

VERNALIZATION AND GIBBERELLIN PHYSIOLOGY OF  
WINTER CANOLA

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## 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<sub>1,3,8,19</sub> and GA<sub>20</sub> concentrations were higher in the vernalized shoots following vernalization. Feeds of [<sup>3</sup>H]GA<sub>20</sub> to vernalized and nonvernalized plants demonstrated higher rates of [<sup>3</sup>H]GA<sub>1</sub> 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 GA<sub>1</sub>.

## 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.

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## 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 [ <sup>3</sup> H]GA <sub>1</sub> and [ <sup>3</sup> H]GA <sub>20</sub> 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 <sub>2</sub> 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 [ <sup>3</sup> H]GA <sub>20</sub> 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 [ <sup>3</sup> H]GA <sub>1</sub> 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 <sub>19</sub> gibberellin.	14
Figure 1-3.	Proposed gibberellin biosynthetic pathway in <i>Brassica</i> from GA <sub>12</sub> -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 <sub>3</sub> .	66
Figure 4-3b.	Dose-response curve of nonvernalized <i>Brassica napus</i> cv. Crystal plants treated with GA <sub>3</sub> .	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 <sub>3</sub> .	67
Figure 4-5.	Photograph of nonvernalized <i>Brassica napus</i> cv. Crystal plants treated with 10 <sup>-1</sup> M GA <sub>3</sub> , GA <sub>1</sub> , GA <sub>4</sub> , GA <sub>5</sub> and 0 M GA.	71
Figure 4-6.	Effect of 0, 10 <sup>-3</sup> , 10 <sup>-2</sup> , and 10 <sup>-1</sup> M GA <sub>1</sub> , GA <sub>3</sub> , GA <sub>4</sub> , and GA <sub>5</sub> 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 <sup>-5</sup> M PP333 at 12 DPV and 10 <sup>-5</sup> M PP333 immediately following vernalization.	74
Figure 4-8.	Effect of 10 <sup>-5</sup> 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 <sup>-4</sup> , 10 <sup>-3</sup> , 10 <sup>-2</sup> , and 10 <sup>-1</sup> M CGA 163'935.	76



Figure 4-10.	Effect of 0, 10 <sup>-4</sup> , 10 <sup>-3</sup> , 10 <sup>-2</sup> , 10 <sup>-1</sup> 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 <sup>-1</sup> 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 <sub>1</sub> and GA <sub>3</sub> and [2H <sub>2</sub> ] GA <sub>1</sub> and GA <sub>3</sub> .	87
Figure 5-2.	Height of vernalized and nonvernalized <i>Brassica napus</i> cv. Crystal plants.	91
Figure 5-3.	Concentrations of GA <sub>19</sub> , GA <sub>20</sub> , GA <sub>1</sub> , GA <sub>3</sub> , and GA <sub>8</sub> 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 [ <sup>3</sup> H]GA <sub>1</sub> and metabolites from SiO <sub>2</sub> partition columns loaded with extracts from vernalized <i>Brassica napus</i> cv. Crystal shoot tips.	100
Figure 6-2. (top)	Percentage [ <sup>3</sup> H]GAs recovered in the EtOAc fractions from SiO <sub>2</sub> partition chromatography after a [ <sup>3</sup> H]GA <sub>1</sub> feed to <i>Brassica napus</i> cv. Crystal plants 6-, 12-, and 15- days post vernalization.	101
Figure 6-2. (bottom)	Percentage [ <sup>3</sup> H]GAs recovered in the EtOAc fractions from SiO <sub>2</sub> partition chromatography after [ <sup>3</sup> H]GA <sub>20</sub> fed to <i>Brassica napus</i> cv. Crystal plants 6-, 12-, and 15- days post vernalization.	101

- Figure 6-3. Elution of [ $^3\text{H}$ ]GA<sub>20</sub> and metabolites from C<sub>18</sub>-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 [ $^3\text{H}$ ]GA<sub>1</sub> and metabolites from C<sub>18</sub>-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<sub>n</sub> = 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<sub>t</sub> = retention time  
SD = short day  
SiO<sub>2</sub> = silicic acid  
TMCS = trimethylchlorosilane

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## 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.

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*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.

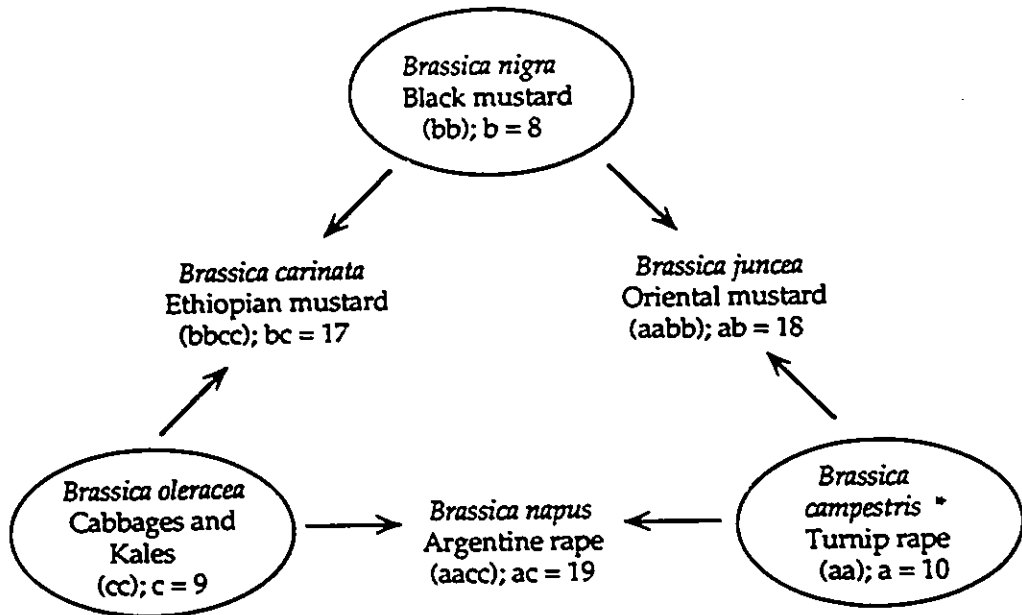


Figure 1-1. Proposed genetic and chromosomal relationships between several *Brassica* species and hybrids (adapted from Williams, 1985 and Bunting, 1986). Lower case letters indicate the nuclear genome such that a = 10 chromosomes, b = 8 chromosomes and c = 9 chromosomes. \* Some authorities consider *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



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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 19<sup>th</sup> 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*

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*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

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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 (GA<sub>3</sub>) (Lang, 1956). However GA<sub>3</sub> 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.

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### *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 GA<sub>12</sub>, a 20-carbon compound from which other 20 or 19 carbon tetra- or penta-cyclic compounds are derived (Fig 1-2). Oxidation of C<sub>20</sub> GAs at C(20) result in the formation of the lactone bridge in the A ring which characterizes the C<sub>19</sub> 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).



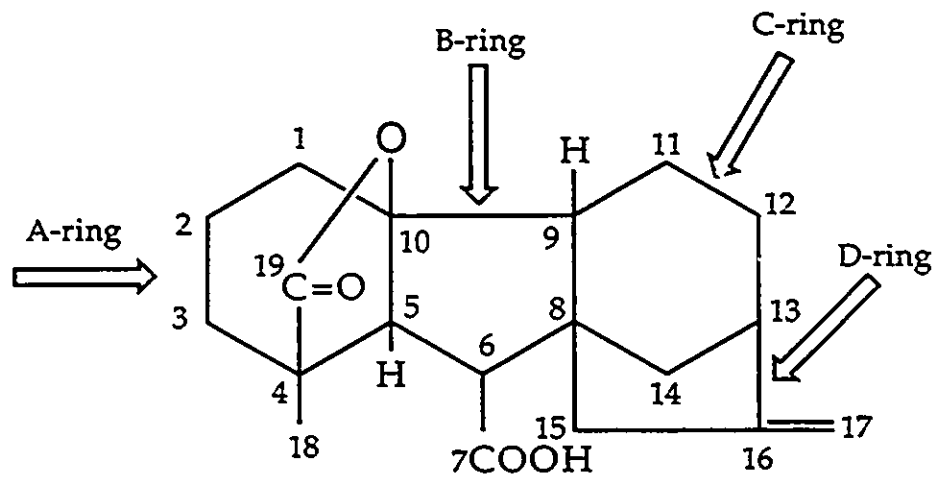


Figure 1-2. A C<sub>19</sub> gibberellin chemical skeleton showing the labelled rings and the numbered carbon atoms.

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The structure of the biologically active GAs, those capable of evoking a physiological response, vary greatly (Pharis *et al.*, 1992). Generally, the C<sub>19</sub>-GAs have a higher activity than the C<sub>20</sub>-GAs (Hoad, 1983). Of the C<sub>19</sub>-GAs, those which are characterized by 3 $\beta$ -hydroxylation, 3 $\beta$ ,13-dihydroxylation and 1,2-unsaturation tend to exhibit maximal biological activity (Hoad, 1983). Conversely, 2 $\beta$ -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 GA<sub>12</sub>-aldehyde, appears to be similar in most plants, the conversion of GA<sub>12</sub>-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).

GA<sub>12</sub>-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 [<sup>2</sup>H<sub>2</sub>]GA<sub>19</sub> to [<sup>2</sup>H<sub>2</sub>]GA<sub>20</sub> and [<sup>2</sup>H<sub>2</sub>]GA<sub>1</sub> (Rood *et al.*, 1990a). Recent feeds of

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[ $^2\text{H}_2$ ]GA<sub>9</sub> 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 GA<sub>53</sub>, GA<sub>44</sub>, GA<sub>19</sub> and GA<sub>20</sub> are formed from GA<sub>12</sub>-aldehyde and eventually give rise to GA<sub>1</sub>, 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 GA<sub>20</sub> to GA<sub>1</sub> involving 3 $\beta$ -hydroxylation is thought to be a pivotal metabolic conversion thereby allowing the regulation of GA<sub>1</sub> biosynthesis (Ingram *et al.*, 1984; Spray *et al.*, 1984). Subsequently, GA<sub>1</sub> can be 2 $\beta$ -hydroxylated to form GA<sub>8</sub>. Ultimately, the rate of formation of 2 $\beta$ -hydroxylated catabolites could be one mechanism regulating the amount of GA<sub>1</sub> 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.

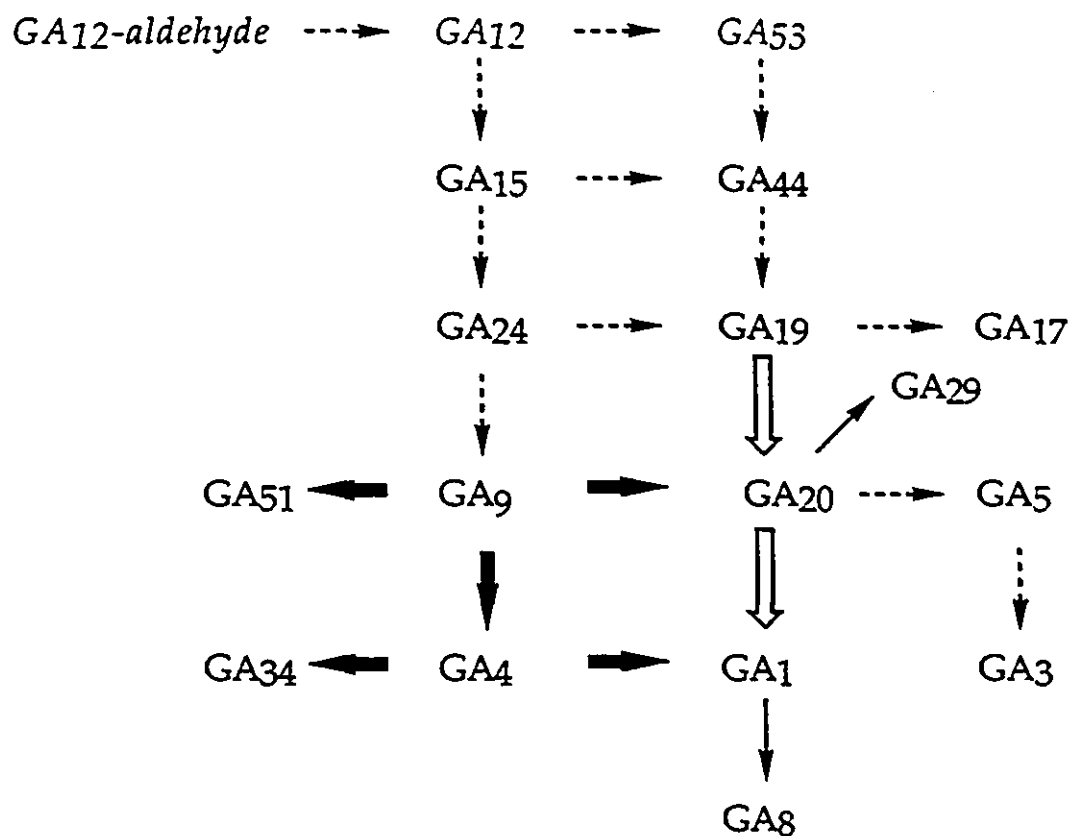


Figure 1-3. Proposed 13-OH and 13-non OH biosynthetic (metabolic) pathways from GA<sub>12</sub>-aldehyde to C<sub>19</sub> GAs for *Brassica*. GA<sub>12</sub> and GA<sub>53</sub> (in italics) have not yet been identified in *Brassica* but have been found in other crucifers. (See Appendix 1 for GA structures.)

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

➡ = demonstrated pathway by metabolic feeds of [<sup>2</sup>H<sub>2</sub>]GA<sub>4</sub> or [<sup>2</sup>H<sub>2</sub>]GA<sub>9</sub> to the rapid cycling *Brassica rapa* genotype *ein* (SB Rood and P Hedden, unpublished).

⇨ = pathway demonstrated by metabolic feeds of [<sup>2</sup>H<sub>2</sub>]GA<sub>19</sub> to the rapid cycling *Brassica rapa* genotype *ein* (Rood *et al.*, 1990a).

→ = proposed pathway based on metabolites identified by GC-MS following [<sup>3</sup>H]GA<sub>20</sub> 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. GA<sub>85</sub> was characterized from the cotyledons of young *Brassica* seedlings and demonstrated a bioactivity that was equal to or greater than that of GA<sub>1</sub> or GA<sub>3</sub>, 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 $\beta$ -hydroxyl forms, GA<sub>85</sub> has a similar inactive analogue, GA<sub>89</sub> (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 C<sub>20</sub> GAs are more abundant in the cold-treated seedlings while the C<sub>19</sub> 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.

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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 (GA<sub>3</sub>) 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 GA<sub>3</sub>. Since this initial observation by Lang, numerous cold-requiring plants have been treated with GA<sub>3</sub> (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 GA<sub>3</sub> 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 GA<sub>7</sub> caused both stem elongation and flower induction, whereas GA<sub>3</sub> resulted in only stem elongation (Michniewicz and Lang, 1962). Similarly, of various GAs tested, only GA<sub>32</sub>, GA<sub>5</sub>, 2,2-dimethyl GA<sub>4</sub>, GA<sub>3</sub> and GA<sub>7</sub> 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 GA<sub>3</sub> 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 GA<sub>3</sub>-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 GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>4</sub>, and GA<sub>5</sub> 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

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following vernalization to determine what metabolites were formed from feeds of [ $^3\text{H}$ ]GA<sub>1</sub> or [ $^3\text{H}$ ]GA<sub>20</sub> 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.

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## 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  $49.6^{\circ}\text{N}$ ) 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  $4^{\circ}\text{C}$ , plants were transferred to a cold room ( $13^{\circ}\text{C} \pm 2^{\circ}$ ) for 72 h (light was from a 150W

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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  $49.6^\circ\text{N}$ ) 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 GA<sub>1</sub>, (from Professor RP Pharis, University of Calgary), gibberellic acid (GA<sub>3</sub>) (119 3093; Eastman Kodak Co., Rochester, New York), GA<sub>4</sub> (Abbott Laboratories, North Chicago, Illinois) or GA<sub>5</sub> (Professor RP Pharis, University of Calgary) was dissolved in 50% (v:v) aqueous ethanol (EtOH) and serially diluted to appropriate concentrations of  $1.0 \times 10^{-1}$ ,  $1.0 \times 10^{-2}$ ,  $1.0 \times 10^{-3}$  M in 50 % EtOH. Applications of 5  $\mu\text{l}$  each were made to the shoot tips of plants using a Hamilton syringe on four consecutive days starting 28 days after planting (DAP).

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### *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  $1.0 \times 10^{-1}$ ,  $1.0 \times 10^{-2}$ ,  $1.0 \times 10^{-3}$  or  $1.0 \times 10^{-4}$  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  $-20^{\circ}\text{C}$  until analysis.

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### 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-<sup>2</sup>H<sub>2</sub>]GA<sub>1</sub>, [17-<sup>2</sup>H<sub>2</sub>]GA<sub>3</sub>, [17-<sup>2</sup>H<sub>2</sub>]GA<sub>4</sub>, [17-<sup>2</sup>H<sub>2</sub>]GA<sub>5</sub>, [17-<sup>2</sup>H<sub>2</sub>]GA<sub>8</sub>, [17-<sup>2</sup>H<sub>2</sub>]GA<sub>9</sub>, [17-<sup>2</sup>H<sub>2</sub>]GA<sub>19</sub>, and [17-<sup>2</sup>H<sub>2</sub>]GA<sub>20</sub> (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-<sup>3</sup>H]GA<sub>1</sub> and [1,2-<sup>3</sup>H]GA<sub>4</sub> (Amersham Corporation, Arlington Heights, Illinois) was added to the discs as chromatographic standards.

Samples were further purified using step-wise elution silica gel (SiO<sub>2</sub>) 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% H<sub>2</sub>O by weight) SiO<sub>2</sub> (2766; SA Scientific

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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  $^3\text{H}$ -distribution was determined by liquid scintillation counting of 0.3 ml aliquots.

Appropriate  $\text{SiO}_2$  fractions were dried and then grouped together using MeOH, filtered using 0.45 $\mu\text{m}$  filters (SJHV004NS; Millipore Waters Chromatography, Mississauga, Ontario), refiltered using C<sub>18</sub> Sep Pak cartridges (51910; Millipore Waters Chromatography) and dried under  $\text{N}_2$  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 C<sub>18</sub> 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  $\text{min}^{-1}$  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  $^3\text{H}$ -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



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temperature under N<sub>2</sub>. Residues were methylated in 100 µl of ethereal diazomethane (Cohen, 1984) at room temperature for 60 min. Subsequently, samples were dried under N<sub>2</sub> and 100 µl of ethereal diazomethane was again added. Methylated samples were dried under N<sub>2</sub> 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 N<sub>2</sub> 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<sup>-1</sup>. 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<sup>-1</sup> to 200°C, a slow rate temperature ramp of 5°C min<sup>-1</sup> to 270°C and a final rapid temperature ramp of 20°C min<sup>-1</sup> 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 GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>4</sub>, GA<sub>5</sub>, GA<sub>8</sub>, GA<sub>9</sub>, GA<sub>19</sub> and GA<sub>20</sub>, 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):

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

where S = ng of standard added to extract, B = % labelled molecules in the internal standard, C = measured intensity of M<sup>+</sup> of unlabelled GA, E = a factor calculated from the relative intensities of ions in the M<sup>+</sup> 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<sup>+</sup> of completely labelled GA, F = the intensity of the ion of m/z equivalent to M<sup>+</sup> of the fully-labelled GA in the M<sup>+</sup> cluster of the unlabeled GA relative to the intensity of M<sup>+</sup>, C = measured intensity of the M<sup>+</sup> 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<sup>+</sup> 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 [<sup>3</sup>H]GA<sub>1</sub> and [<sup>3</sup>H]GA<sub>20</sub> in Vernalized and Nonvernalized Plants

#### *Application of [<sup>3</sup>H]GAs*

Metabolism of [<sup>3</sup>H]GAs were examined in two separate experiments. In one experiment, 2.1 KBq of either [1,2-<sup>3</sup>H]GA<sub>1</sub> (from Amersham; specific activity: about 1.2 TBq per mmol) or [2,3-<sup>3</sup>H]GA<sub>20</sub> (from Professor RP Pharis,

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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-<sup>3</sup>H]GA<sub>1</sub> or [2,3-<sup>3</sup>H]GA<sub>20</sub> 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 [<sup>3</sup>H]GAs to putative conjugated [<sup>3</sup>H]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 N<sub>2</sub>, aliquots of each sample were

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analyzed by reversed-phase C<sub>18</sub> HPLC (as previously described) to determine the relative distributions of the [<sup>3</sup>H]GA precursor <sup>3</sup>H-metabolites. Tentative identification of <sup>3</sup>H-metabolites was based on comparison of chromatographic Rts of authentic [<sup>3</sup>H]GA standards and Rts described by Koshioka *et al.* (1983). Further, previous identification of [<sup>2</sup>H]GA metabolites has already been made following similar feeds to *Brassica* (Rood *et al.*, 1990a). Metabolite recovery was expressed as percent of total recovered <sup>3</sup>H.

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 C<sub>18</sub>  $\mu$ Bondapak reversed-phase high pressure liquid chromatography.

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

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## 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). GA<sub>1</sub> and its biosynthetic precursors, GA<sub>20</sub> and GA<sub>19</sub>, were extracted, chromatographically purified, and quantified by gas-chromatography-selected ion monitoring (GC-SIM), using [<sup>2</sup>H<sub>2</sub>]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. GA<sub>1</sub> concentrations also increased acropetally along the plant axis in reproductive plants. During early silique filling, GA<sub>1</sub> concentrations were highest in siliques and progressively lower in flowers, inflorescence stalks (peduncles plus pedicels), stems, leaves, and roots. Concentrations of GA<sub>19</sub> and GA<sub>20</sub> showed similar patterns of distribution except in leaves, in which concentrations were higher, but variable. Immature siliques were qualitatively rich in endogenous GAs and GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>4</sub>, GA<sub>8</sub>, GA<sub>9</sub>, GA<sub>17</sub>, GA<sub>19</sub>, GA<sub>20</sub>, GA<sub>24</sub>, GA<sub>29</sub>, GA<sub>34</sub>, GA<sub>51</sub> and GA<sub>53</sub> were identified by GC-SIM. In whole siliques, GA<sub>19</sub>, GA<sub>20</sub>, GA<sub>1</sub> and GA<sub>8</sub> 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.

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## 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 GA<sub>1</sub> 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 GA<sub>1</sub> and two of its biosynthetic precursors, GA<sub>20</sub> and GA<sub>19</sub>. Additionally, the major endogenous GAs in the GA-rich immature siliques (including seeds) were investigated.

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## 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.

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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-<sup>2</sup>H<sub>2</sub>] GA<sub>19</sub>, [17,17-<sup>2</sup>H<sub>2</sub>] GA<sub>20</sub>, [17,17-<sup>2</sup>H<sub>2</sub>]GA<sub>1</sub>, and [17,17-<sup>2</sup>H<sub>2</sub>]GA<sub>8</sub> 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 (SiO<sub>2</sub>) partition chromatography followed by reversed-phase C<sub>18</sub> 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 SiO<sub>2</sub> and C<sub>18</sub> 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<sub>5,7,12,13,25,27,36,44,70,77,85</sub> and 2β-OH GA<sub>53</sub>, 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.

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## Results and Discussion

### *GA Distribution in Vegetative Seedlings*

GA<sub>1</sub> 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 GA<sub>1</sub> concentrations, while stem concentrations were somewhat lower. Thus, within the plant axis, GA<sub>1</sub> concentration increased acropetally. A similar axial distribution of GA<sub>1</sub> has been recently reported for *Pisum* (Smith *et al.*, 1992).

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

Two immediate metabolic precursors of GA<sub>1</sub>, GA<sub>20</sub> and GA<sub>19</sub>, were also most concentrated in shoot apical regions and progressively less concentrated in stems and roots (Fig. 3-1). Although GA<sub>20</sub> and GA<sub>19</sub> concentrations were both consistently lower than GA<sub>1</sub>, GA<sub>19</sub> concentrations were greater than GA<sub>20</sub> concentrations in the plant axis. However, unlike the high concentrations of GA<sub>20</sub> that have been reported in the upper leaves of pea (Smith *et al.*, 1992), concentrations of GA<sub>20</sub> in canola leaves were lower than those of GA<sub>1</sub> (Table 3-1). Additionally, GA<sub>19</sub> 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

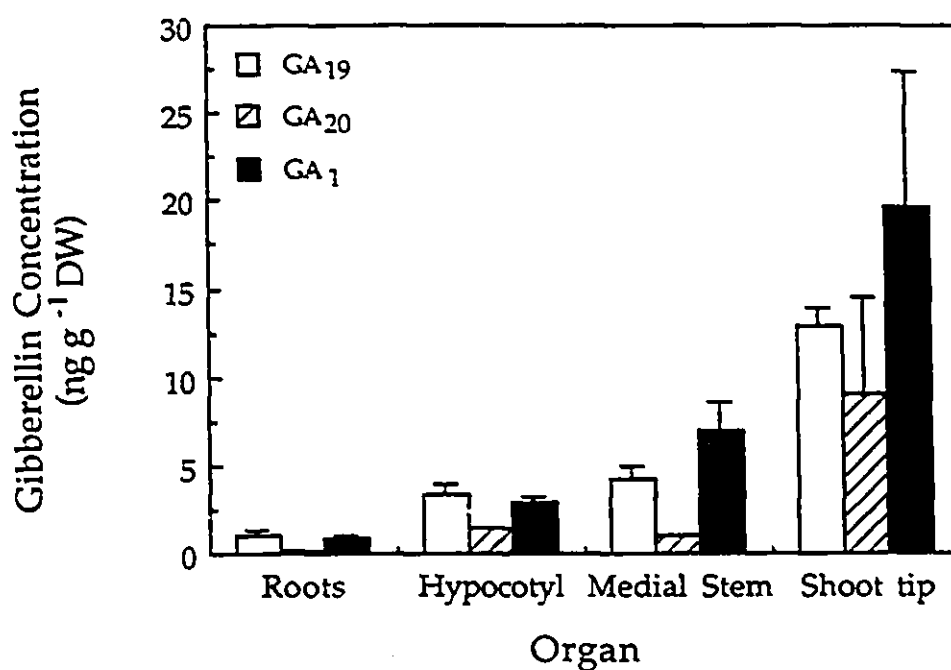


Figure 3-1. Gibberellin concentration ( $\text{ng g}^{-1}$  dry weight) in different organs of 28 day old (vegetative) *Brassica napus* (canola cv. Westar) plants. Values represent the means + s.e. of two replicates.

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 <sup>-1</sup> 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 GA<sub>19</sub>, GA<sub>20</sub>, and GA<sub>1</sub> 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. GA<sub>1</sub> 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, GA<sub>19</sub>, GA<sub>20</sub> and particularly GA<sub>1</sub> concentrations progressively declined with age (Fig. 3-3). Consequently, the concentrations of these free GAs, and particularly free GA<sub>1</sub>, were very low in the mature siliques. García-Martínez *et al.* (1987) also found



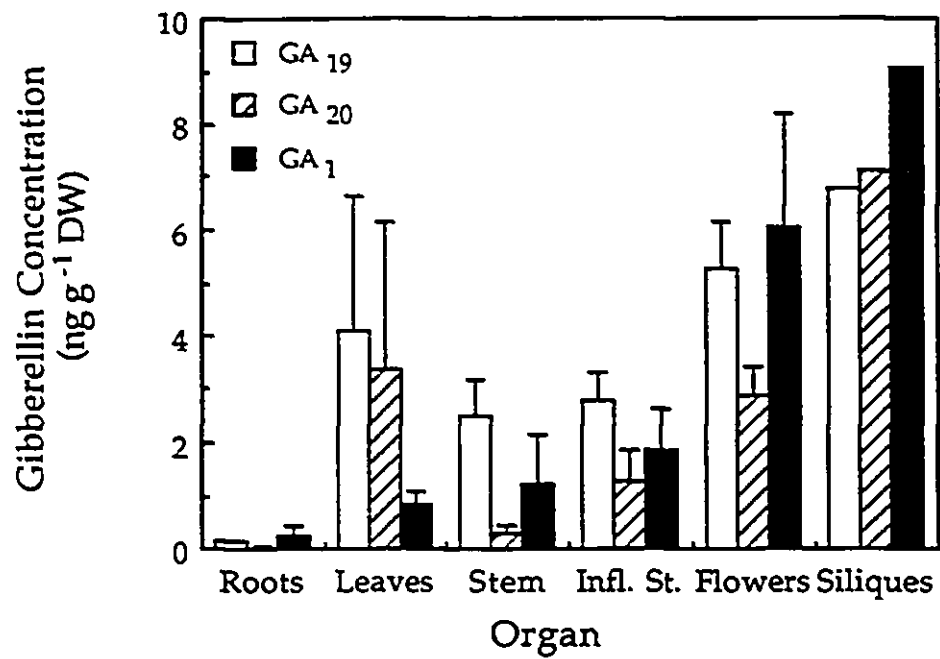


Figure 3-2. Gibberellin concentrations ( $\text{ng g}^{-1}$  dry weight) in different organs of 64 day old *Brassica napus* (canola cv. Westar) plants during early silique-filling. 'Infl. St.' indicates the inflorescence stalk that includes peduncles plus pedicels. Values represent the means of five 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 GA<sub>19</sub>, GA<sub>20</sub>, GA<sub>1</sub> and GA<sub>8</sub> 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. GA<sub>19</sub>, GA<sub>20</sub>, GA<sub>1</sub> and GA<sub>8</sub> concentrations were, respectively, 18.2-, 9.9-, 13.6-, and 2.6-fold higher in immature than mature seeds. The mature seeds contained more GA<sub>20</sub> 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 GA<sub>19</sub>, GA<sub>20</sub> and GA<sub>1</sub> 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.*,

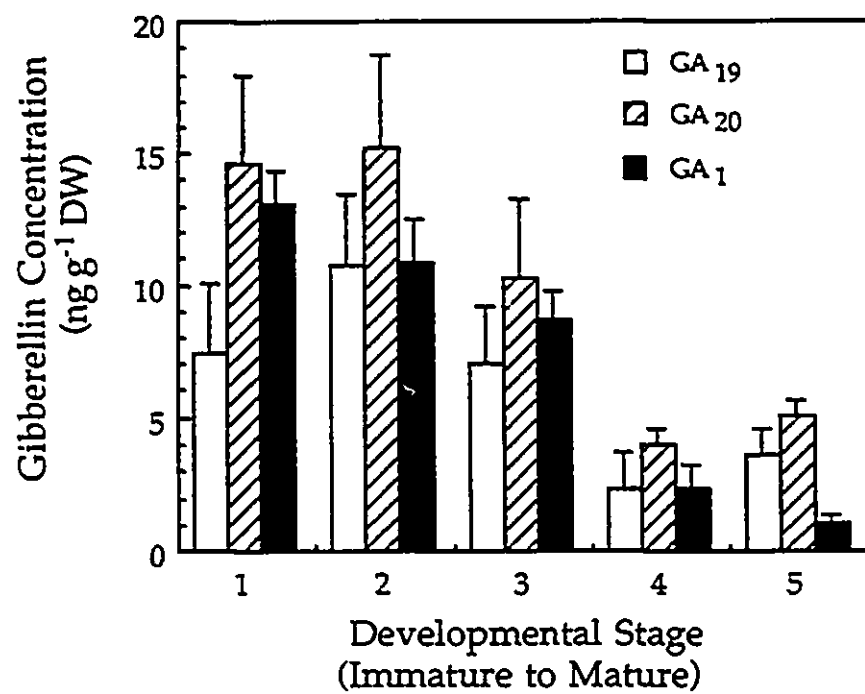


Figure 3-3. Gibberellin concentrations ( $\text{ng g}^{-1}$  dry weight) of *Brassica napus* (canola cv. Westar) siliques at different developmental stages. Values represent means of four replicates + s.e. for tissue harvested from plants grown in field tests at Lethbridge in 1989 and 1990.

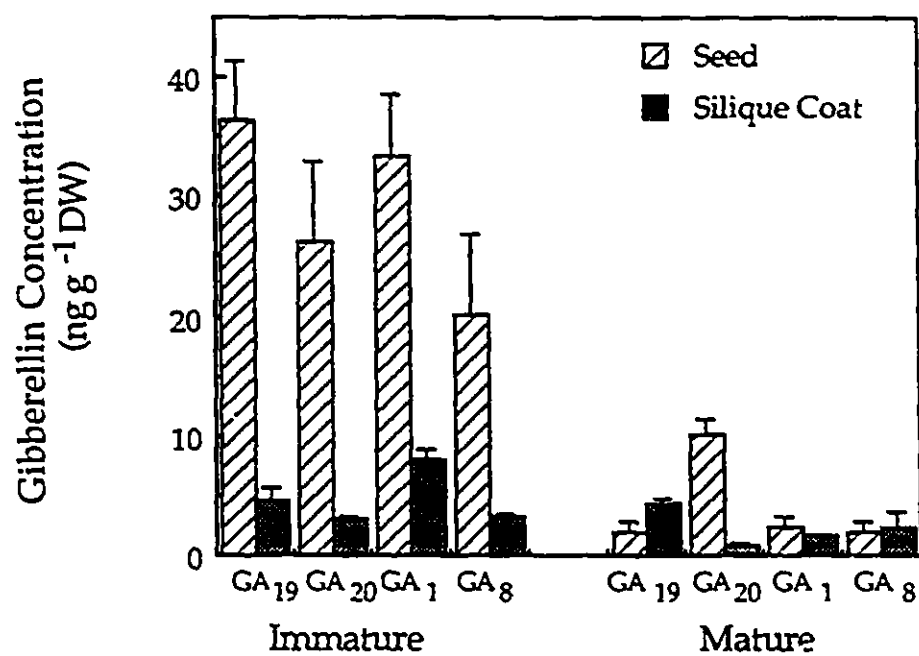


Figure 3-4. Gibberellin concentration ( $\text{ng g}^{-1}$  dry weight) of immature (stage 1) and mature (stage 5) *Brassica napus* (canola cv. Westar) seeds and silique coats. Values represent the means of two to five replicates + s.e. from plants grown in field tests at Lethbridge in 1990.

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

<sup>a</sup> Kovats Retention Index. Samples were analyzed on a DB-5 capillary column.

<sup>b</sup> MeGA

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1989). The three additional GAs, GA<sub>4</sub>, GA<sub>9</sub> and GA<sub>53</sub> 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<sub>5,7,12,13,25,27,36,44,70,77,85</sub> and 2 $\beta$ -OH GA<sub>53</sub>.

Based on the ease of detection by GC-SIM, GA<sub>4</sub>, GA<sub>9</sub>, GA<sub>24</sub>, and GA<sub>51</sub> were apparently most abundant, GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>20</sub>, and GA<sub>34</sub> were intermediate, and GA<sub>8</sub>, GA<sub>17</sub>, GA<sub>19</sub>, GA<sub>29</sub> and GA<sub>53</sub> 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 GA<sub>4</sub>, GA<sub>9</sub>, GA<sub>24</sub>, GA<sub>34</sub> and GA<sub>51</sub>, 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), GA<sub>4</sub>, and its precursor, GA<sub>9</sub>, are abundant in immature siliques. GA<sub>4</sub> 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 GA<sub>1</sub> biosynthesis and in some conditions elevated GA<sub>1</sub> 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

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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 (GA<sub>3</sub>) was applied to the shoot tip of 2-, 4-, 6-week partially-vernalized canola plants to determine whether GA<sub>3</sub>-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 GA<sub>3</sub> 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, GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>4</sub> or GA<sub>5</sub> at concentrations ranging from 10<sup>-3</sup> to 10<sup>-1</sup> 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: GA<sub>3</sub> > GA<sub>1</sub> > GA<sub>4</sub> > GA<sub>5</sub>. 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.

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## 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 GA<sub>3</sub> (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 (GA<sub>3</sub>) 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  $\mu$ l of either a 1.0,  $1.0 \times 10^{-1}$ ,  $1.0 \times 10^{-2}$ , or  $1.0 \times 10^{-3}$  M GA<sub>3</sub> 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  $\mu$ l of  $1.0 \times 10^{-1}$ ,  $1.0 \times 10^{-2}$ ,  $1.0 \times 10^{-3}$  M GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>4</sub>, and GA<sub>5</sub> 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  $\mu$ l of  $1.0 \times 10^{-3}$  M GA<sub>3</sub> 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.

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## 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).



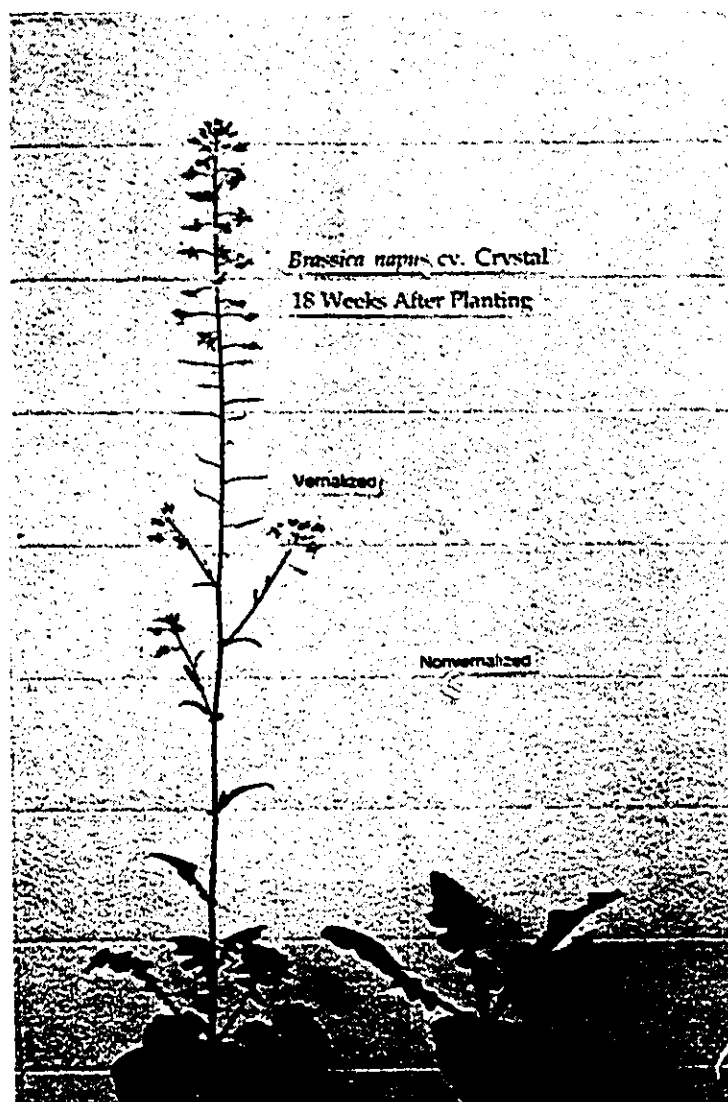


Figure 4-1. Nonvernalized (right) and 8-week vernalized (left) *Brassica napus* cv. Crystal plants, 126 days after planting (42 days post-vernalization).

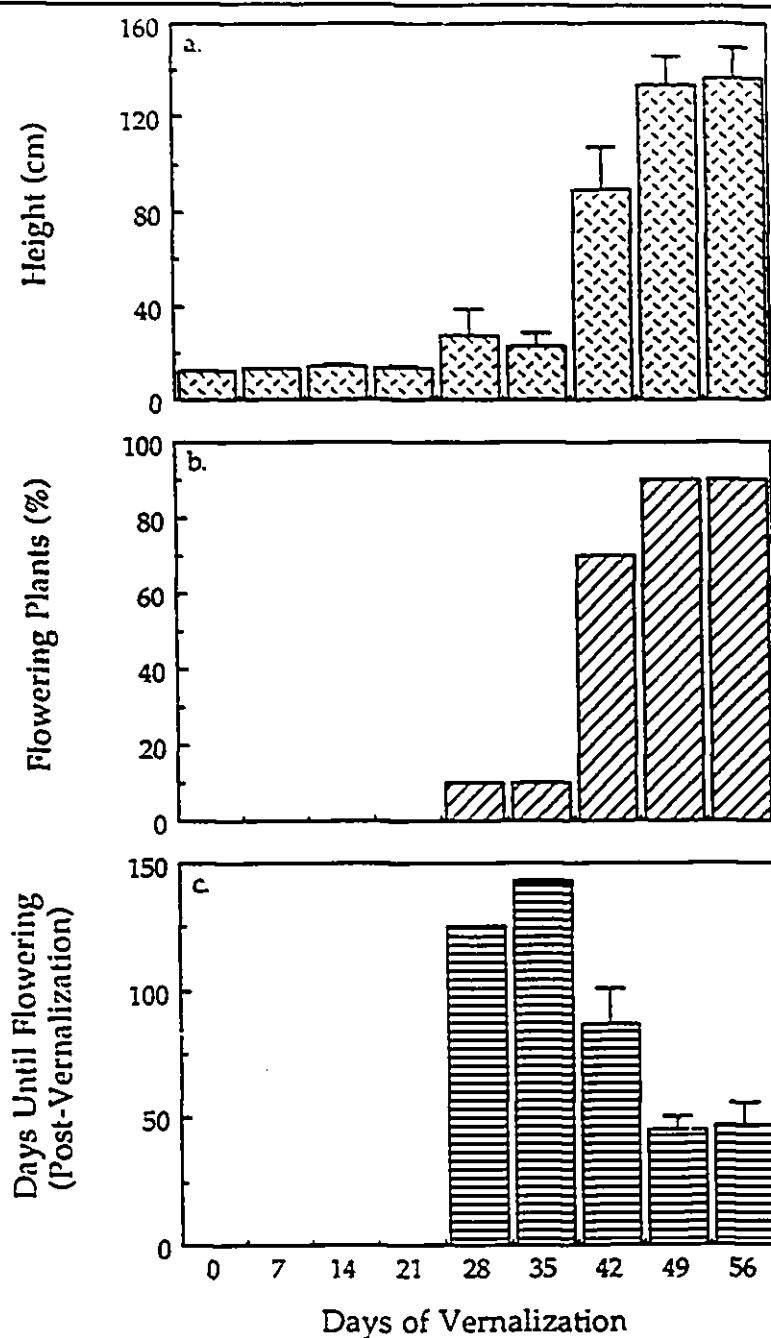


Figure 4-2. Plant elongation (a), percentage of flowering plants (b) and days post-vernalization to reach anthesis (c) in *Brassica napus* cv. Crystal plants exposed to lengths of vernalization at 4°C ranging from 0 to 56 days. Values represent the means + s.e. of ten plants.

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*Effect of GA Application to Nonvernalized Winter Canola*

Application of GA<sub>3</sub> 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 GA<sub>3</sub> on stem elongation is well documented for numerous plants (Phinney, 1985; Wittwer and Bukovac, 1958; Zeevaart, 1983). In some cold-requiring plants, GA<sub>3</sub> 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).

GA<sub>3</sub> 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 GA<sub>3</sub> 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 GA<sub>3</sub> in *Thlaspi* plants vernalized at different temperatures, prompting the suggestion that thermoinduced stem growth may occur in two separate

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processes: initiation and regulation of growth. In another system involving the GA-induced initiation and control of hydrolytic enzyme activity, sensitivity to exogenous GA<sub>3</sub> was increased by pretreating deembryonated seeds with low temperatures prior to GA<sub>3</sub> 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 GA<sub>3</sub>-treated canola plants, GA<sub>3</sub> 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 GA<sub>3</sub>-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 GA<sub>3</sub>. In kohlrabi (*Brassica oleracea*), GA<sub>3</sub> 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, GA<sub>3</sub> 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 GA<sub>3</sub> prior to complete vernalization had

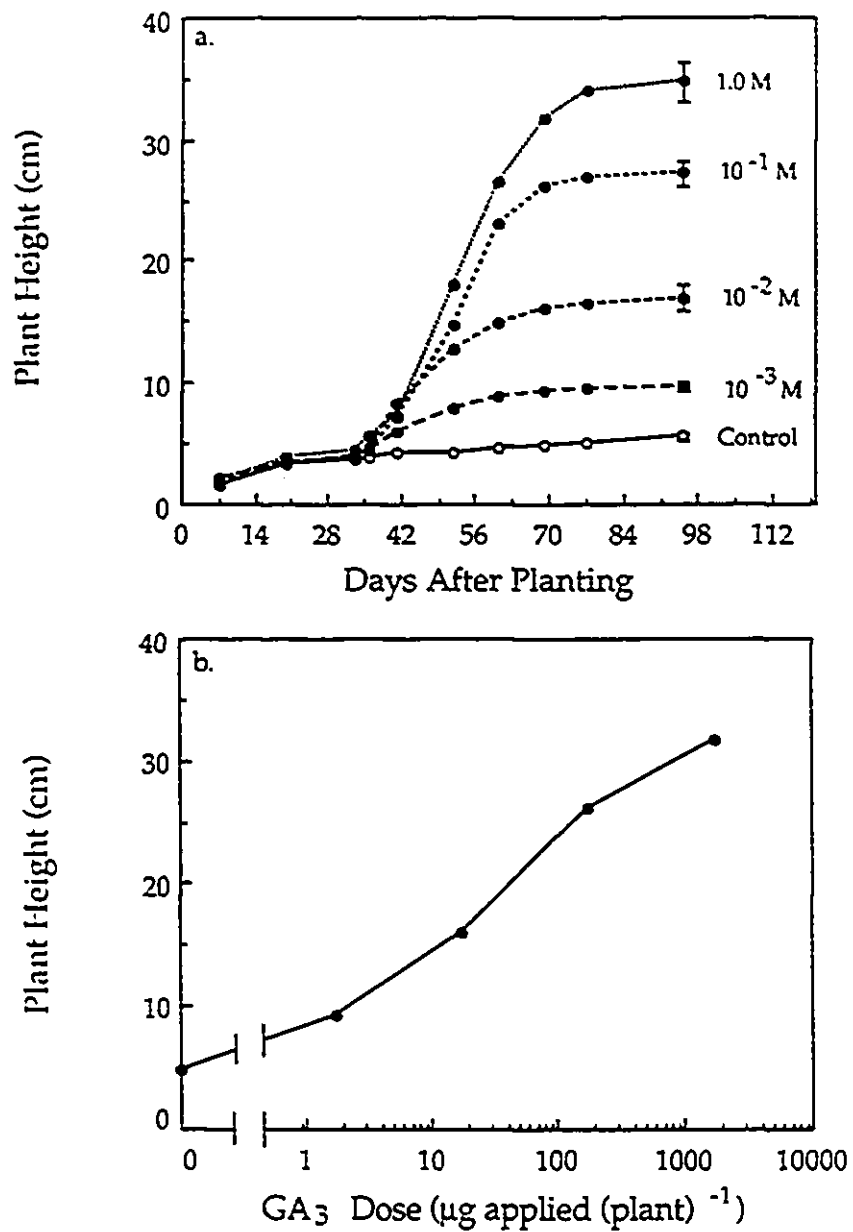


Figure 4-3. (a) Height of nonvernalized *Brassica napus* cv. Crystal plants treated with several concentrations of GA<sub>3</sub>, 28 days after planting. Values represent the means  $\pm$  s.e. of ten plants. (b) Dose-response curve of Crystal nonvernalized plants treated with GA<sub>3</sub>.

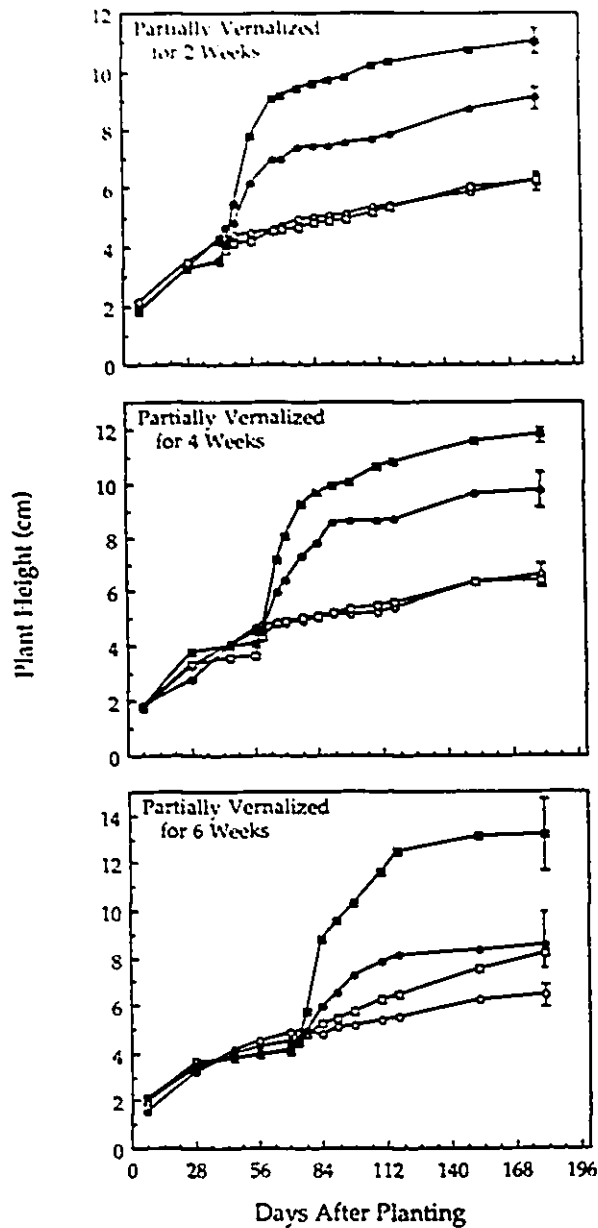


Figure 4-4. Response of 2-, 4-, and 6-week partially-vernallized and nonvernalized *Brassica napus* cv. Crystal plants to exogenous GA<sub>3</sub>. No plants from any of the treatments flowered. Legend: O = Nonvernalized; □ = Partially-vernallized; ● = Nonvernalized + GA<sub>3</sub>; ■ = Partially-vernallized + GA<sub>3</sub>. Values represent the means  $\pm$  s.e. of ten plants.

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accelerated flowering (Leshem and Steiner, 1968). Thus, the timing of GA<sub>3</sub> 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 GA<sub>3</sub> 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, GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>4</sub> and GA<sub>5</sub> 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: GA<sub>3</sub> > GA<sub>1</sub> ≥ GA<sub>4</sub> > GA<sub>5</sub> (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 GA<sub>5</sub> 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 GA<sub>5</sub> or 2,2 di-Me GA<sub>4</sub> were induced to flower. Suge and Rappaport (1968) also demonstrated that GA<sub>3</sub> 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,

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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 GA<sub>4</sub>, although GA<sub>5</sub> was also effective at inducing flowering. In two other cold-requiring plants, *Myosotis* and *Centaureium*, GA<sub>7</sub> and GA<sub>1</sub>, and GA<sub>3</sub>, GA<sub>1</sub>, GA<sub>4</sub>, GA<sub>5</sub> and GA<sub>9</sub>, 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



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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).

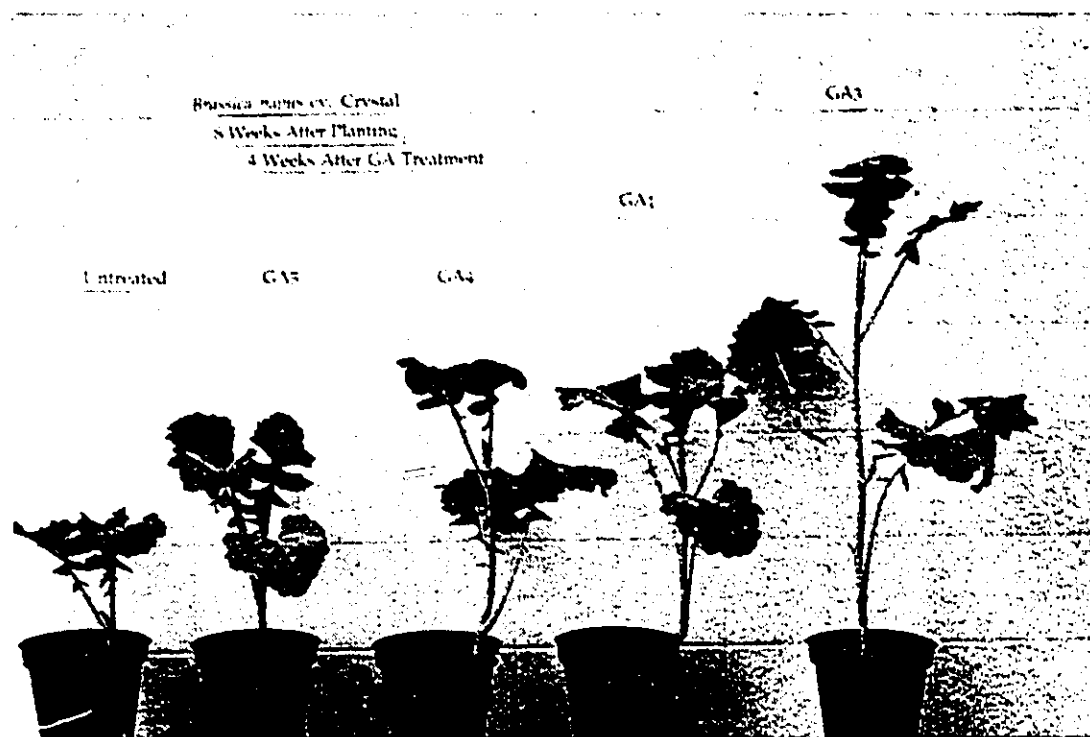


Figure 4-5. Nonvernalized *Brassica napus* cv. Crystal plants, 30 days following GA application (from left to right): untreated,  $10^{-1}$  M GA5, GA4, GA1, and GA3.

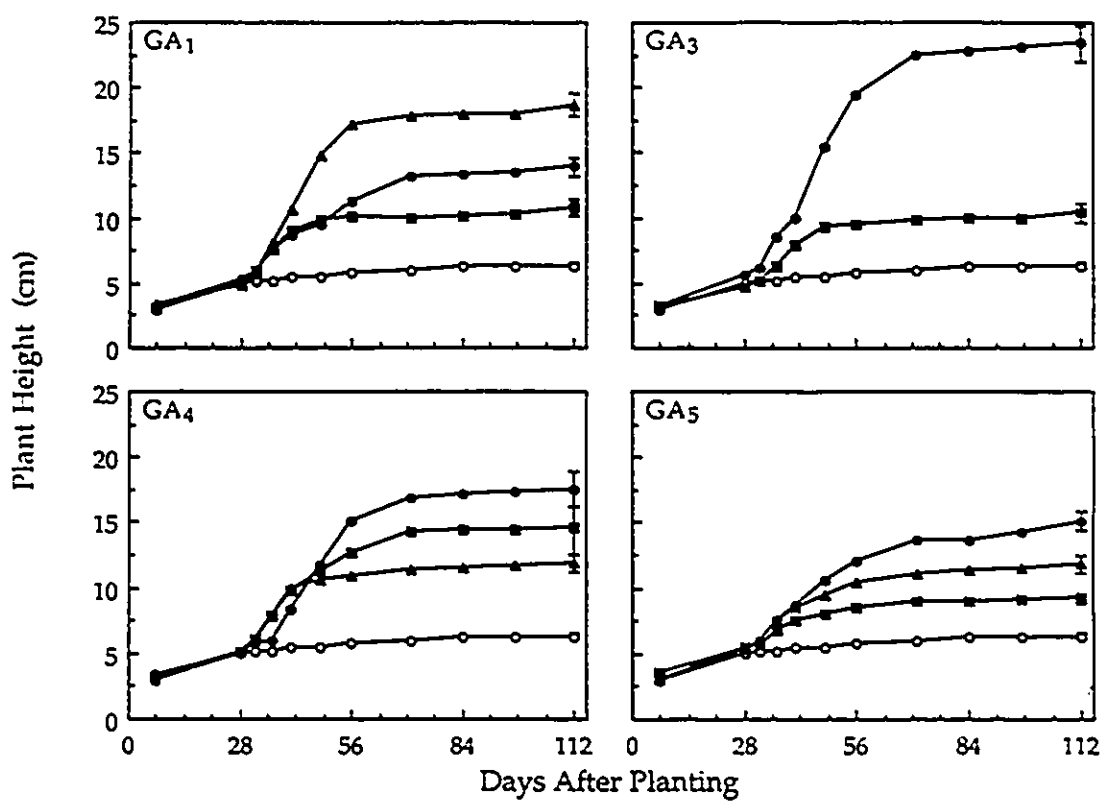


Figure 4-6. Effect of 0 (O), 10<sup>-3</sup> (■), 10<sup>-2</sup> (▲), and 10<sup>-1</sup> (●) M GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>4</sub>, and GA<sub>5</sub> on plant height in nonvernalized *Brassica napus* cv. Crystal plants, 70 days after GA application. None of the plants flowered. Values represent the means  $\pm$  s.e. of ten plants.

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*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 $\beta$ -hydroxylase which catalyzes the formation of GA<sub>1</sub> from GA<sub>20</sub> (Rademacher *et al.*, 1992). Barley plants treated with CGA 163'935 had reduced levels of endogenous GA<sub>1</sub>, while levels of the immediate biosynthetic precursor, GA<sub>20</sub>, were elevated (Adams *et al.*, 1991). Similarly in rice, endogenous GA<sub>1</sub> levels decreased following application of prohexadione, ar. acylcyclohexadione, while levels of precursors such as GA<sub>19</sub>

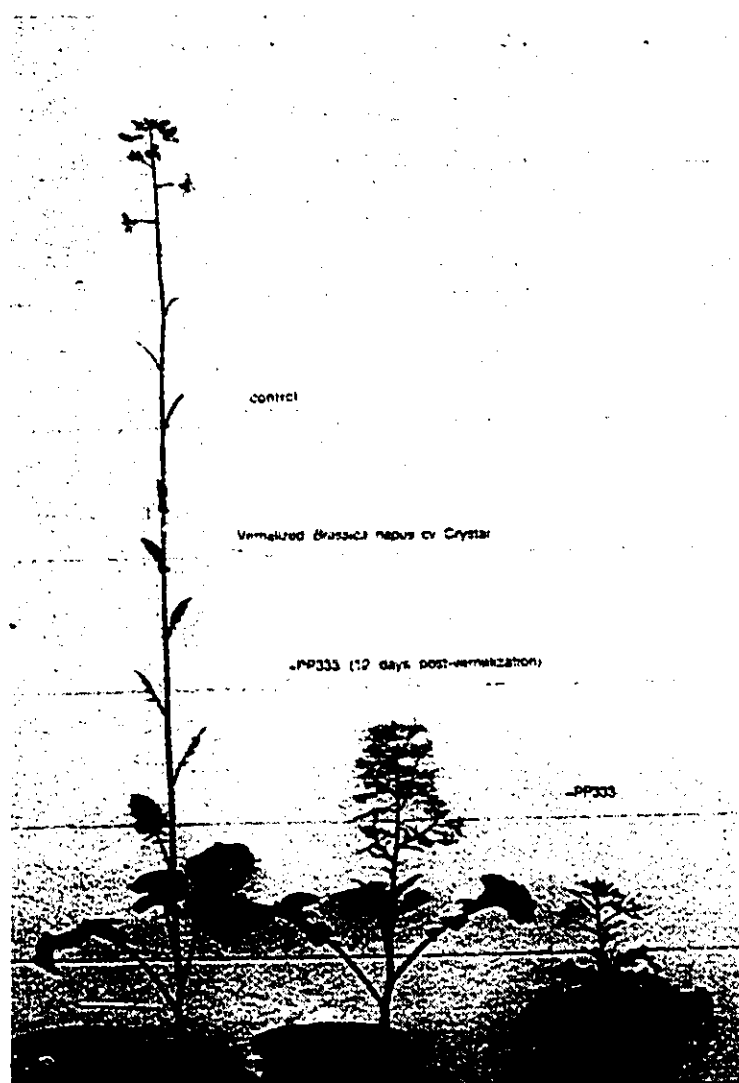


Figure 4-7. Ten week vernalized *Brassica napus* cv. Crystal untreated plants (left), PP333 treated 12 DPV (center) and PP333 treated immediately following vernalization (right), 28 days following PP333 root drench.

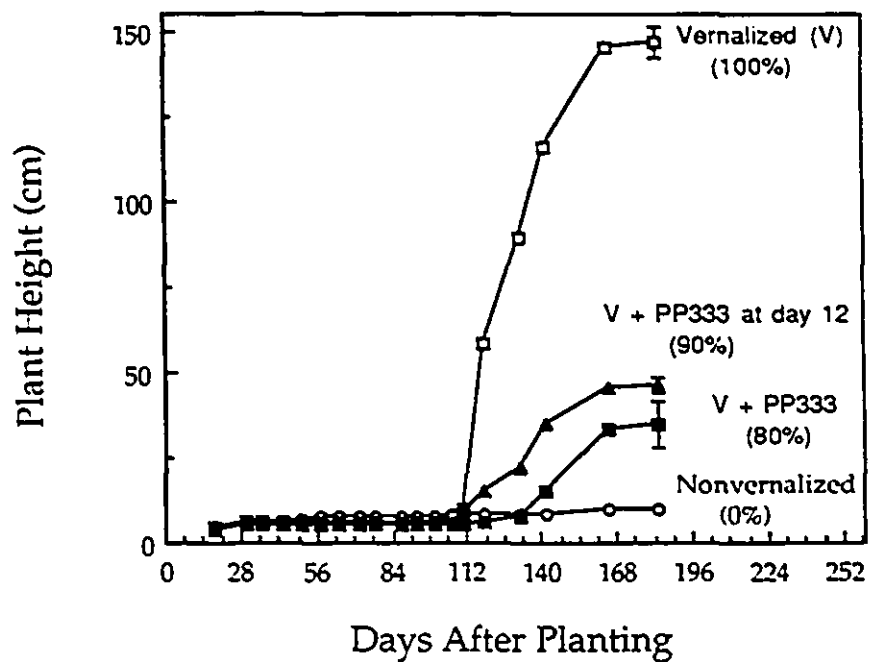


Figure 4-8. Effect of  $10^{-5}$  M paclobutrazol on plant height of 70 day vernalized *Brassica napus* cv. Crystal, 182 days after planting. The percentage of flowering planting 84 days post vernalization is shown on the right in parenthesis. The values represent the means  $\pm$  s.e. of ten plants.

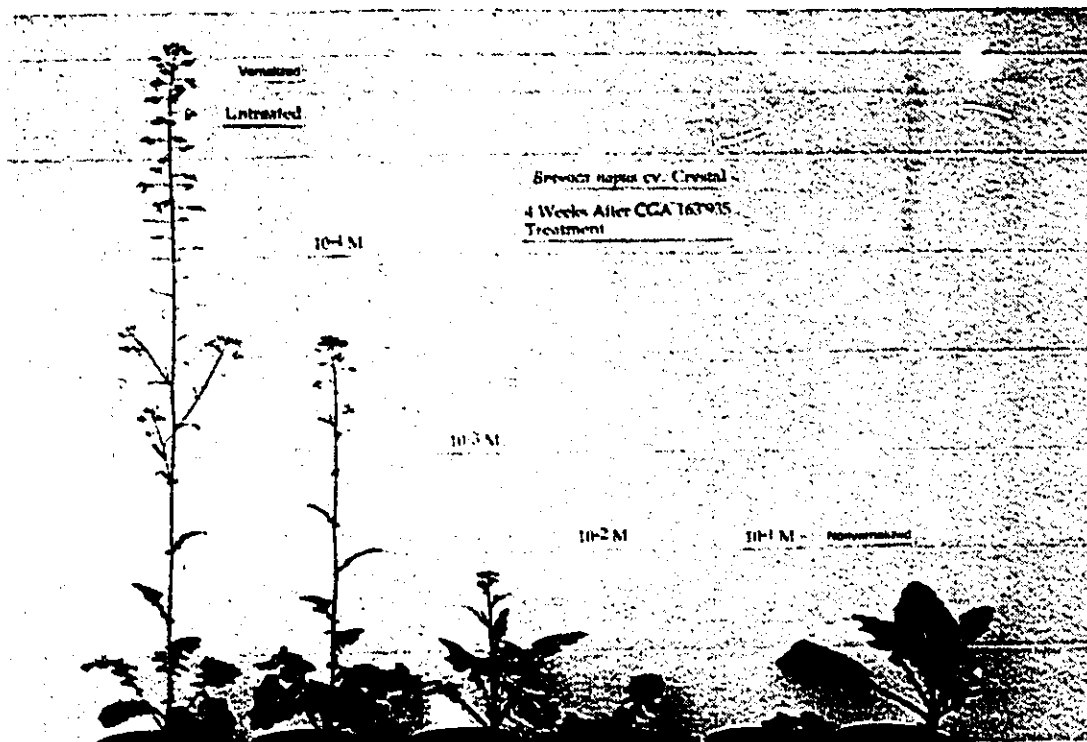


Figure 4-9. Seventy day vernalized *Brassica napus* cv. Crystal plants (from left to right) treated with 0, 10<sup>-4</sup>, 10<sup>-3</sup>, 10<sup>-2</sup>, 10<sup>-1</sup> M CGA 163'935, 28 days following foliar application. The winter canola plant on the extreme right is nonvernalized and untreated.

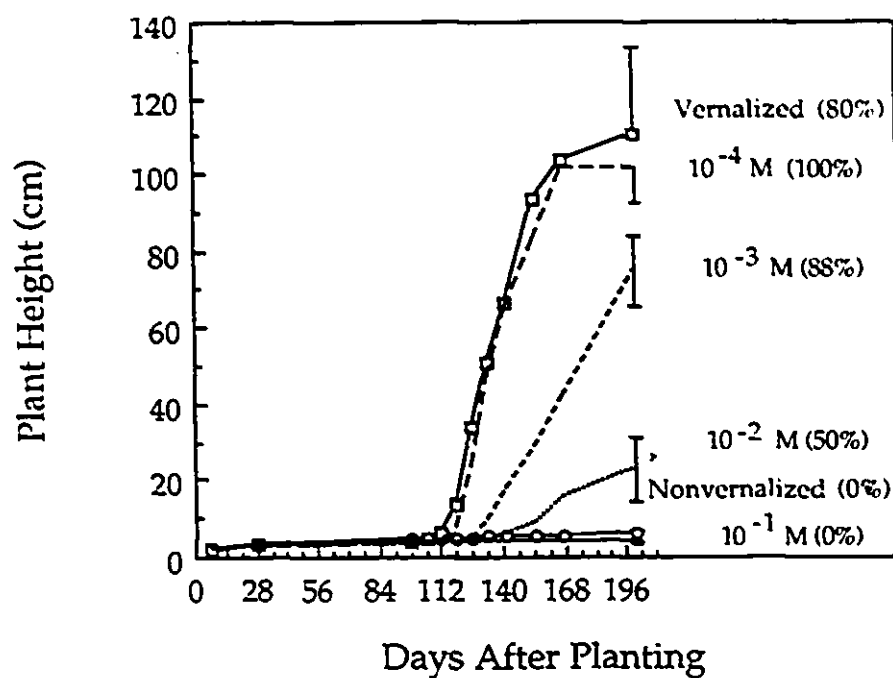


Figure 4-10. Effect of 0,  $10^{-4}$ ,  $10^{-3}$ ,  $10^{-2}$ ,  $10^{-1}$  M CGA 163'935 on plant height of 70 day vernalized *Brassica napus* cv. Crystal, 168 days after planting. Values represent the means  $\pm$  s.e. of nine plants. The percentage of flowering planting 70 days post vernalization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



and GA<sub>20</sub> 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,

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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 GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>4</sub>, and GA<sub>5</sub>. 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

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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*, GA<sub>1</sub> 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).

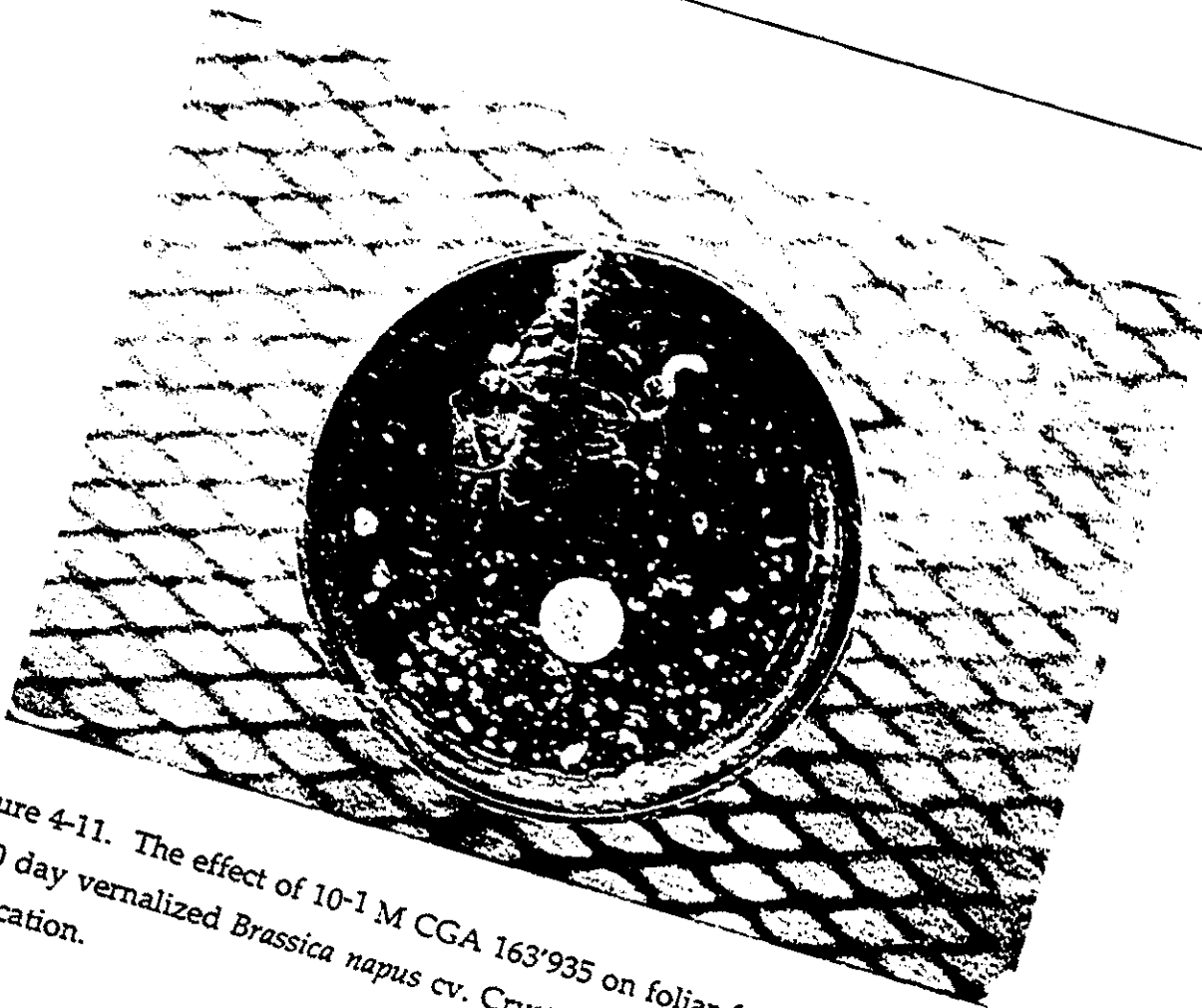


Figure 4-11. The effect of 10<sup>-1</sup> M CGA 163'935 on foliar form and plant height of 70 day vernalized *Brassica napus* cv. Crystal plants, 28 days following foliar application.

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**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 C<sub>18</sub> reversed-phase HPLC and quantified by gas chromatography-mass spectrometry, selected ion monitoring (GC-MS, SIM) using [<sup>2</sup>H<sub>2</sub>]GA<sub>1,3,4,5,8,9,19</sub>, 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<sub>1,3,8,19,20</sub> 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 GA<sub>19</sub> formation. The greatest increases in GA concentrations, including GA<sub>1</sub> and GA<sub>3</sub>, coincided with the onset of stem elongation and floral differentiation, suggesting that these GAs are involved in the regulation of these responses.

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## 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 GA<sub>3</sub> treatment and long-day (LD) photoperiod while those exposed to a short-day (SD) photoperiod and GA<sub>3</sub> 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 [<sup>2</sup>H<sub>2</sub>]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.



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## 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-<sup>2</sup>H<sub>2</sub>]GA<sub>1</sub>, [17-<sup>2</sup>H<sub>2</sub>]GA<sub>3</sub>, [17-<sup>2</sup>H<sub>2</sub>]GA<sub>4</sub>, [17-<sup>2</sup>H<sub>2</sub>]GA<sub>8</sub>, [17-<sup>2</sup>H<sub>2</sub>]GA<sub>9</sub>, [17-<sup>2</sup>H<sub>2</sub>]GA<sub>19</sub>, [17-<sup>2</sup>H<sub>2</sub>]GA<sub>20</sub> 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-<sup>2</sup>H<sub>2</sub>]GA<sub>5</sub> 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)<sup>-1</sup>).

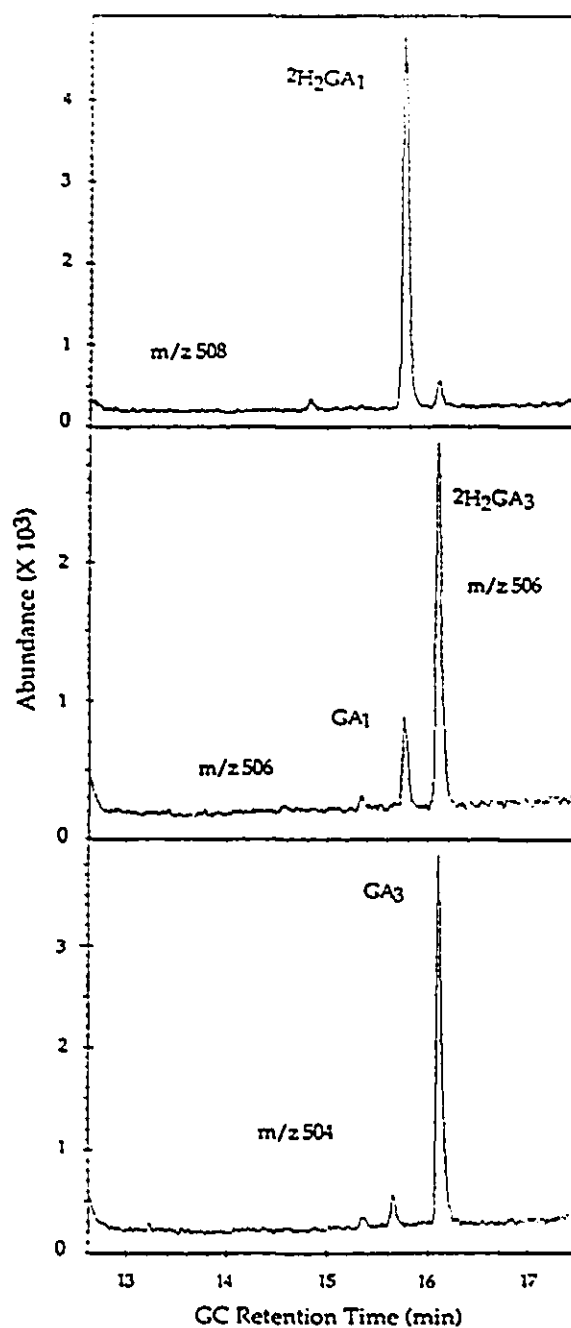


Figure 5-1. Gas chromatography-mass spectrometry selected ion monitoring (GC-MS SIM) profiles of the molecular ions from methyl ester trimethylsilyl ether derivatives of endogenous GA<sub>1</sub> and GA<sub>3</sub> and internal standards of [<sup>2</sup>H<sub>2</sub>] GA<sub>1</sub> and [<sup>2</sup>H<sub>2</sub>]GA<sub>3</sub>.

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## 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 GA<sub>4</sub> 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 GA<sub>3</sub> > GA<sub>19</sub> > GA<sub>1</sub> = GA<sub>8</sub> > GA<sub>20</sub>. 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. GA<sub>1</sub> at 8 DPV, ANOVA,  $df = 1/2$ ,  $F = 37.36$ ,  $p = 0.025$ ) at all harvests (Fig. 5-3). Concentrations of GA<sub>1</sub> and GA<sub>3</sub> were significantly (e.g. GA<sub>3</sub> 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-

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/2.3- (for GA<sub>1</sub>/ GA<sub>3</sub>), 15.8-/9.6-, and 5.2/4.6-fold higher at 0-, 8- and 18-DPV, respectively. Similarly, GA<sub>8</sub> 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 GA<sub>1</sub>, GA<sub>19</sub> and GA<sub>20</sub>, were both significantly increased (e.g. GA<sub>19</sub> 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 GA<sub>1</sub>, 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 GA<sub>19</sub>. Future studies should include analyses of other GAs including GA<sub>44</sub>, GA<sub>53</sub> and GA<sub>12</sub>, 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*

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*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.

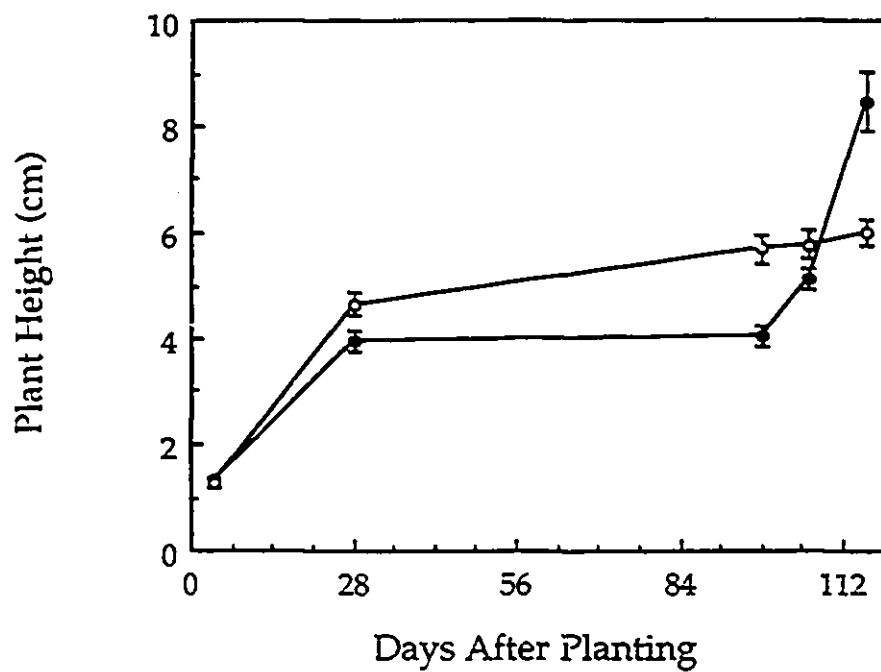


Figure 5-2. Height of *Brassica napus* cv. Crystal vernalized (●) and nonvernalized (○) plants. Plants were vernalized for 70 days from day 28 until day 98.

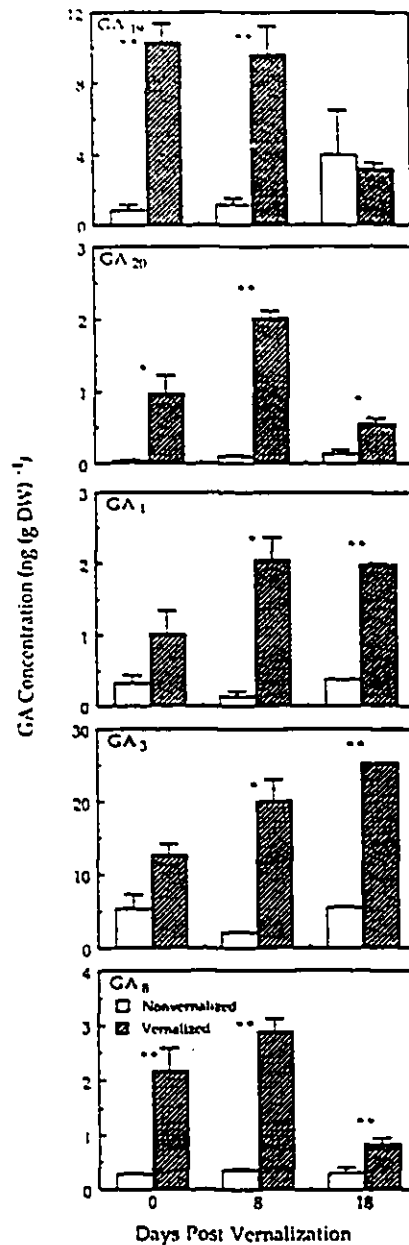


Figure 5-3. The concentrations of GA<sub>19</sub>, GA<sub>20</sub>, GA<sub>1</sub>, GA<sub>3</sub>, and GA<sub>8</sub> in shoot tips from vernalized and nonvernalized *Brassica napus* cv. Crystal plants at 0, 8 and 18 days post-vernialization. The \*, \*\* indicates that values are significantly different 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 [<sup>3</sup>H]GA<sub>1</sub> AND [<sup>3</sup>H]GA<sub>20</sub> 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 [<sup>3</sup>H]GA<sub>20</sub> or [<sup>3</sup>H]GA<sub>1</sub> 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 C<sub>18</sub> reversed-phase high pressure liquid chromatography. Vernalized plants contained significantly higher amounts of <sup>3</sup>H recovered in the EtOAc-soluble fractions following [<sup>3</sup>H]GA<sub>1</sub> or [<sup>3</sup>H]GA<sub>20</sub> feeds. Conversely, nonvernalized plants had significantly higher amounts of <sup>3</sup>H eluted with MeOH, a fraction that probably contained [<sup>3</sup>H]GA glucosyl conjugates. Of the recovered EtOAc-soluble radioactivity, approximately 46% and 56% were associated with the exogenous precursors, [<sup>3</sup>H]GA<sub>1</sub> and [<sup>3</sup>H]GA<sub>20</sub>, respectively. Additional peaks of radioactivity corresponded to the Rts of [<sup>3</sup>H]GA<sub>1</sub> and [<sup>3</sup>H]GA<sub>29</sub> following the [<sup>3</sup>H]GA<sub>20</sub> feed and [<sup>3</sup>H]GA<sub>8</sub> following the [<sup>3</sup>H]GA<sub>1</sub> feed. Although there were no qualitative differences in [<sup>3</sup>H]GA<sub>1</sub> or [<sup>3</sup>H]GA<sub>20</sub> metabolism between vernalized and nonvernalized plants, vernalized plants generally had decreased rates of EtOAc-insoluble <sup>3</sup>H metabolite formation. Furthermore, progressive increases in the formation of [<sup>3</sup>H]GA<sub>1</sub> from [<sup>3</sup>H]GA<sub>20</sub> 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 GA<sub>1</sub> biosynthesis and particularly the 3 $\beta$ -hydroxylation of GA<sub>20</sub> to GA<sub>1</sub> 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 GA<sub>20</sub> are also likely to be responsible for the control of growth and developmental responses following vernalization in winter canola.

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## Introduction

Prior to GA<sub>12</sub>-aldehyde formation, GA biosynthesis appears to be similar in most plants (Graebe, 1987; Sponsel, 1987). However, the conversion from GA<sub>12</sub>-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, GA<sub>1</sub> and two of its precursors, GA<sub>19</sub> and GA<sub>20</sub>, 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 GA<sub>1</sub> 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 [<sup>2</sup>H<sub>2</sub>]GA<sub>19</sub> to [<sup>2</sup>H<sub>2</sub>]GA<sub>20</sub> and [<sup>2</sup>H<sub>2</sub>]GA<sub>1</sub> (Rood *et al.*, 1990a). Furthermore, [<sup>3</sup>H]GA<sub>20</sub> feeds to *ein* result in the apparent formation of [<sup>3</sup>H]GA<sub>1</sub>, [<sup>3</sup>H]GA<sub>29</sub> and [<sup>3</sup>H]GA<sub>8</sub> (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 GA<sub>1</sub> in stem elongation, metabolic studies focussed

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of the metabolism of [ $^3\text{H}$ ]GA<sub>1</sub> and [ $^3\text{H}$ ]GA<sub>20</sub>, GA<sub>20</sub> being the immediate precursor of GA<sub>1</sub>. 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-<sup>3</sup>H]GA<sub>1</sub> (specific activity: about 1.2 TBq per millimole) or [2,3-<sup>3</sup>H]GA<sub>20</sub> (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-<sup>3</sup>H]GA<sub>1</sub> or [2,3-<sup>3</sup>H]GA<sub>20</sub> 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 [<sup>3</sup>H]GA Metabolites*

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

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(Chapter 2). Dried samples were loaded onto glass fiber discs that were placed on a 5 g SiO<sub>2</sub> 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 [<sup>3</sup>H]GA<sub>7</sub>, [<sup>3</sup>H]GA<sub>8</sub>, [<sup>3</sup>H]GA<sub>20</sub>, and [<sup>3</sup>H]GA<sub>29</sub> and 40 ml of MeOH which eluted <sup>3</sup>H-metabolites that were insoluble in the above solvent steps (putative [<sup>3</sup>H]GA conjugates) (see Chapter 2). Fractions were combined into EtOAc- and MeOH-soluble groups and recovery of [<sup>3</sup>H]GAs was determined as per Chapter 2. Subsequently, samples were separated on HPLC and quantitative and qualitative [<sup>3</sup>H]GA metabolite assessments were performed (refer to Chapter 2).

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## Results and Discussion

### *Metabolite Distribution Following Silica Gel Separation*

Following the [ $^3\text{H}$ ]GA<sub>1</sub> and [ $^3\text{H}$ ]GA<sub>20</sub> 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 [ $^3\text{H}$ ]GA-precursor and acidic [ $^3\text{H}$ ]GA metabolites (free [ $^3\text{H}$ ]GAs) from the more polar EtOAc-insoluble  $^3\text{H}$ -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 [ $^3\text{H}$ ]GAs eluted in the 95% EtOAc, and EtOAc-insoluble  $^3\text{H}$ -metabolites were eluted with MeOH. The increased polarity of the EtOAc-insoluble metabolites suggests the presence of [ $^3\text{H}$ ]GA-conjugates and those probably represented glucosyl conjugates (Schneider, 1983).

The distributions of  $^3\text{H}$  in extracts from both the [ $^3\text{H}$ ]GA<sub>1</sub> and [ $^3\text{H}$ ]GA<sub>20</sub> 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 [ $^3\text{H}$ ]GAs between vernalized and nonvernalized winter canola shoots. Following 24 h of metabolism after [ $^3\text{H}$ ]GA<sub>20</sub> application, nonvernalized plant extracts contained 69%, 66%, and 69% EtOAc-soluble  $^3\text{H}$  at 6-, 12- and 15-DPV, respectively, while vernalized plant extracts had 75%, 71% and 75%.

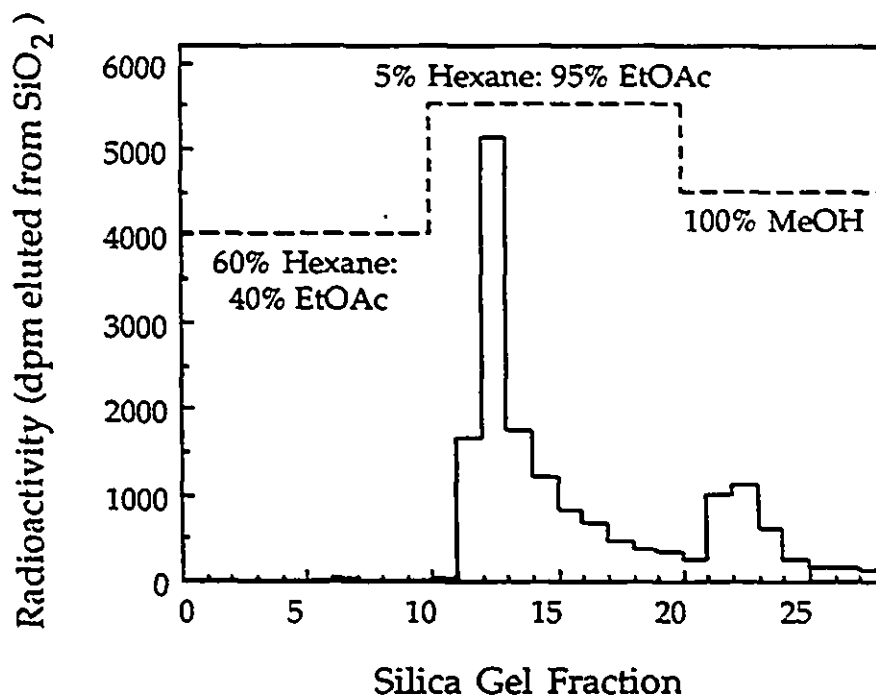


Figure 6-1. Elution of radioactivity from step-elution SiO<sub>2</sub> partition columns loaded with extracts from vernalized *Brassica napus* cv. Crystal plants fed [<sup>3</sup>H]GA<sub>1</sub>.

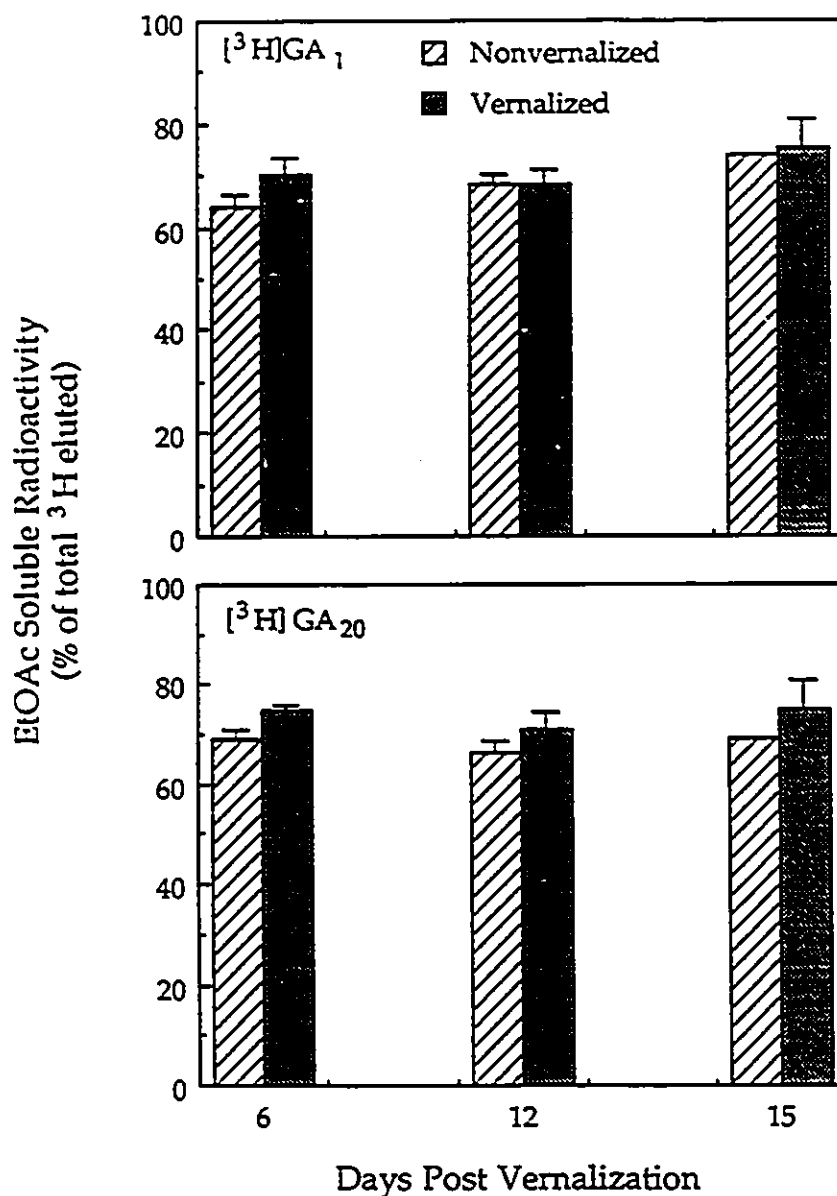


Figure 6-2. Percentage of total radioactivity eluted with EtOAc from step-elution  $\text{SiO}_2$  partition column loaded with extracts from vernalized and nonvernalized *Brassica napus* cv. plants fed  $[^3\text{H}]\text{GA}_1$  (top) and  $[^3\text{H}]\text{GA}_{20}$  (bottom) at 6-, 12-, and 15-days post-vernalization. Values are means  $\pm$  s.e. of 5 (6-, 12-DPV) and 2 (15-DPV) replicates.



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Appreciable amounts of EtOAc-insoluble radioactivity eluted in the MeOH from both vernalized and nonvernalized extracts. Since the relative amounts of EtOAc-soluble  $^3\text{H}$ -metabolites were significantly different and a complementary relationship exists between the amounts of EtOAc-soluble and EtOAc-insoluble  $^3\text{H}$ -metabolites, there was also a significant difference in the amounts of EtOAc-insoluble  $^3\text{H}$ -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  $^3\text{H}$ -conjugate formation.

#### *Metabolite Distribution Following [ $^3\text{H}$ ]GA<sub>20</sub> or [ $^3\text{H}$ ]GA<sub>1</sub> Feeds*

A number of metabolites are commonly produced from GA<sub>20</sub>, including GA<sub>1</sub>, epi-GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>5</sub>, GA<sub>8</sub>, and GA<sub>29</sub> and their glucosyl ester or ether counterparts (Graebe, 1987; MacMillan, 1990). Based on HPLC elution, the principal  $^3\text{H}$ -metabolite formed after [ $^3\text{H}$ ]GA<sub>20</sub> feeds to either vernalized or nonvernalized winter canola plants was [ $^3\text{H}$ ]GA<sub>1</sub> (Fig. 6-3). In vernalized winter canola plants [ $^3\text{H}$ ]GA<sub>20</sub> turnover was increased to produce significantly more [ $^3\text{H}$ ]GA<sub>1</sub> (Table 6-1). At 6-DPV vernalized plants had increased rates of formation of [ $^3\text{H}$ ]GA<sub>1</sub> and the apparent rate of 3 $\beta$ -hydroxylation of [ $^3\text{H}$ ]GA<sub>20</sub> to [ $^3\text{H}$ ]GA<sub>1</sub> 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 [ $^3\text{H}$ ]GA<sub>20</sub> to [ $^3\text{H}$ ]GA<sub>1</sub> was 6.7% to 39.4% higher in the vernalized compared to nonvernalized samples. Thus, the rate of 3 $\beta$ -

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hydroxylation was increased in vernalized in winter canola plants. This increase was determined following feeds of relatively high specific activity [ $^3\text{H}$ ]GAs and hence, induced effects resulting from exogenous GA application should have been minimal. The positioning of  $^3\text{H}$ -label in the A-ring of the gibberellin structure also leads to  $^3\text{H}$  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 GA<sub>20</sub> concentrations. The total turnover from endogenous GA<sub>20</sub> to GA<sub>1</sub> would be the combined product of available GA<sub>20</sub> level and metabolic rate. Thus, the difference in  $3\beta$ -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 [ $^3\text{H}$ ]GAs in either the vernalized or nonvernalized plant tissue, corresponded to the Rt of [ $^3\text{H}$ ]GA<sub>29</sub>, another logical metabolite produced from [ $^3\text{H}$ ]GA<sub>20</sub> (Fig. 6-3). There were no significant differences in the apparent rate of formation of [ $^3\text{H}$ ]GA<sub>29</sub> in vernalized compared to nonvernalized plants (Table 6-1).

Generally, vernalized plants had reduced formation rates of all EtOAc-insoluble  $^3\text{H}$ -metabolites. There were significant differences in the amounts of EtOAc-insoluble  $^3\text{H}$ -metabolites produced following [ $^3\text{H}$ ]GA<sub>20</sub> feeds at 6-DPV (Table 6-1; e.g. GA<sub>1</sub>-conjugate at 6-DPV, ANOVA,  $df = 1/8$ ,  $F = 5.717$ ,  $p = 0.044$ ). The largest  $^3\text{H}$ -EtOAc insoluble metabolite peak was near the Rt of authentic [ $^3\text{H}$ ]GA<sub>20</sub>, the intermediate peak was near [ $^3\text{H}$ ]GA<sub>1</sub>, and smallest peak was near [ $^3\text{H}$ ]GA<sub>29</sub>. These peaks probably represent GA glucosyl

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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 [ $^3\text{H}$ ]GA<sub>20</sub> and more [ $^3\text{H}$ ]GA<sub>20</sub> and [ $^3\text{H}$ ]GA<sub>1</sub> 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 [<sup>3</sup>H]GA<sub>20</sub> 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 C<sub>18</sub> HPLC column. The eluant was MeOH:H<sub>2</sub>O 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>					
	GA <sub>20</sub>	EtOAc Fraction		MeOH Fraction		
		GA <sub>1</sub>	GA <sub>29</sub>	GA <sub>20</sub>	GA <sub>1</sub>	GA <sub>29</sub>
<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).

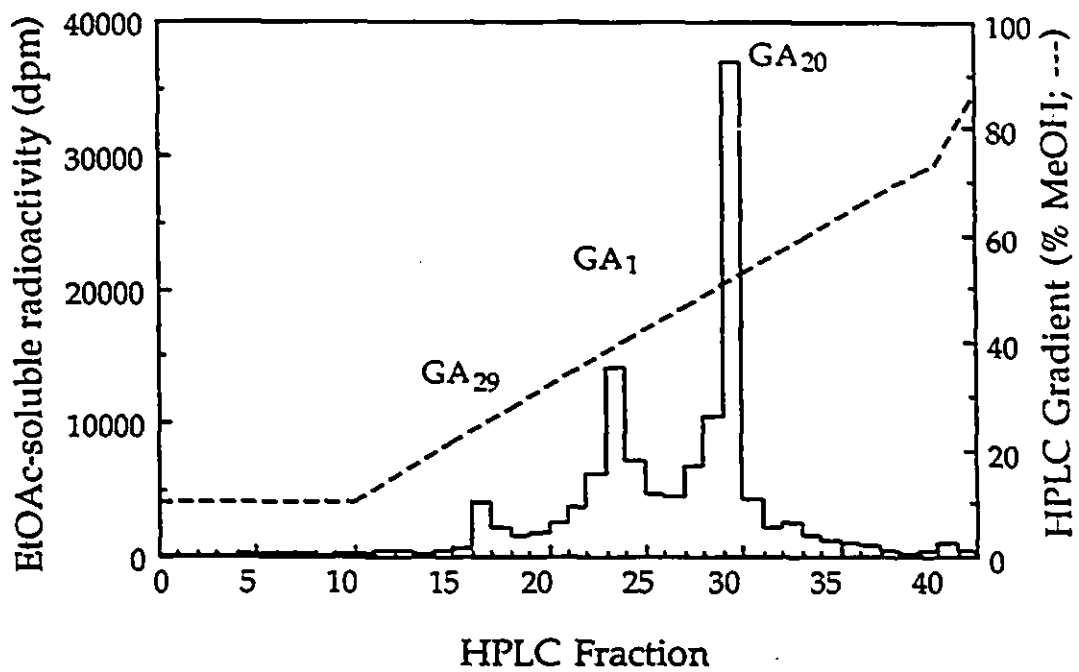


Figure 6-3. Elution of radioactivity from reversed-phase C18 HPLC column loaded with EtOAc-soluble fractions following a feed of [ $^3\text{H}$ ]GA<sub>20</sub> to vernalized *Brassica napus* cv. Crystal plants 12-days post-vernalization.

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The sole  $^3\text{H}$ -labelled EtOAc-soluble metabolite indicated by  $\text{C}_{18}$  reversed-phase HPLC from either nonvernalized or vernalized winter canola plants fed  $^3\text{H}$ GA<sub>1</sub> was  $^3\text{H}$ GA<sub>8</sub> (Figure 6-4). However, there were no significant differences in the rates of the  $2\beta$ -hydroxylation conversion of the  $^3\text{H}$ GA<sub>1</sub> to  $^3\text{H}$ GA<sub>8</sub> in vernalized compared to nonvernalized plants (Table 6-2). Two peaks of  $^3\text{H}$ -metabolites were detected from the EtOAc-insoluble  $\text{SiO}_2$  fractions. The larger peaks had an  $R_t$  that was near the  $R_t$  of authentic  $^3\text{H}$ GA<sub>1</sub>, while the other peak had a  $R_t$  that was similar to that of authentic  $^3\text{H}$ GA<sub>8</sub>, suggesting that these two metabolites were glucosyl conjugates of  $^3\text{H}$ GA<sub>1</sub> and  $^3\text{H}$ GA<sub>8</sub>, 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  $2\beta$ -hydroxylation is not substantially influenced by vernalization, nor is the apparent rate of GA<sub>1</sub> or GA<sub>8</sub> 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  $^3\text{H}$ GA<sub>1</sub> and  $^3\text{H}$ GA<sub>20</sub> 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).  $^3\text{H}$ GA<sub>20</sub> can be  $2\beta$ -hydroxylated to form  $^3\text{H}$ GA<sub>29</sub>, but is apparently more abundantly  $3\beta$ -hydroxylated to form  $^3\text{H}$ GA<sub>1</sub>. Subsequently,  $^3\text{H}$ GA<sub>1</sub> is likely  $2\beta$ -hydroxylated to form  $^3\text{H}$ GA<sub>8</sub>. GA conjugates of all of the above metabolites are probably also formed. In other plant systems additional GA<sub>20</sub> metabolites exist, particularly GA<sub>3</sub> via a GA<sub>5</sub> intermediate (MacMillan, 1990). However, the formation of  $^2\text{H}_2$ GA<sub>3</sub> from  $^2\text{H}_2$ GA<sub>20</sub> has not been confirmed in *Brassica* (SB Rood and DW

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Pearce, personal communication).

This study demonstrates increased [ $^3\text{H}$ ]GA<sub>1</sub> biosynthesis and reduced  $^3\text{H}$ -conjugate formation from [ $^3\text{H}$ ]GA<sub>20</sub> in winter canola plants following vernalization. There were no significant differences in conversion rates of [ $^3\text{H}$ ]GA<sub>1</sub> to [ $^3\text{H}$ ]GA<sub>8</sub>. In another cold-requiring crucifer, *Thlaspi arvense*, feeds of [ $^{14}\text{C}$ ]GA<sub>12</sub>-aldehyde showed little difference in metabolism but, [ $^{14}\text{C}$ ]ent-kaurenoic acid (KA) feeds produced significant differences in metabolism suggesting that conversion of KA to GA<sub>12</sub>-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 [ $^{14}\text{C}$ ]ent-kaurene (Moore and Moore, 1991).

The increase in GA<sub>1</sub> 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 GA<sub>1</sub> 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 GA<sub>1</sub> and GA<sub>3</sub> (Chapter 4), suggest that GA<sub>1</sub> biosynthesis may be involved in the events such as stem elongation induced by vernalization. However, since the changes in GA<sub>20</sub> 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 GA<sub>20</sub> to GA<sub>1</sub> 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 GA<sub>20</sub>, the precursor investigated in the present study.



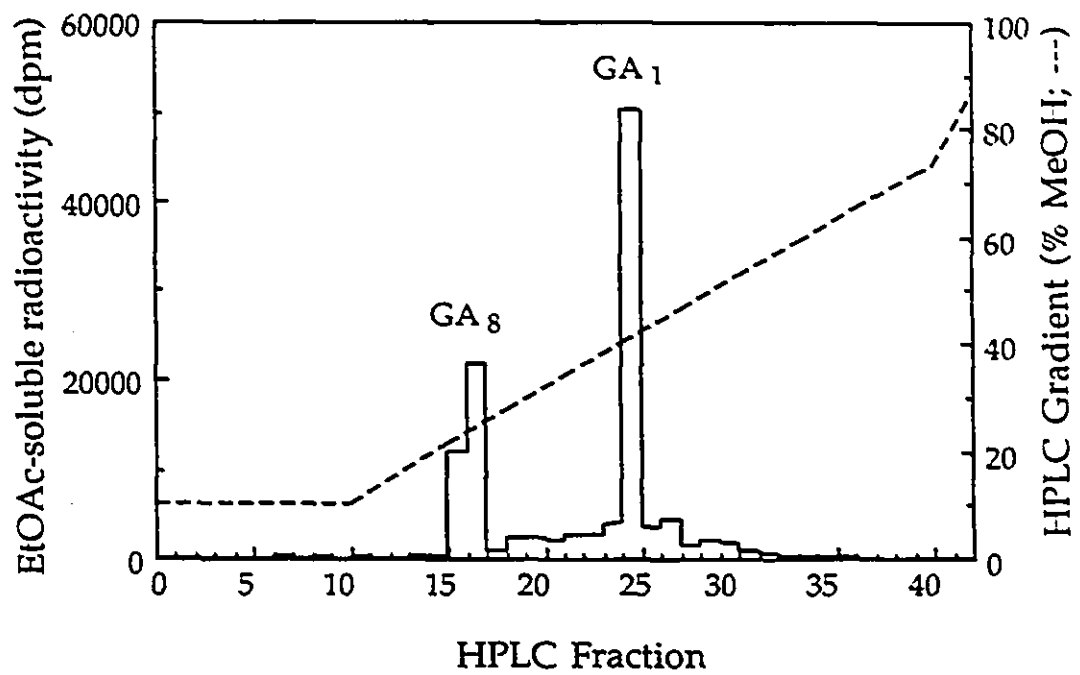


Figure 6-4. Elution of radioactivity from reversed-phase C<sub>18</sub> HPLC column loaded with EtOAc-soluble fractions following a feed of [<sup>3</sup>H]GA<sub>1</sub> to vernalized *Brassica napus* cv. Crystal plants 12-days post-vernalization.

Table 6-2. Distribution of principal metabolites from [ $^3\text{H}$ ]GA<sub>1</sub> 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 C<sub>18</sub> HPLC column. The eluant was MeOH:H<sub>2</sub>O 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 <sub>1</sub>	GA <sub>8</sub>	GA <sub>1</sub>	GA <sub>8</sub>
<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  $^3\text{H}$ -metabolites formed from [ $^3\text{H}$ ]GA<sub>1</sub> or [ $^3\text{H}$ ]GA<sub>20</sub>. 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

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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, GA<sub>1</sub> (and GA<sub>3</sub>), 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<sub>1,3,8,19</sub> and GA<sub>20</sub> 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 GA<sub>19</sub>.

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 GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>4</sub>, and GA<sub>5</sub> 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 GA<sub>1</sub> biosynthesis, CGA 163'935, suggests that GA<sub>1</sub> and probably GA<sub>3</sub> 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, GA<sub>1</sub> and GA<sub>3</sub>, 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 [<sup>3</sup>H]GA<sub>20</sub>. The rate of conversion of [<sup>3</sup>H]GA<sub>20</sub> to [<sup>3</sup>H]GA<sub>1</sub>, endogenous GA<sub>1</sub> being the putative bioeffector of elongation, was slightly greater in the vernalized plants. However, subsequent rates of conversion of [<sup>3</sup>H]GA<sub>1</sub> to [<sup>3</sup>H]GA<sub>8</sub> were similar in both plants. While the slightly faster synthesis of GA<sub>1</sub> 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.

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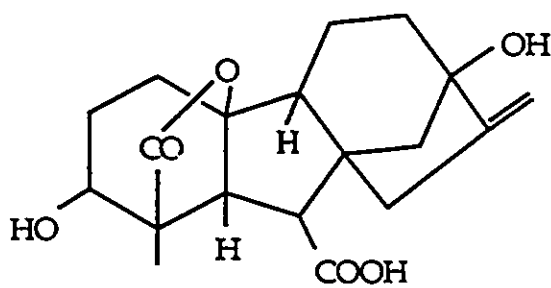
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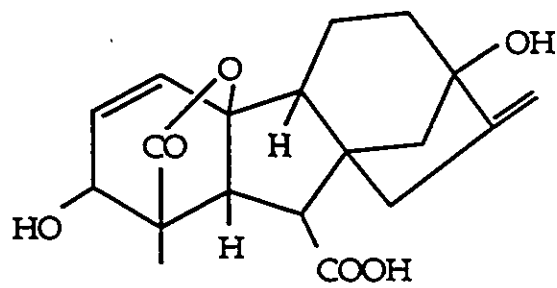
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## APPENDIX 1

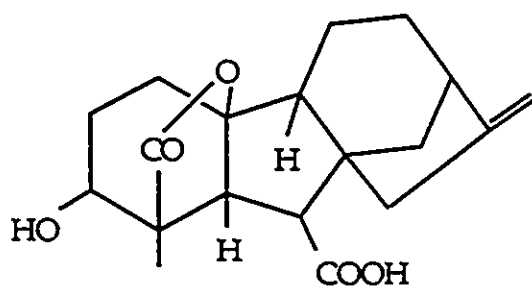
## GIBBERELLIN STRUCTURES



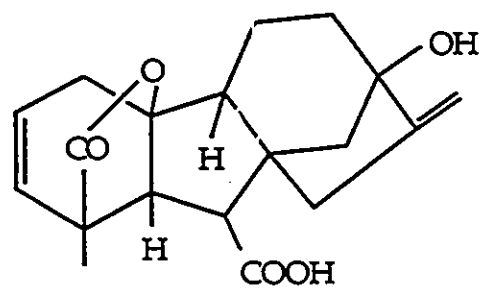
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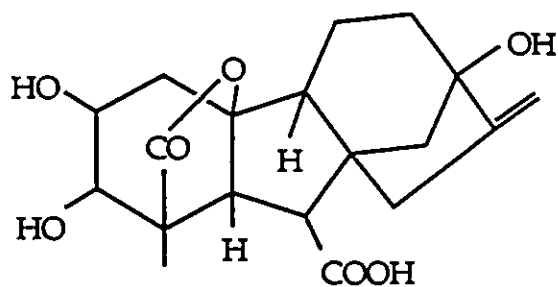
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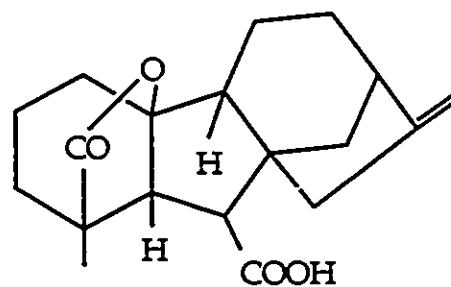
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GA5

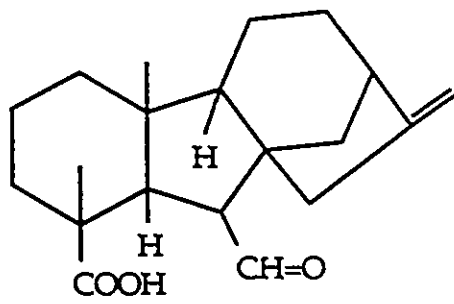


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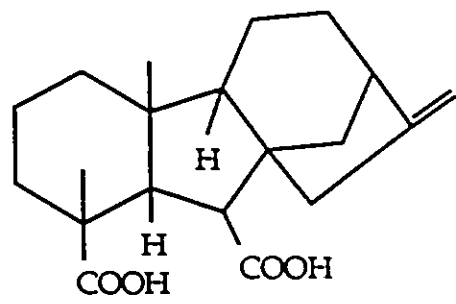


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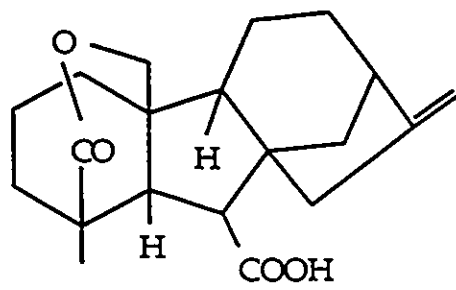




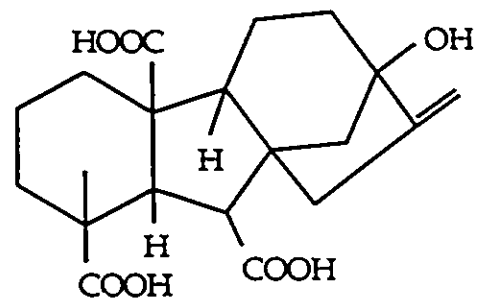
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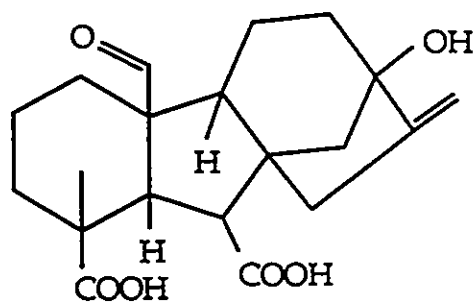
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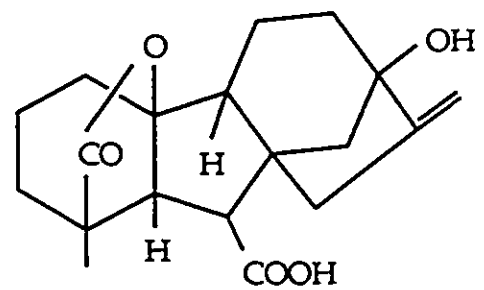
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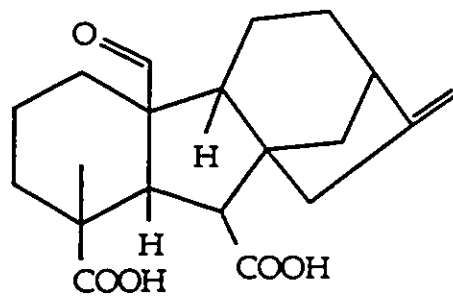
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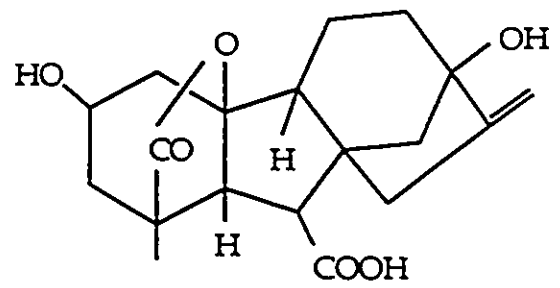
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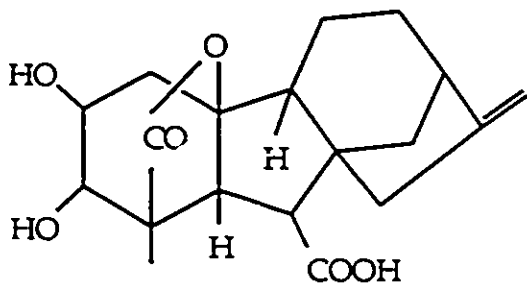
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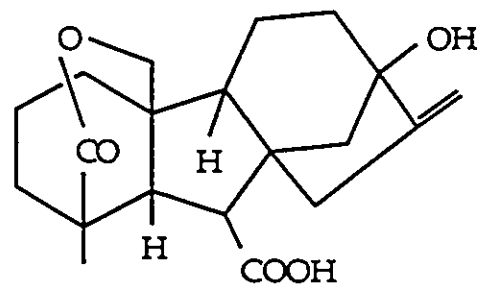
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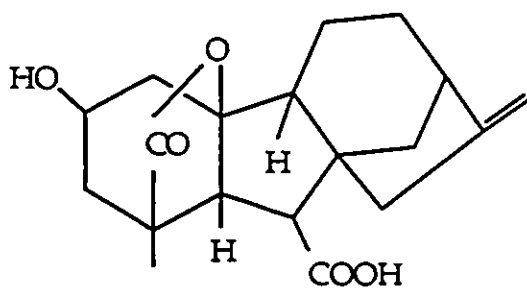
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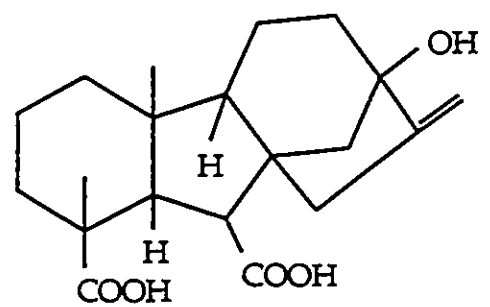
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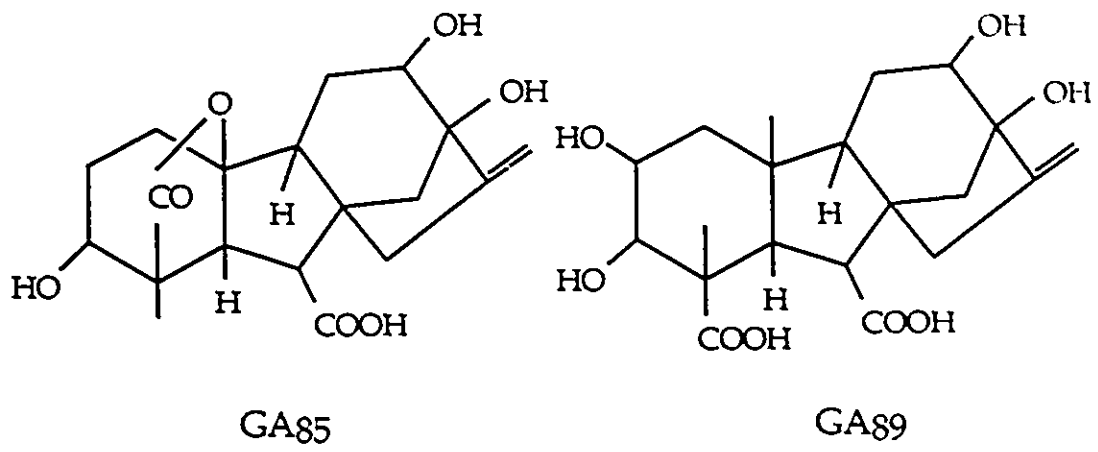
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GA51

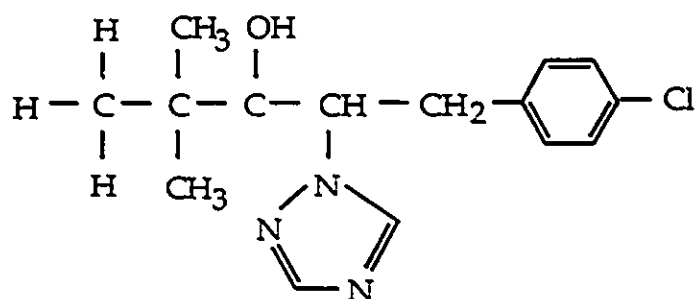


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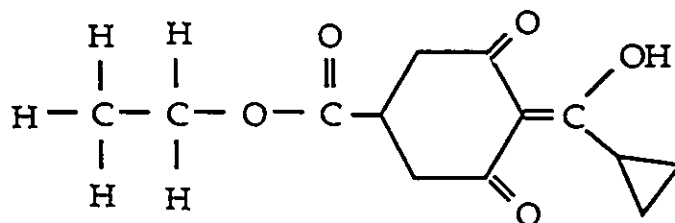


## APPENDIX 2

## PLANT GROWTH RETARDANT STRUCTURES



Paclobutrazol (PP333) - [(2RS, 3RS)-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