAs such as GA₁ and GA₃ (Chapter 4), suggest that GA₁ biosynthesis may be involved in the events such as stem elongation induced by vernalization. However, since the changes in GA₂₀ metabolism in the present study are rather small, this biosynthetic step is probably not be the primary regulatory step induced by vernalization. Although, biosynthetic regulation of GA₂₀ to GA₁ in winter canola could occur at a specific developmental period which did not coincide with the experimental sampling period. Hazebroek and Metzger (1990) and Moore and Moore (1991) have demonstrated differential metabolism early in the GA biosynthetic pathway suggesting that regulation accompanying vernalization may occur

prior to the biosynthesis of GA₂₀, the precursor investigated in the present study.

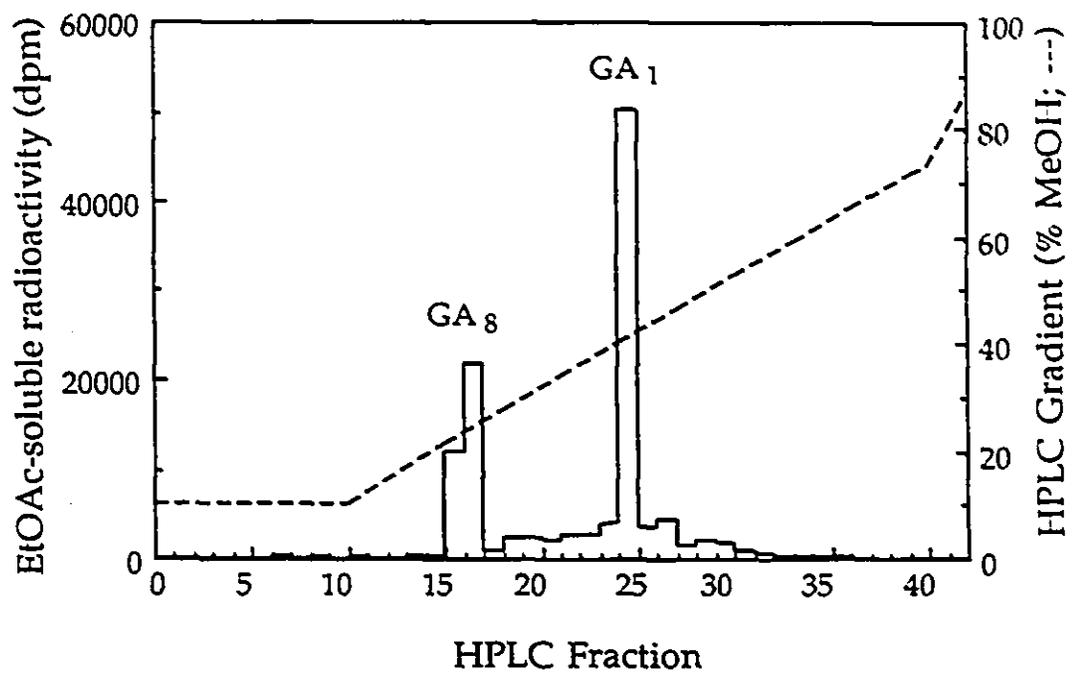


Figure 6-4. Elution of radioactivity from reversed-phase C₁₈ HPLC column loaded with EtOAc-soluble fractions following a feed of [³H]GA₁ to vernalized *Brassica napus* cv. Crystal plants 12-days post-vernalization.

Table 6-2. Distribution of principal metabolites from [^3H]GA₁ feeds to vernalized and nonvernalized *Brassica napus* cv. Crystal plants at 6-, 12- and 15- days post-vernalization. EtOAc- and MeOH-soluble silica gel fractions were eluted on reversed-phase C₁₈ HPLC column. The eluant was MeOH:H₂O with 1% acetic acid, 10 min at 10% MeOH, 38 min of a linear gradient to 73% MeOH and 5 min of a linear gradient from 73 to 100% MeOH at a flow of 1.7 ml/min. Values are means \pm s.e. from 5 (6-, 12-DPV) or 2 (15-DPV) replicates.

Days After Vernalization	<u>% of Total Recovered Radioactivity</u>			
	EtOAc Fraction		MeOH Fraction	
	GA ₁	GA ₈	GA ₁	GA ₈
<i>Nonvernalized</i>				
6	47.5 \pm 3.6*	14.0 \pm 2.4	23.1 \pm 1.0	13.4 \pm 2.2
12	45.4 \pm 6.4	6.4 \pm 2.7	19.4 \pm 1.7	10.4 \pm 1.2
15	67.5 \pm 0.7*	3.2 \pm 1.6	22.3 \pm 0.6	1.8 \pm 0.7
<i>Vernalized</i>				
6	61.7 \pm 2.9*	8.3 \pm 1.1	19.6 \pm 4.4	6.5 \pm 0.6
12	52.3 \pm 2.6	15.1 \pm 1.2	19.0 \pm 3.2	10.4 \pm 1.2
15	59.1 \pm 0.3*	12.8 \pm 9.9	19.5 \pm 7.1	3.4 \pm 0.4

* significant difference between nonvernalized and vernalized treatments (ANOVA).

CONCLUSIONS

Winter canola (*Brassica* spp.) is a winter annual oilseed that has an obligate requirement for vernalization or chilling for subsequent stem elongation and flowering. Since the phytohormone gibberellin (GA) plays a regulatory role in the regulation of stem elongation and flowering in numerous plants, including *Brassica*, the primary focus of this Thesis was to investigate the GA physiology of winter canola following vernalization. A preliminary study using an annual spring canola cultivar was performed to investigate the overall distribution of endogenous GAs within vegetative and reproductive *Brassica* plants. Studies using winter canola incorporated classical approaches to the study of hormone physiology to assess the involvement of GAs in events that follow vernalization. Included were exogenous plant growth regulator studies involving the application of GAs or 'anti-GA' plant growth retardants, quantification of endogenous GAs and analysis of ³H-metabolites formed from [³H]GA₁ or [³H]GA₂₀. Collectively, these approaches demonstrate a positive correlation between GAs and stem elongation, although the relationship between GAs and flowering is less clear.

Analyses of the distribution of GAs within plants are important for accurate assessment of endogenous GA status. Often extracts from entire plants are used for analyses and unfortunately if a large proportion of the sample consists of relatively GA-deficient tissue, the overall GA concentration may not be meaningful. Studies of tissue from both vegetative and reproductive *Brassica* plants indicated that GA distribution is not uniform within the plant. GA concentrations are lowest in the roots and increase acropetally, becoming highest in the shoot tips. Reproductive structures, such as siliques, are both quantitatively and qualitatively rich in

endogenous GAs. Thus, there are changes in both the types and amounts of GAs present in different organs during different developmental stages. Since the shoot tips had abundant concentrations of 13-hydroxylated GAs that are precursors of the putative bioeffector, GA₁ (and GA₃), subsequent studies with winter canola focussed only on this tissue.

Although bioassay results from other cold-requiring plants have suggested that GA-like substances increase following vernalization, none of the previous studies have actually compared GA concentrations using definitive physiochemical methods and quantitative internal standards. In winter canola, endogenous concentrations of GA_{1,3,8,19} and GA₂₀ were significantly higher in tissue from vernalized plants relative to nonvernalized plant tissue of the same age. This suggests that increased GA content may be responsible for stem elongation and possibly flowering following vernalization. Furthermore, since all of the measured GAs that occur late in the GA biosynthetic pathway demonstrated elevated concentrations, the point at which regulation may occur is likely to be prior to GA₁₉.

The involvement of GAs in the regulation of stem elongation is further substantiated by the results from application of plant growth regulators. Stem elongation was induced by exogenous GA₁, GA₃, GA₄, and GA₅ following application to the shoot tips of nonvernalized plants. However, nonvernalized plants could not be induced to flower. This suggests that nonvernalized plants could be partially GA-deficient, and hence normally fail to elongate. Since partially-vernalized plants were more sensitive (responsive) to exogenous GA, the chilling treatment may also enhance the plant's sensitivity to GA (exogenous or endogenous).

Experimentally, decreased endogenous GA concentrations can sometimes

be achieved by the application of plant growth retardants (PGRs) that block GA biosynthesis, such as paclobutrazol (PP333) or CGA 163'935. Vernalized winter canola plants treated with these PGRs displayed reduced shoot elongation and delayed flowering. This provides further evidence for a regulatory role for GA in the control of stem elongation and flowering following vernalization. Furthermore, the results from the combination of an early biosynthetic block, PP333, and a late block of GA₁ biosynthesis, CGA 163'935, suggests that GA₁ and probably GA₃ are important for the regulation of stem elongation. Additionally, since flower development was delayed by both PGRs, perhaps GAs of the late 13-hydroxylated pathway, GA₁ and GA₃, are involved in the regulation of flowering (rate), while other GAs are responsible for floral initiation.

Metabolic differences were also present between vernalized and nonvernalized winter canola plants following feeds of [³H]GA₂₀. The rate of conversion of [³H]GA₂₀ to [³H]GA₁, endogenous GA₁ being the putative bioeffector of elongation, was slightly greater in the vernalized plants. However, subsequent rates of conversion of [³H]GA₁ to [³H]GA₈ were similar in both plants. While the slightly faster synthesis of GA₁ could be correlated to elongation, it is less likely that the minor increases in conversion would enable the major anatomical switch from the vegetative to the reproductive condition. Kaurenoid biosynthetic regulation is reportedly increased in other thermoinduced plants, and it is possible that similar early GA biosynthetic events could also be important in winter canola flowering.

These studies using winter canola to investigate GA physiology following vernalization have demonstrated a positive correlation between endogenous GA content and stem elongation. The regulatory role of GAs is further substantiated by exogenous plant growth regulator treatments and metabolic

feeds. However, the role of GA in the control of flowering in winter canola is less direct. Flowering is a complex process and is probably regulated by a collection of factors rather than a single controlling substance. To better understand the involvement of GA in the process of flowering, a molecular approach that investigates specific genotypes with altered flowering responses could help solve the flowering puzzle.

LITERATURE CITED

- Adams R, Weiler EW, Kerber E, Pfister K, Schär H-P. (1991) Studies on the action of the new growth retardant CGA 163'935. Proceedings of the British Crop Protection Conference - Weeds 3: 1133-1138.
- Ali A, Machado VS. (1982) Use of gibberellic acid to hasten flowering in rutabaga. Can J Plant Sci 62: 823-826.
- Amrein J, Rufener J, Quadranti M. (1989) The use of CGA 163'935 as a growth regulators in cereals and oilseed rape. Proceedings of the British Crop Protection Conference - Weeds 1: 89-94.
- Bowman JG. (1984) Commercial oilseed rape breeding. Aspects of Applied Biology (6) Agronomy, physiology, plant breeding and crop protection of oilseed rape. pp 31-36.
- Bunting ES. (1984) Oilseed rape in perspective: with particular reference to crop expansion and distribution in England 1973-1983. Aspects of Applied Biology (6) Agronomy, physiology, plant breeding and crop protection of oilseed rape. pp 11-21.
- Bunting ES. (1986) Oilseed rape in perspective. In: DH Scarsbrick and RW Daniels (eds), Oilseed rape. Collins, London, pp 1-31.
- Chailakhyan MKh. (1937) Concerning the hormonal nature of plant development processes. C.R. (Dokl) Acad Sci SSSR 16: 227.
- Chailakhyan MKh. (1957) Effect of gibberellins on growth and flowering in plant. Dokl Akad Nauk SSSR 117: 1077-1080.
- Chailakhyan MKh. (1958) [Hormonal factors of plant flowering] In Russian. Fiziol. Rast. 5: 541-560.

-
- Chailakhyan MKh. (1968) Internal factors of plant flowering. *Ann Rev Plant Physiol* 19: 1-36.
- Chailakhyan MKh, Lozhnikova VN. (1962) Gibberellin-like substances and vernalization of plants. *Soviet Plant Physiology* 9: 15-22.
- Chailakhyan MKh. (1970) Hormonal regulation of plant flowering in different photoperiodic groups. *In: DJ Carr (ed), Plant Growth Substances 1970. Proceedings of the 7th International Conference on Plant Growth Substances, Canberra, Australia. Springer Verlag, Heidelberg, pp 745-752.*
- Chailakhyan MKh. (1975) Substances of plant flowering. *Biol Plant (Praha)* 17: 1-11.
- Chakravarti SC. (1958) Gibberellic acid and vernalization. *Nature* 182: 1612-1613.
- Chouard P. (1960) Vernalization and its relations to dormancy. *Ann Rev Plant Physiol* 11: 191-238.
- Cohen JD. (1984) Convenient apparatus for the generation of small amounts of diazomethane. *J Chromatog* 303: 193-196.
- Cronquist A. (1988) The evolution and classification of flowering plants. The New York Botanical Garden, Bronx, New York.
- Curtis OF, Chang HT. (1930) The relative effectiveness of the crown as contrasted with that of the rest of the plant upon flowering in celery plants. *Amer J Bot* 17: 1047-1048.
- de Jong GJ. (1981) Polder Projects. *In: ES Bunting (ed), Production and utilization of protein in oilseed crops. Nijhof, Den Haag.*

-
- Durley RC, Crozier A, Pharis RP, McLaughlin GE. (1972) Chromatography of 33 gibberellins on a gradient eluted silica gel partition column. *Phytochemistry* 11: 3029-3033.
- Evans LT. (1969) A short history of the physiology of flowering. In: LT Evans (ed), *The induction of flowering some case histories*. Cornell University Press, Ithaca, New York, pp 1- 13.
- Evans LT. (1971) Flower induction and the flower concept. *Ann Rev Plant Physiol* 22: 365-394.
- Evans LT, King RW, Chu A, Mander LN, Pharis RP. (1990) Gibberellin structure and florigenic activity in *Lolium temulentum*, a long-day plant. *Planta* 182: 97-106.
- Fujioka S, Yamane H, Spray CR, Gaskin P, MacMillan J, Phinney BO, Takahashi N. (1986) Qualitative and quantitative analyses of gibberellins in vegetative shoots of normal, *dwarf-1*, *dwarf-2*, *dwarf-3*, and *dwarf-5* seedlings of *Zea mays* L. *Plant Physiol* 88: 1367-1372.
- Gaskin P, MacMillan J, Firn RD, Pryce RJ. (1971) 'Parafilm' a convenient source of *n*-alkanes for the determination of gas chromatographic retention indices. *Phytochemistry* 10: 1155-1157.
- García-Martínez JL, Santes C, Croker SJ, Hedden P. (1991) Identification and distribution of gibberellins in fruits of *Pisum sativum* L. cv. Alaska during pod development. *Planta* 184:53-60.
- García-Martínez JL, Sponsel VM, Gaskin P. (1987) Gibberellins in developing fruits of *Pisum sativum* cv. Alaska: Studies on their role in pod growth and seed development. *Planta* 170:130-137.
- Graebe J. (1987) Gibberellin biosynthesis and control. *Ann Rev Plant Physiol* 38: 419-465.
-

-
- Grossmann K. (1992) Plant growth retardants: their mode of action and benefit for physiological research. *In*: CM Karssen, LC Van Loon and D Vreugdenhil (eds), *Progress in Plant Growth Regulation*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 788-797.
- Hänsel H. (1953) Vernalisation of winter rye by negative temperatures and the influence of vernalisation upon the lamina length of the first and second leaf in winter rye, spring barley and winter barley. *Ann Bot* 17: 417-432.
- Harada H. (1962) Etude des substances naturelles de croissance en relation avec la floraison - Isolement d'une substance de montaison. *Rev Gén Bot* 69: 201-297.
- Harborne JB. (1988) *Introduction to ecological biochemistry*, 3rd Edition. Academic Press, London.
- Harper FR, Berkenkamp B. (1975) Revised growth-stage key for *Brassica campestris* and *B. napus*. *Can J Plant Sci* 55: 657-658.
- Hazebroek JP, Metzger JD. (1990) Thermoinductive regulation of gibberellin metabolism in *Thlaspi arvense* L. *Plant Physiol* 94: 157-165.
- Hedden P. (1983) *In vitro* metabolism of gibberellins. *In*: A Crozier (ed), *The biochemistry and physiology of gibberellins* (Vol 1). Praeger Publishers, New York, pp 99-149.
- Hedden P, Croker SJ, Rademacher W, Jung J. (1989) Effects of the triazole plant growth retardant BAS 111..W on gibberellin levels in oilseed rape, *Brassica napus*. *Physiol Plant* 75: 445-451.
- Heide OM. (1970) Seed-stalk formation and flowering in cabbage I. Day-length, temperature, and time relationships. *Scientific Reports of the Agricultural College of Norway* 27: 1-20.

-
- Hoad GV. (1983) Gibberellin bioassays and structure-activity relationships. In: A Crozier (ed), The biochemistry and physiology of gibberellins (Vol. 2). Praeger Publishers, New York, pp 57-94.
- Hodgson AS. (1978) Rapeseed adaptation in Northern New South Wales. I Phenological responses to vernalization, temperature and photoperiod by annual and biennial cultivars of *Brassica campestris* L., *Brassica napus* L., and Wheat cv. Timgalen. Aust J Agric Res 29: 693-710.
- Ingram TJ, Reid JB, Murfet IC, Gaskin P, Willis CL, MacMillan J. (1984) Internode length in *Pisum*. The *Le* gene controls the 3 β -hydroxylation of gibberellin A₂₀ to gibberellin A₁. Planta 160: 455-463.
- Joseph C, Seigneuret, Touraud G, Billot J. (1983) The free gibberellins of *Cichorium intybus* L. Root: Identification and changes during vernalization. Z fur Pflanzen 110: 401-407.
- Jung J, Rademacher W. (1983) Plant growth regulating chemicals. In: LG Nickell (ed), Plant Growth Regulating Chemicals, Volume 1. CRC Press Inc., Boca Raton, Florida, pp 253-271.
- Kamiya Y, Nakayama I, Kobayashi M. (1992) Useful probes to study the biosynthesis of gibberellins. In: CM Karssen, LC Van Loon and D Vreugdenhil (eds), Progress in Plant Growth Regulation. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 555-565.
- Kaufman PB, Ghosheh NS, Nakosteen L, Pharis RP, Durley RC (1976) Analysis of native gibberellins in the internodes, nodes, leaves, and inflorescences of developing *Avena* plants. Plant Physiol 58: 131-134.
- Kerber E, Leypoldt G, Seiler A. (1989) CGA 163'935, a new plant growth regulator for small grain cereals, rape and turf. Proceedings of the British Crop Protection Conference - Weeds 1: 83-88.

-
- Kimber DS. (1984) Progress in the introduction of low glucosinolate winter varieties. Aspects of Applied Biology (6) Agronomy, physiology, plant breeding and crop protection of oilseed rape. pp 23-30.
- King SR and Bacon RK. (1992) Vernalization requirement of winter and spring oat genotypes. Crop Sci 32: 677-680.
- Klebs G. (1903) Willkürliche entwicklungsänderungen bei pflanzen. Ein Beitrag zur Physiologie der Entwicklung. Jena, G. Fischer.
- Kneen B. (1992) The rape of canola. New Canada Publications, Toronto, 230 p.
- Kobayashi M, Yamaguchi I, Murofushi N, Ota Y, Takahashi N. (1988) Fluctuation and localization of endogenous gibberellins in rice. Agric Biol Chem 52: 1189-1194.
- Koshioka M, Harada J, Takeno K, Noma M, Sass T, Ogiyama K, Taylor JS, Rood SB, Legge RL, Pharis RP. (1983) Reversed-phase C₁₈ high-performance liquid chromatography of acidic and conjugated gibberellins. J Chromatog 256:101-115.
- Lang A. (1952) Physiology of flowering. Ann Rev Plant Physiol 3: 265-306.
- Lang A. (1956) Induction of flower formation in biennial *Hyoscyamus* by treatment with gibberellin. Naturwissenschaften 43: 284-185.
- Lang A. (1957) The effect of gibberellin upon flower formation. Proc Nat Acad Sci (Washington) 43: 709-717.
- Lang A. (1965) Physiology of flower initiation. In: W Ruhland (ed), Encyclopedia of Plant Physiology. Springer-Verlag, Berlin, pp 1380-1536.
- Lang A and Reinhard E. (1961) Gibberellins and flower formation. Adv Chem Series 28: 71-79.
-

-
- Lesham Y, Steiner S. (1968) Effect of gibberellic acid and cold treatment on flower differentiation and stem elongation of cauliflower (*Brassica oleracea* var. *Botrytis*). Israel J Agric Res 18: 133-134.
- Lin J-T, Stafford AE. (1987) Comparison of the endogenous gibberellins in the shoots and roots of vernalized and non-vernalized Chinese spring wheat seedlings. Phytochemistry 26: 2485-2488.
- MacMillan J. (1990) Metabolism of gibberellin A₂₀ and A₉ in plants: Pathways and enzymology. In: RP Pharis and SB Rood (eds), Plant Growth Substances 1988. Springer-Verlag, Berlin, pp 307-313.
- Mandel RM, Rood SB, Pharis RP. (1992) Bolting and floral induction in annual and cold-requiring biennial *Brassica* spp.: Effects of photoperiod and exogenous gibberellin. In: CM Karssen, LC Van Loon and D Vreugdenhil (eds), Progress in Plant Growth Regulation. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 371-379.
- Mander LN. (1992) The chemistry of gibberellins: An overview. Chem Rev 92: 573-612.
- Margara J. (1963) Rôle éventuel de gibbérélines endogènes dans le développement du Colza. Physiol Vég 1: 315-324.
- Martínez-García, García-Martínez. (1992) Effect of the growth retardant LAB 198 999, an acylcyclohexanedione compound, on epicotyl elongation and metabolism of gibberellin A₁ and A₂₀ in cowpea. Planta 188: 245-251.
- Martinez-Zapater JM, Somerville CR. (1990) Effect of light quality and vernalization on late-flowering mutants of *Arabidopsis thaliana*. Plant Physiol 92: 770-776.
- Melchers G. (1939) Die blühhormone. Ber dtsh bot ges 57: 29-48.

-
- Metzger JD (1985) Role of gibberellins in the environmental control of stem growth in *Thlaspi arvense* L. *Plant Physiol* 78: 8-13.
- Metzger JD. (1988) Localization of the site of perception of thermoinductive temperatures in *Thlaspi arvense* L. *Plant Physiol* 88: 424-428.
- Metzger JD. (1988a) Hormones and reproductive development. In: PJ Davies (ed), *Plant hormones and their role in plant growth and development*. Kluwer Academic Publishers, New York, pp 431-462.
- Metzger JD. (1990) Gibberellins and flower initiation in herbaceous angiosperms. In: RP Pharis and SB Rood (eds), *Plant Growth Substances* 1988. Springer-Verlag, Berlin, pp 476-485.
- Metzger JD. (1990b) Comparison of biological activities of gibberellins and gibberellin-precursors native to *Thlaspi arvense* L. *Plant Physiol* 94: 151-156.
- Metzger JD, Dusbabek K. (1991) Determination of the cellular mechanisms regulating thermo-induced stem growth in *Thlaspi arvense* L. *Plant Physiol* 97: 630-637.
- Metzger JD, Mardaus MC. (1986) Identification of endogenous gibberellins in the winter annual weed *Thlaspi arvense* L. *Plant Physiol* 80: 396-402.
- Michniewicz M, Lang A. (1962) Effect of nine different gibberellins on stem elongation and flower formation in cold-requiring and photoperiodic plants grown under noninductive conditions. *Planta* 58: 549-563.
- Michniewicz M, Kriesel K, Rozej B. (1981) Role of endogenous growth regulators in vernalization of seeds of radish (*Raphanus sativus* L.) *Acta Soc Bot Pol* 50: 653-662.
- Moore TC and Moore JA. (1991) Induction of *ent*-kaurene biosynthesis by low temperature in dwarf peas. *J Plant Growth Regul* 10:91-95.

-
- More, D. (1992) Celebrating 25 years of progress. Canola Digest (Canola Council of Canada Publication) 26: 2.
- Morrison MJ, Andrews CJ. (1992) Variable increases in cold hardiness induced in winter rape by plant growth regulators. J Plant Growth Regul 11: 113-117.
- Murofushi N, Durley RC, Pharis RP. (1977) Preparation of radioactive gibberellins A₂₀, A₅, and A₈. Agric Biol Chem 41: 1075-1079.
- Murofushi N, Honda I, Hirasawa, Yamaguchi I, Takahashi N, Phinney BO. (1991) Gibberellins from the seed, tassel, cob and silk of maize. Agric Biol Chem 55: 435-439.
- Murofushi N, Yang Y-Y, Yamaguchi I, Schneider G, Kato Y. (1992) Liquid chromatography/atmospheric pressure chemical ionization mass spectrometry of gibberellin conjugates. In: CM Karssen, LC Van Loon and D Vreugdenhil (eds), Progress in Plant Growth Regulation. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 900-904.
- Myers LF, Christian KR, Kirchner RJ. (1982) Flowering responses of 48 lines of oilseed rape (*Brassica* spp.) to vernalization and daylength. Aust J Agric Res 33: 927-936.
- Nakamura E. (1961) Seed vernalization of the cabbages (*Brassica oleracea* spp.) I. Effect of seed-vernalization on the flowering in cabbage. J Japan Soc Hort Sci 30: 57-62.
- Nakayama I, Kobayashi M, Kamiya Y, Abe H, Sakurai A. (1992) Effects of a plant-growth regulator, prohexadione-calcium (BX-112), on the endogenous levels of gibberellins in rice. Plant Cell Physiol 33: 59-62.
- Napp-Zinn K. (1963) Über den einfluss von genen und gibberellinen auf die blütenbildung van *Arabidopsis thaliana*. Ber dtsch bot Ges 76: 77.
-

-
- Napp-Zinn K. (1969) *Arabidopsis thaliana* (L.) Heynh. In: LT Evans (ed), The induction of flowering some case histories. Cornell University Press, Ithaca, New York, pp 291-304.
- Napp-Zinn K. (1984) Light and vernalization. In: D Vince-Prue, B Thomas, KE Cockshull (eds), Light and flowering process. Academic Press, London, pp 75-88.
- Napp-Zinn K. (1987) Vernalization - Environmental and genetic regulation. In: JG Atherton (ed), Manipulation of flowering. Butterworths, London, pp 123-132.
- Netzer MH, Tittone E, Merrien A. (1986) Initiation florale du colza d'hiver *Brassica napus* L. Jet Neuf en conditions contrôlées. Centre technique interprofessionnel des oleagineux métropolitains. Informations techniques 96: 3-9.
- Ogawa Y, Fujiyama G, Iwai S. (1990) Promoting effect of gibberellin A₃ on flowering induced by chilling in *Pharbitis nil* Choisy. J Japan Soc Hort Sci 59: 371-376.
- Paszkowski W. (1983) The Canadian canola industry. Proceedings of the 6th International Rapeseed Conference, Paris, Volume 1, pp 3-8.
- Pharis RP, Evans LT, King RW, Mander LN. (1987) Gibberellins, endogenous and applied, in relation to flower induction in the long-day plant *Lolium temulentum*. Plant Physiol 84: 1132-1138.
- Pharis RP, Zhang R, Bau-Jen Jiang I, Dancik BP, Yeh FC. (1992) Differential efficacy of gibberellins in flowering and vegetative shoot growth, including heterosis and inherently rapid growth. In: CM Karssen, LC Van Loon and D Vreugdenhil (eds), Progress in Plant Growth Regulation. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 13-33.
-

-
- Pharis RP, King RW. (1985) Gibberellins and reproductive development in seed plants. *Ann Rev Plant Physiol* 36: 517-568.
- Phinney BO. (1985) Gibberellin A₁ dwarfism and shoot elongation in higher plants. *Biol Plant* 27: 172-179.
- Phinney BO, Spray C. (1990) Dwarf mutants of maize - Research tools for the analysis of growth. *In: RP Pharis and SB Rood (eds), Plant Growth Substances 1988*. Springer-Verlag, Berlin, pp 65-73.
- Purvis ON. (1961) The physiological analysis of vernalisation. *In: W Ruhland (ed), Encyclopedia of Plant Physiology* (16). Springer Verlag, Berlin, pp 76-122.
- Rademacher W, Temple-Smith KE, Griggs DL, Hedden P. (1992) The mode of action of acylcyclohexanediones - a new type of growth retardant. *In: CM Karssen, LC Van Loon and D Vreugdenhil (eds), Progress in Plant Growth Regulation*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 571-577.
- Rademacher W, Jung J, Graebe JE, Schwenen L. (1984) On the mode of action of tetracyclis and triazole growth retardants. *British Plant Growth Regulator Group, Monograph* 11: 1-11.
- Reda F, Larsen P, Rasmussen OE. (1978) Levels of growth regulating substance during vernalization of winter wheat. *Physiol Plant* 42: 109-113.
- Reid DM, Beall FD, Pharis RP. (1991) Environmental cues in plant growth. *In: FC Steward and RGS Bidwell (eds) Plant physiology A treatise Vol X: Growth and Development*. Academic Press, San Diego, pp 65-185.
- Reid JB. (1990a) Phytohormone mutants in plant research. *J Plant Growth Regul* 9: 97-111.

-
- Reid JB. (1990b) Gibberellin synthesis and sensitivity mutant in *Pisum*. In: RP Pharis and SB Rood (eds), Plant Growth Substances 1988. Springer-Verlag, Berlin, pp 74-83.
- Robbins WH. (1957) Gibberellic acid and the reversal of adult *Hedera* to a juvenile state. *Am J Bot* 44: 743-746.
- Rogler CE, Hackett WP. (1975) Phase change in *Hedera helix*: Induction of the mature to juvenile phase change by gibberellin A₃. *Physiol Plant* 34: 141-147.
- Rood SB, Mandel R, Pharis RP. (1989a) Endogenous gibberellins and shoot growth and development in *Brassica napus*. *Plant Physiol* 89: 269-273.
- Rood SB, Pearce D, Pharis RP. (1987) Identification of endogenous gibberellins from oilseed rape. *Plant Physiol* 85: 605-607.
- Rood SB, Pearce D, Williams PH, Pharis RP. (1989b) A gibberellin-deficient *Brassica* mutant - *rosette*. *Plant Physiol* 89: 482-487.
- Rood SB, Pharis RP, Koshioka M. (1983) Reversible conjugation of gibberellins *in situ* in maize. *Plant Physiol* 73: 340-346.
- Rood SB, Smienk S, Pearce D, Pharis RP. (1987) Gibberellins and the regulation of height growth. In: AR Cooke (ed), 1987 Joint PGRSA-JSCRIP Proceedings of the 14th Annual Plant Growth Regulator Society of America Meeting, Honolulu, Hawaii, pp 139-144.
- Rood SB, Williams PH, Pearce D, Murofushi N, Mander LN, Pharis RP. (1990a) A mutant gene that increases gibberellin production in *Brassica*. *Plant Physiol* 93: 1168-1174.
- Rood SB, Zanewich KP, Bray D. (1990b) Growth and development of *Brassica* genotypes differing in endogenous gibberellin content. II. GA content, growth analyses and cell sizes. *Physiol Plant* 79: 679-685.

-
- Salisbury FB, Ross CW. (1992) *Plant Physiology*, 4th Edition. Wadsworth Publishing Company, Belmont, CA.
- Schneider G. (1983) Gibberellin conjugates. *In*: A Crozier (ed), *The biochemistry and physiology of gibberellins* (Vol 1). Praeger Publishers, New York. pp 389-456.
- Schneider G, Jensen E, Spray CR, Phinney BO. (1992) Hydrolysis and reconjugation of gibberellin A₂₀ glucosyl ester by seedling of *Zea mays* L. *Proc Natl Acad Sci USA* 89: 8045-8048.
- Sheng C, Bhaskar KV, Chu W-LA, Mander LN, Pearce DW, Pharis RP, Young S. (1992a) Identification of a novel gibberellin (GA₈₅) in very young seedlings of *Brassica campestris* cv. Tobin. *Biosci Biotech Biochem* 56: 564-566.
- Sheng C, Bhaskar V, Mander LN, Pearce DW, Pharis RP, Young S. (1992b) Identification of gibberellins Ag₉ from *Brassica campestris*. *Phytochemistry* 31: 4055-4057.
- Singh SP, Paleg LG. (1984) Low temperature induction of hormonal sensitivity in genotypically gibberellic acid-insensitive aleurone tissue. *Plant Physiol* 74: 437-438.
- Smith VA, Knatt CJ, Gaskin P, Reid JB. (1992) The distribution of gibberellins in vegetative tissues of *Pisum sativum* L. I. Biological and biochemical consequences of the *le* mutation. *Plant Physiol* 99: 368-371.
- Song KM, Osborn TC, William PH. (1988) *Brassica* taxonomy based on nuclear restriction fragment length polymorphisms (RFLPs). 1. Genome evolution of diploid and amphidiploid species. *Theor Appl Genet* 75: 784-794.

-
- Song K, Osborn TC, Williams PH. (1990) *Brassica* taxonomy based on nuclear restriction fragment length polymorphisms (RFLPs). 3. Genome relationships in *Brassica* and related genera and the origin of *B. oleracea* and *B. rapa* (syn *campestris*). *Theor Appl Genet* 79: 497-506.
- Sponsel VM, Hoad GV, Beeley LJ. (1977) The biological activities of some new gibberellins (GAs) in six plant bioassays. *Planta* 135: 143-147.
- Sponsel VM. (1987) Gibberellin biosynthesis and metabolism. In: PJ Davies (ed), *Plant hormones and their role in plant growth and development*. Kluwer Academic Publishers, New York, pp 43-75.
- Spray CR, Phinney BO, Gaskin P, Gilmour SJ, MacMillan J. (1984) Internode length in *Zea mays* L. The dwarf-1 mutation controls the 3 β -hydroxylation of gibberellin A₂₀ to gibberellin A₁. *Planta* 160: 464-468.
- Steel GC, Torrie JH. (1960) *Principles and procedures of statistics*. McGraw-Hill Book Company, New York.
- Strickberger MW. (1976) *Genetics*, 2nd Edition. Macmillan Publishing, New York.
- Suge H. (1970) Changes of endogenous gibberellins in vernalized radish plants. *Plant Cell Physiol* 11: 720-735.
- Suge H. (1984) Re-examination on the role of vernalization and photoperiod in the flowering of *Brassica* crops under controlled environment. *Japan J Breed* 34: 171-180.
- Suge H. (1986) Reproductive development of higher plants as influenced by brassinolide. *Plant Cell Physiol* 27: 199-205.
- Suge H, Rappaport L. (1968) Role of gibberellins in stem elongation and flowering in radish. *Plant Physiol* 43: 1208-1214.
-

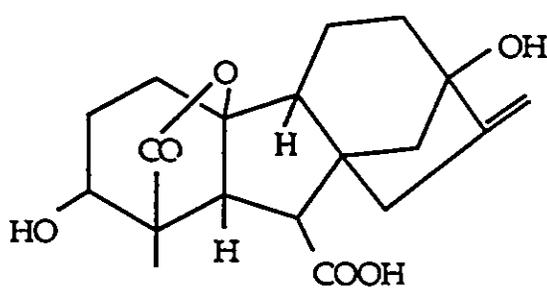
-
- Suge H, Takahashi H. (1982) The role of gibberellins in the stem elongation and flowering of Chinese cabbage, *Brassica campestris* var. *pekinensis* in their relation to vernalization and photoperiod. Rep Inst Agr Tohoku Univ 33: 15-34.
- Takahashi N, Yamaguchi I, Yamane H. (1986) Gibberellins. In: N Takahashi (ed), Chemistry of Plant Hormones. CRC Press Inc., Boca Raton, Florida, pp 57-151.
- Takahashi N. (1990) Endogenous plant hormones in rice in relation to the regulation of its life cycle. In: RP Pharis and SB Rood (eds), Plant Growth Substances 1988. Springer-Verlag, Berlin, pp 11-20.
- Takahashi N, Kobayashi M. (1991) Organ-specific gibberellins in rice: Roles and biosynthesis. In: N Takahashi, BO Phinney and J MacMillan (eds), Gibberellins. Springer-Verlag, New York, pp 9-21.
- Takahashi N, Yamaguchi I, Yamane H. (1986) Gibberellins. In: Takahashi N (ed) Chemistry of Plant Hormones. CRC Press, Inc., Boca Raton, pp 57-151.
- Talon M, Koornneef M, Zeevaart JAD. (1990a) Endogenous gibberellins in *Arabidopsis thaliana* and possible steps blocked in the biosynthetic pathways of the semidwarf *ga4* and *ga5* mutants. Proc Nat Acad Sci USA 87: 7983-7987.
- Talon M, Zeevaart JAD. (1990b) Gibberellins and stem growth as related to photoperiod in *Silene armeria* L. Plant Physiol 92: 1094-1100.
- Thompson KF and Hughes WG. (1986) Breeding and varieties. In: DH Scarsbrick and RW Daniels (eds), Oilseed rape. Collins, London, pp 32-82.
- Tommey AM, Evans EJ. (1991) Temperature and daylength control flower initiation in winter oilseed rape (*Brassica napus* L.). Ann Appl Biol 118: 201-208.

-
- Trewavas AJ. (1982) Growth substance sensitivity: The limiting factor in plant development. *Physiol Plant* 55: 60-72.
- Trewavas AJ, Jones AM. (1981) Consequences of hormone-binding studies for plant growth substance research. *What's new in plant physiology* 12(2): 5-8.
- U N. (1935) Genome analysis in the *Brassica*, with special reference to the experimental formation of *Brassica napus*. *Japanese Journal of Botany* 7: 389-452.
- Van Marrewijk NPL. (1976) Artificial cold treatment, gibberellin application and flowering response of kohlrabi (*Brassica oleracea* L. var. *Gongylodes* L.) *Scientia Horticulturae* 4: 367-375.
- Weibel RO. (1960) Effect of gibberellin on the vernalization period of winter wheat. *Agronomy Journal* 52: 122-123.
- Wellensiek SJ. (1961) Leaf vernalization. *Nature* 192: 1097-1098.
- Wellensiek SJ. (1962) Dividing cells as the locus for vernalization. *Nature* 195: 307-308.
- Wellensiek SJ. (1964) Dividing cells as the prerequisite for vernalization. *Plant Physiol.* 38: 832-835.
- Whelan EDP, Schaalje GB. (1992) Vernalization of embryonic callus from immature embryos of winter wheat. *Crop Sci* 32: 78-80.
- Whitney EN, Hamilton EV, Rolfes SR. (1990) *Understanding nutrition*. West Publishing Company, St. Paul, MN, USA.
- Wiebe HJ. (1990) Vernalization of vegetable crops - A review. *Acta Horticulture* 267: 323-328.

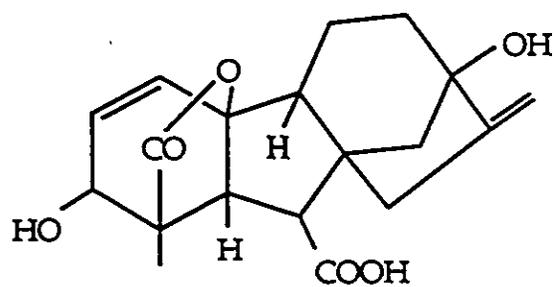
-
- Williams PH. (1985) Crucifer genetics cooperative resource book. University of Wisconsin-Madison, Madison, Wisconsin.
- Wittwer SH, Bukovac MJ. (1957) Gibberellin effects on temperature and photoperiodic requirements for flowering of some plants. *Science* 126: 30-31.
- Wittwer SH, Bukovac MJ. (1958) The effects of gibberellin on economic crops. *Economic Botany* 12: 213-255
- Zanewich KP, Rood SB, Williams PH. (1990) Growth and development of *Brassica* genotypes differing in endogenous gibberellin content. I. Leaf and reproductive development. *Physiol Plant* 79: 673-678.
- Zanewich KP, Rood SB, Southworth CE and Williams PH. (1991) Dwarf mutants of *Brassica*: Responses to applied gibberellins and gibberellin content. *J Plant Growth Regul* 10: 121-127.
- Zeevaart JAD. (1968) Vernalization and gibberellins in *Lunaria annua*. In: F Wightman and G Setterfield (eds), *Biochemistry and physiology of plant growth substances*, Proceedings of the sixth international conference of plant growth substances, Runge Press Ltd, Ottawa, pp 1357-1370.
- Zeevaart JAD. (1983) Gibberellins and flowering. In: A Crozier (ed), *The biochemistry and physiology of gibberellins* (Vol 2). Praeger Publishers, New York, pp 333-374.

APPENDIX 1

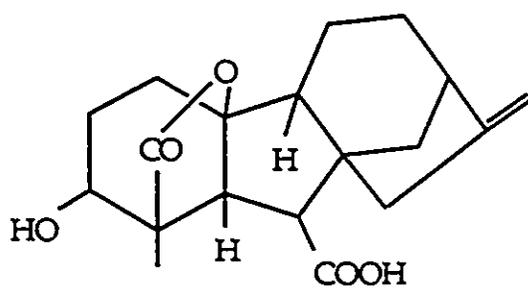
GIBBERELLIN STRUCTURES



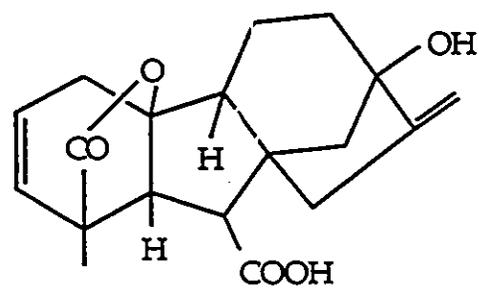
GA1



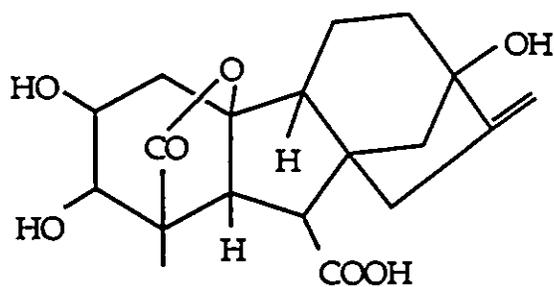
GA3



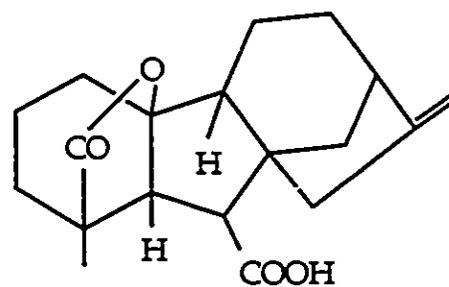
GA4



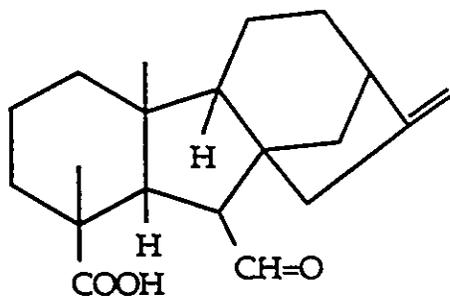
GA5



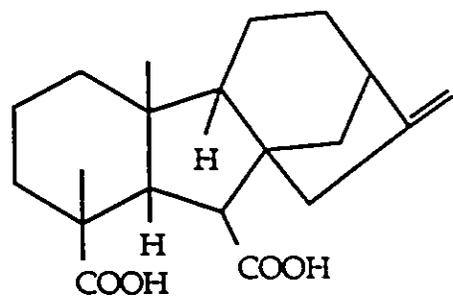
GA8



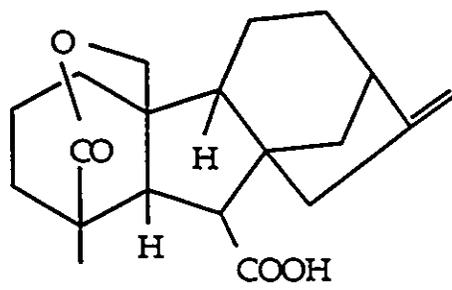
GA9



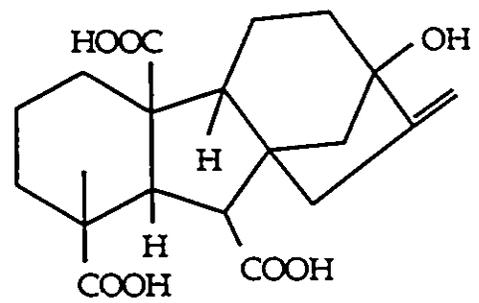
GA12-aldehyde



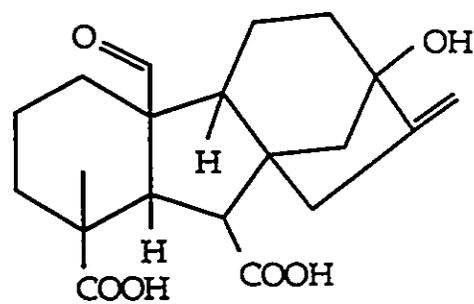
GA12



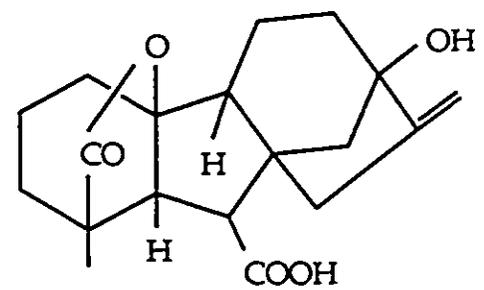
GA15



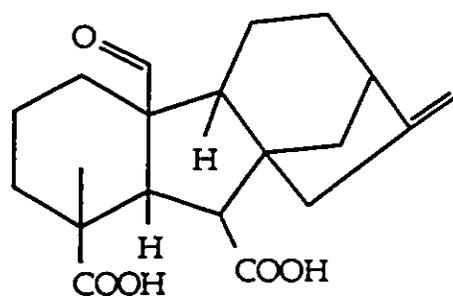
GA17



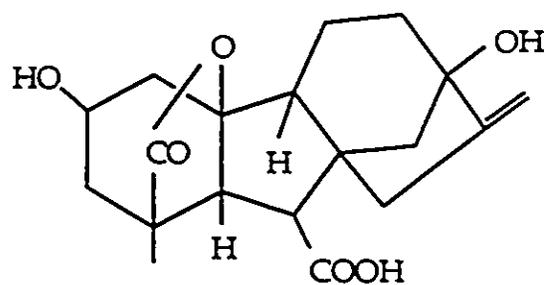
GA19



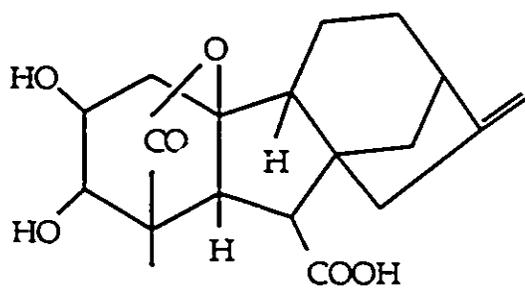
GA20



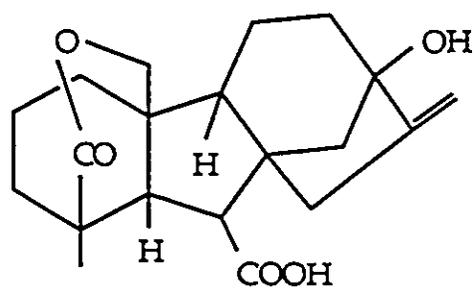
GA24



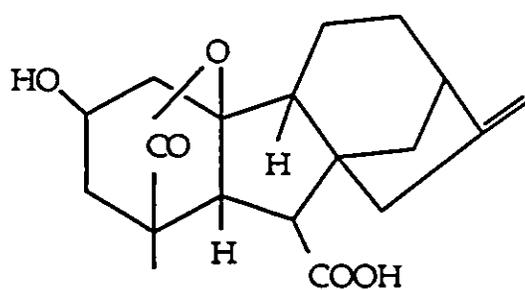
GA29



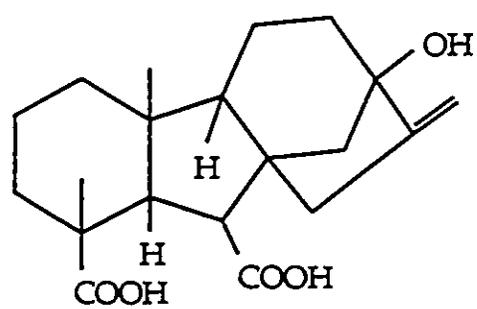
GA34



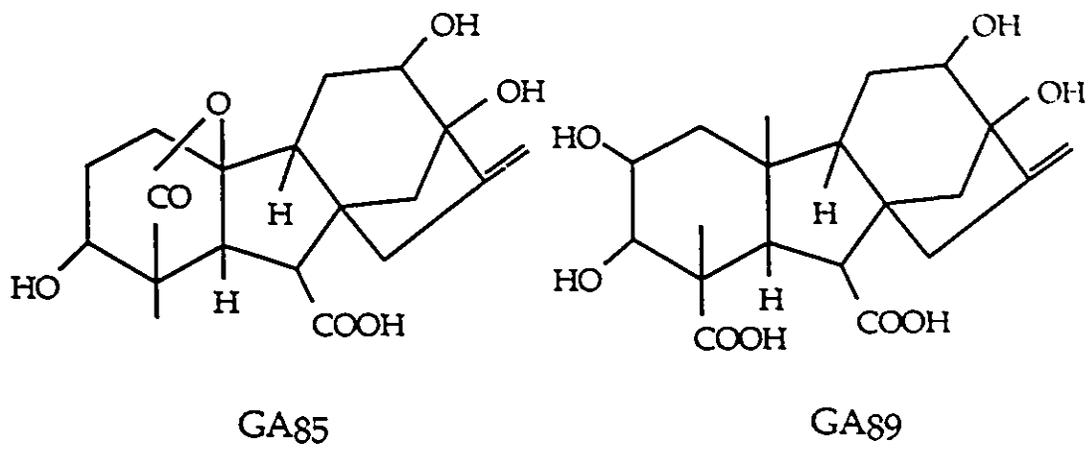
GA44



GA51

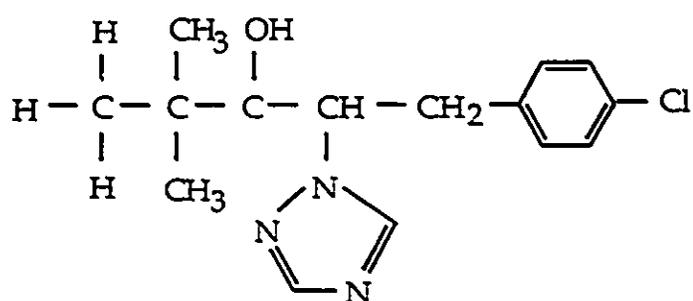


GA53

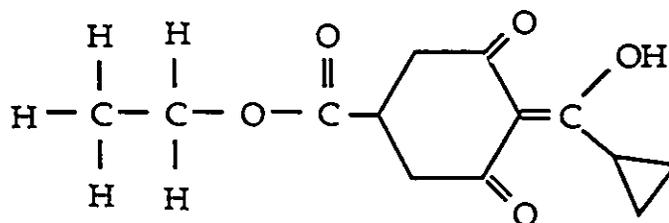


APPENDIX 2

PLANT GROWTH RETARDANT STRUCTURES



Paclobutrazol (PP333) - [(2*RS*, 3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1*H*-1, 2, 3-triazol-1-yl)penta-3-ol]



CGA 163'935 (cimectacarb) - ethyl 4-cyclopropyl (hydroxy) methylene-3,5-dioxocyclohexane-carboxylate