REGULATION OF GIBBERELLIN METABOLISM BY ENVIRONMENTAL FACTORS IN *ARABIDOPSIS THALIANA*

DISSERTATION

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By

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ABSTRACT

Environmental factors such as light and temperature play an important role in plant development. The bolting response associated with flowering in *Arabidopsis* and other rosette species is regulated by gibberellin (GA). Presumably there are one or more blocks in the GA biosynthetic pathway that are removed following induction of flowering by either long-days (LD) or vernalization. The objective of this research was to identify steps in biosynthesis regulated by photoperiod and vernalization in *Arabidopsis thaliana* by comparing the biological activities of native GAs and GA precursors under inductive and non-inductive condition.

A T-DNA tagged dominant mutant with a cold requirement line (CR) and a recombinant inbred line selected for a LD requirement (CS933) were used. Quantitative analysis of the content of kaurenoic acid (KA) in these two environmental responsive types when plants were subjected to inductive conditions did not support the hypothesis that the GA biosynthesis pathway is regulated in the conversion of KA to 7-OH KA in *Arabidopsis*.

For 20 carbon GAs, a common feature that distinguished between active and inactive compounds was the oxidation state of C-20. GA precursors and GAs containing a C-20 methyl group (GA_{12}) were not active, while GAs containing a C-20

aldehyde (GA_{24} and GA_{36}) had substantial biological activity when applied to non-induced plants of both response types. This result suggests that the limiting step in GA biosynthesis pathway is located at the step in which the C-20 carbon is oxidized.

In addition, for both 19 and 20 carbon GAs, C13 hydroxylation resulted in reduced biological activity when compared to the non-C13 hydroxylated homologs. These results suggest that 1) under non-inductive conditions C-20 oxidation is the rate-limiting step in the synthesis of bioactive GAs; 2) both vernalization and LD regulate GA biosynthesis at the same control point; 3) the non-C13 hydroxylation pathway leading to GA_4 may be more important in regulating stem growth than the early C13 hydroxylation pathway leading to GA_1.

Dedicated to my parents and my wife.

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FIELDS OF STUDY

Major Field: Horticulture and Crop Science
Studies in Plant Hormone and Plant Physiology

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LITERATURE REVIEW

Gibberellin Overview

The Research History of Gibberellins

Gibberellins (GAs) are an important class of plant hormones that have been extensively studied over the past fifty years. GAs have been shown to have effects on seed germination, stem elongation, flower induction, anther development, and seed growth. The following historical review was based on a review written by Phinney in 1983. Gibberellins were first discovered in the late 1890's. Hori first reported a disease-causing fungus that affected many popular rice cultivars. When infected at an early stage of development, plants generally had less root growth and more elongated narrow leaves. The infected plants were taller than the normal ones and were often infertile. The widespread occurrence of this disease had caused the annual rice production in Japan to drop by more than 40% and imposed serious threats to Japan's economy (Sawada, 1912).

The cause of the so called "bakanae" symptom on rice did not become clear until Sawada (1912) suggested that the stem elongation on rice might be related to the stimulus originating from the mycelium. Kurosawa later confirmed the suggestion in 1926 by applying sterile filtrate of cultured *Gibberella fugjikuroi* to rice and successfully mimicking the "bakanae" symptom. He also found that the disease could be spread via the filtrate to a broad range of crops such as maize, oat and sesame. The term *gibberellin* was first used by Yabuta to describe the substance isolated from fungus culture. Moreover, Yabuta and Takeishi (1936) identified procedures to isolate and purify the gibberellin. In their investigations, they found that applying a purified extract of culture filtrate stimulated seedling elongation of various crops including rice, barley, buckwheat, soybean, gourd, tomato, cucumber, and morning glory (Yabuta and Takeishi, 1936; 1938).

After World War II, scientists from western countries began gibberellin research. In 1955, Stodola et al. reported that a new gibberellin compound, gibberellin-X, was isolated from fermentation of fungus culture. At the same time, a new gibberellin compound called gibberellic acid was also isolated from fungus filtrate by Curtis and Cross (1955). After an exchange of sample between Stodola and Grove, they surprisingly discovered that gibberellin-X and gibberellic acid were identical and named the compound gibberellic acid. It is now known that the gibberellic acid is GA_3.

The structure of gibberellic acid was proposed by Cross et al. (1956) and confirmed by Grove (1961) and Hanson (1968). There were no reports on gibberellin-

like substances found in higher plants until 1956. Radley (1956) purified a gibberellic acid-like substance from pea seedlings. Later a comprehensive research program in gibberellins in higher plants was conducted by Phinney's group (Phinney et al., 1957). According to their results, gibberellin-like substances were found in extracts from nine genera representing seven different families of flowing plants. Finally MacMillan and Suter (1958) reported the identification of gibberellin A_1 (GA_1) from immature seeds of runner bean. These findings from higher plants helped set the foundation for plant physiologists to explore further into the complex world of gibberellin.

Structures and Characteristics of Gibberellins

Gibberellins are a group of diterpenoid acids in which more than 126 members have been identified (Hedden and Phillips, 2001). All gibberellins contain a typical *ent*-gibberellane structure (Fig 1.1). Based on their structures, GAs can be divided into two groups: C-20 GAs and C-19 GAs, which contain either 20-carbons or 19-carbons, respectively (Fig. 1.2). The C-20-GAs are characterized by the presence of carbon-20 that can exist as methyl-, hydroxymethyl-, aldehyde-, or a carboxylic acid. The C-19 GAs, on the other hand, have a carboxylic acid at carbon-19 that bonds to carbon-10 to form a five carbon lactone bridge.

GAs can exist either free or as conjugated forms in nature. Even though there have been numerous kinds of GAs found in higher plants, only a few of them are biologically active in plants. Most of the conjugated GAs are those in which the GA is

linked to glucose with either an ether or ester bond (Rademacher, 1992). These conjugated GAs have little or no biological activity in higher plants.

To be biologically active, GAs must have the following characteristics: 1) an intact gibberellane ring system; 2) a carboxyl group at C-7; and 3) a 5-membered lactone ring in ring A. Other GAs found within plants are precursors or deactivation

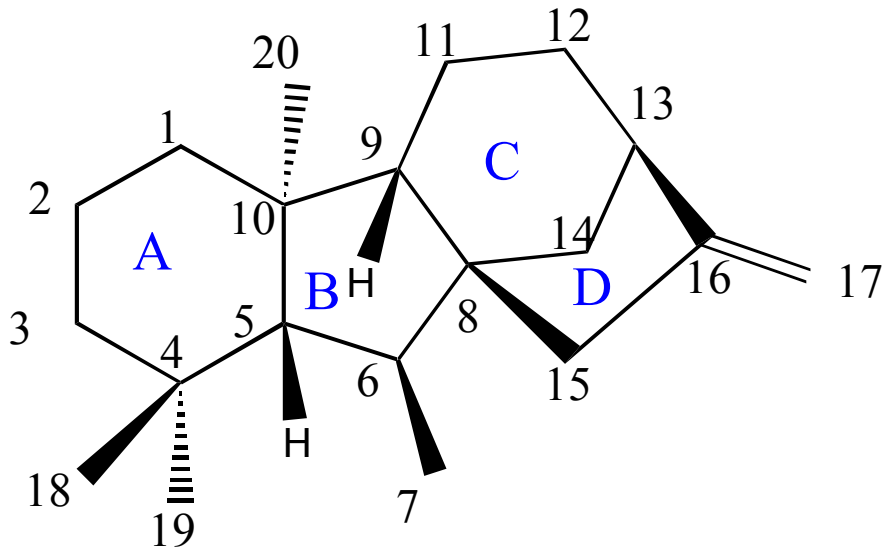


Figure 1.1 The Structure of *ent*-gibberellane. *ent*-Gibberellane has a four-ring structure. Each ring is defined in the order of A, B, C and D, respectively. Various GAs are generated by oxidation or hydroxylation reactions. The degree of the hydroxylation at C-2, 3 and 13 determine the bioactivity of the GA.

products of the active GAs. It is worth noting that single gene dwarf mutant lines and some chemical retardants that inhibit specific metabolic reactions were often used to determine which GA(s) is (are) the active hormone for general plant growth and development.

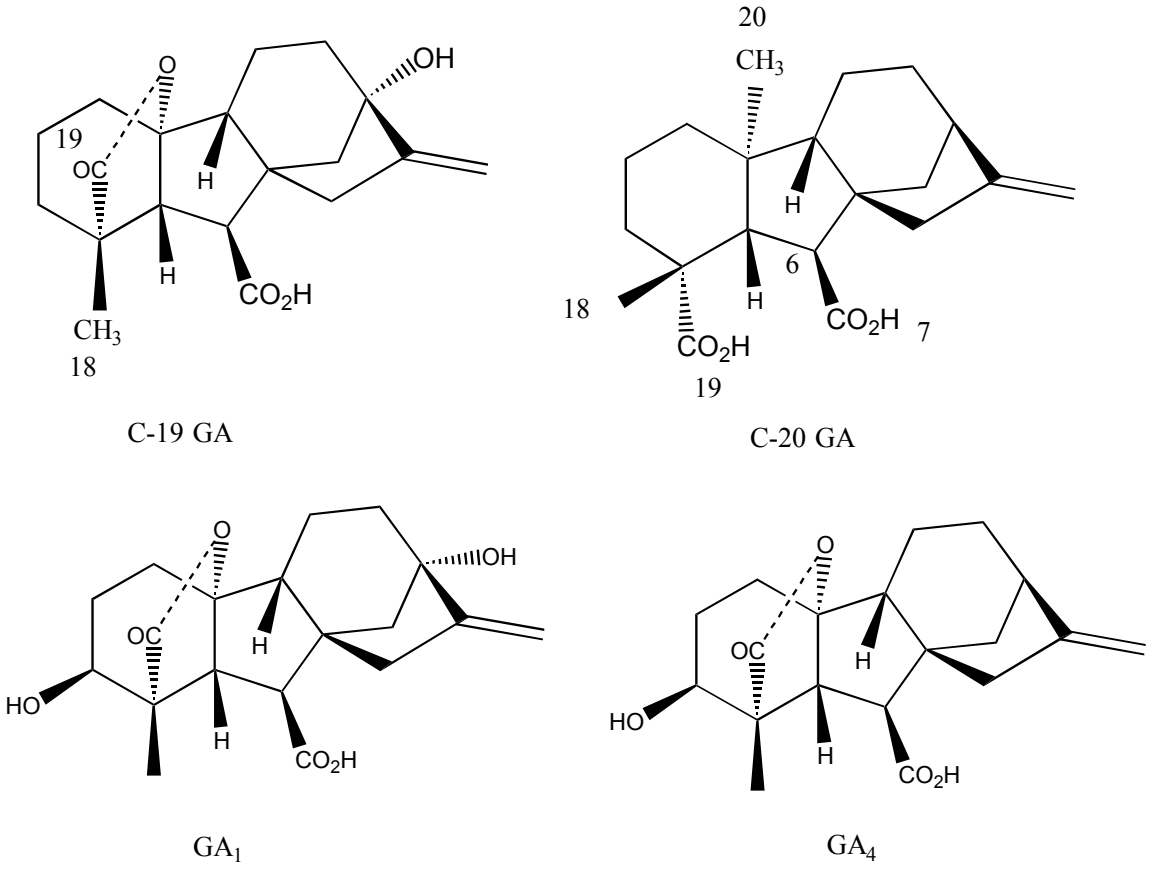


Figure 1.2 Structures of C-20 and C-19 GAs. GA_1 and GA_4, both C-19 GAs, are the two most bioactive form of GAs in plants. C-20 GAs have 20 carbons on the ring structure whereas C-19 GAs lack a carbon at the 20^{th} position in the ring.

Biosynthetic Pathway of GAs

Several materials have been used for the investigation of GA biosynthetic pathways including the fungus *Gibberella fujikuroi* and immature seeds of pumpkin, pea, and maize (Bensen et al., 1995; Blechschmidt et al., 1984; Rojas et al., 2001; Spray et al., 1984). The biosynthetic pathway of GA can be divided into three parts. The biosynthetic pathway of GAs starts with the conversion of geranylgeranyl diphosphate to *ent*-kaurene. *ent*-Kaurene is sequentially oxidized to GA_{12}-aldehyde. All of the various GAs are then derived from the GA_{12}-aldehyde. In general, transformation of mevalonic acid (MVA) to GA_{12}-aldehyde is the same in *G. fujikuroi* and all higher plants.

It has been demonstrated that MVA is the basic precursor of GAs based on feeding experiments using ^{14}C-labeled MVA in fungus culture and liquid endosperm of immature seeds such as cucumber and pumpkin (Birch et al., 1958; 1959; Graebe et al., 1965). MVA is first converted to mevalonate-5-pyrophosphate (MVAPP) by mevalonic kinase (Fig. 1.3). Isopentenyl pyrosphosphate (IPP) is formed as a result of decarboxylation of MVPP, followed by the isomerization of IPP to generate dimethylallyl pyrophosphate (DMAPP), which is the basic unit for terpene biosynthesis. A C20-intermediate geranylgeranyl pyrophosphate (GGPP) is generated condensing two molecules of IPP using GGPP synthetase as a catalyst. GGPP is considered the first compound of all diterpenes.

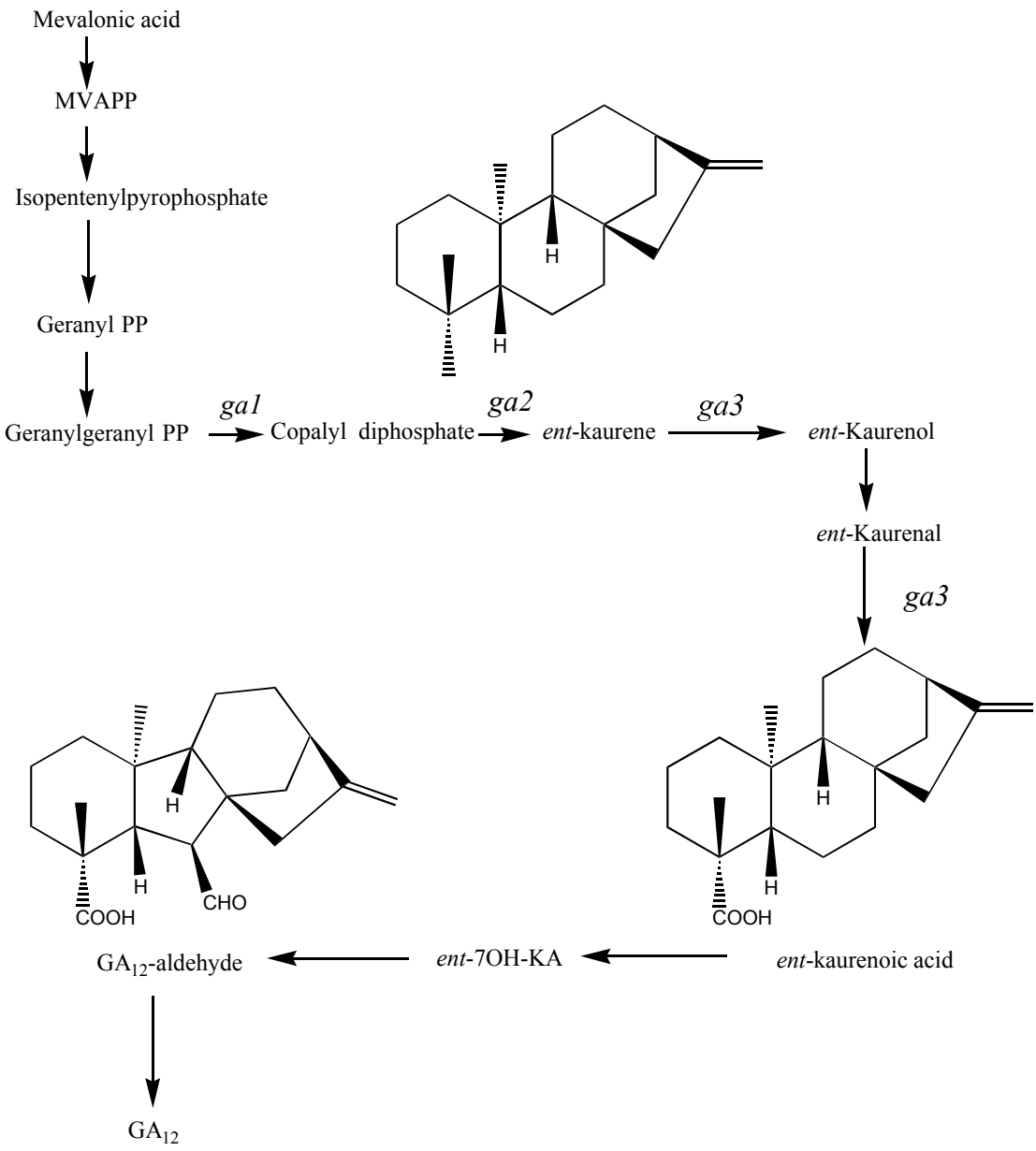


Figure 1.3 Illustration of the GA biosynthesis pathway. Mevalonic acid is the base component to synthesize geranylgeranylphyrohosphate (GGPP). GGPP is further converted into a four-ring structure of *ent*-kaurene. The oxidation reactions that occur in the endoplasmic reticulum convert *ent*-kaurene to GA_{12}-aldehyde.

The cyclization of GGPP to *ent*-kaurene is the first step in the GA biosynthesis pathway (Hedden and Kamiya, 1997; Sponsel, 1995). Two distinctive enzymes have been shown to be involved with these reactions. The *ent*-kaurene synthetase A enzyme catalyses the conversion of GGPP to a copalyl pyrophosphate (CPP), whereas the *ent*-kaurene synthetase B helps the further conversion of CPP to the tetracyclic diterpene, *ent*-kaurene. Both enzymes have been suggested to be renamed as *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS), respectively (MacMillan et al., 1997). The gene encoding *ent*-copalyl diphosphate synthase has been cloned from several plant species including *Arabidopsis*, maize and pea (Sun and Kamiya, 1994; Bensen et al., 1995; Swain et al., 1995; Zhu et al., 1997).

The gene, *GA1*, which encodes CPS, has been cloned from *Arabidopsis* (Sun and Kamiya, 1994). Their research showed that a deletion of the *GA1* in the mutant line resulted in a dwarf phenotype in *Arabidopsis*. Studies on the promoter of the *GA1* also showed the highest expression of this gene in the shoot apex, root tips and in vascular tissue of leaves (Silverstone et al., 1997a). Unlike CPS, the gene encoding *ent*-kaurene synthase (KS) has been cloned only from endosperm of pumpkin seeds (Yamaguchi et al., 1996). The transcript of KS was found in shoot tips, cotyledon and seedlings of pumpkin. High levels of enzymatic activity for both CPS and KS have been observed in developing chloroplasts of wheat seedlings and leucoplasts of pumpkin endosperm (Aach et al., 1997). Little enzyme activity was observed in mature chloroplasts.

Much less is known about the reaction in the second stage of GA biosynthesis compared to the other two stages. The second stage of the GA biosynthesis pathway starts from a sequential oxidation of *ent*-kaurene, *ent*-kaurenal to *ent*-kaurenoic acid. The enzymes are microsomal and require NADPH and oxygen as substrates in the reaction. That the reaction can be inhibited by carbon monoxide and reversed by light (450nm) suggests that a cytochrome P-450 related enzyme might be involved in this reaction (Hasson and West, 1976). Cytochrome P-450 enzymes have long been proposed to play a significant role in plant metabolism including the biosynthesis of lignin phenolics, membrane sterols, indole alkaloids, glucosinolates, jasmonic acid and brassinosteroids (Bolwell et al., 1994; Chapple, 1998; Donaldson and Luster, 1991; Durst, 1991; Riviere and Cabanne, 1987; Schuler, 1996). Several GA-deficient dwarf mutants including *lh-2* in pea, *dx* in rice and D3 in maize show defective activity of *ent*-kaurene oxidase (Swain and Reid, 1992; Swain et al., 1993; Swain et al., 1995; Ogawa et al., 1996; Winkler and Helentjaris, 1995). The enzyme activity of *ent*-kaurene oxidase was relative low in the cell-free extract of immature *lh-2* seeds. The deduced amino acid sequence indicated that D3 might encode a member of cytochrome P-450 monooxygenase. Cytochrome P-450 enzymes may also serve as a hydroxylase in the oxidation of *ent*-kaurenoic acid to generate *ent*-7α-hydroxykaurenoic acid (Dennis and West, 1967; Murphy and West, 1969). The second stage of GA biosynthesis ends with the contraction of the B-ring of *ent*-7α-hydroxykaurenoic acid to GA_{12}-aldehyde, which is the first GA formed in all systems.

The *ent*-kaurenoic acid oxidase gene has been isolated from both *Arabidopsis* and barley in recent years (Helliwell et al., 1999; 2001). Using a modified expression system in yeast, Helliwell et al. were able to show that two CYP88A proteins from *Arabidopsis* and one from barley convert KA to GA_{12} (Helliwell et al., 1999). DNA sequences from both *Arabidopsis* and barley were compared against the *Dwarf3* gene in maize. Sequence analysis showed that the maize *Dwarf3* also encodes *ent*-kaurenoic acid oxidase.

The processes that are involved prior to the formation of GA_{12} in the GA biosynthesis pathway are almost the same among different plant species. Series of oxidation reactions on carbon-20 lead to its elimination from the molecule as CO_2 and form C_{19}-GAs (Fig 1.4). The oxidation at C-7 from an aldehyde to a carboxylic acid is the first reaction in the third stage of the GA biosynthetic pathway. Some studies indicated that either monooxygenase or dioxygenase catalyzed this reaction in different plant material (Hedden, 1992; Grosselindemann et al., 1998; Lange et al., 1994; Lange, 1997). Further hydroxylation at C-13 of GA_{12} to generate GA_{53} is common in higher plants.

The subsequent successive oxidation of GA_{53} and the elimination of C-20 results in the formation of C-19 GAs. The following discussion will be focused on the last stage of the GA metabolism in *Arabidopsis thaliana,* which was the species that I used in my studies.

The advantages of using *Arabidopsis* as a model plant in plant molecular and genetic research has been described (Meyerowitz, 1987). The most popular approach

to study the GA biosynthesis pathway in *Arabidopsis* has been the characterization of candidate genes isolated and cloned from dwarf mutants. There have been more than twenty GAs genes identified using this approach. They are the components in the three parallel pathways: the early 13-hydroxylation pathway, the non-3, 13-hydroxylation pathway and the early 3-hydroxylation pathway (Kende and Zeevaart, 1997; Hedden, 1999). All three pathways are connected so that hydroxylation can occur at different levels of oxidation at C-20 (Fig 1.4).

The early 13-hydroxylation pathway begins at the conversion of GA_{12} to GA_{53} with continuous oxidation to generate the C-19 GAs, GA_{20} through GA_{44} and GA_{19}. The GA 20-oxidase not only carries out the oxidation in the early-13 hydroxylation pathway, but also is involved in the same reaction in the other two parallel pathways. In the non-3, 13-hydroxylation pathway, GA_{12} is oxidized to GA_9 via GA_{15} and GA_{24}. While in the early 3-hydroxylation pathway, GA_4 is obtained from a successive oxidation of GA_{37} through GA_{36}.

Quantitative analysis showed that the accumulation of C-20 GAs such as GA_{12}, GA_{53}, GA_{24} and GA_{36} were observed in *ga5* mutant plants, whereas the levels of C-19 GAs such as GA_9, GA_{51}, GA_4, GA_{34}, GA_{20}, GA_1, GA_{29} and GA_8 were lower in *ga5* mutants than in the wild type plants (Talon et al., 1990b). This strongly suggested that a single dioxygenase catalyzes C-20 hydroxylation and the elimination of C-20 to generate C-19 GAs. The *ga4* mutants had reduced levels of 3-hydroxy-GAs (GA_{37}, GA_{36}, GA_4, GA_{13}, and GA_{34}) and 3,13-dihydroxy-GAs (GA_1 and GA_8). They, however, showed accumulation of 13-hydroxy-GAs (GA_{44}, GA_{19}, GA_{17}, and GA_{20})

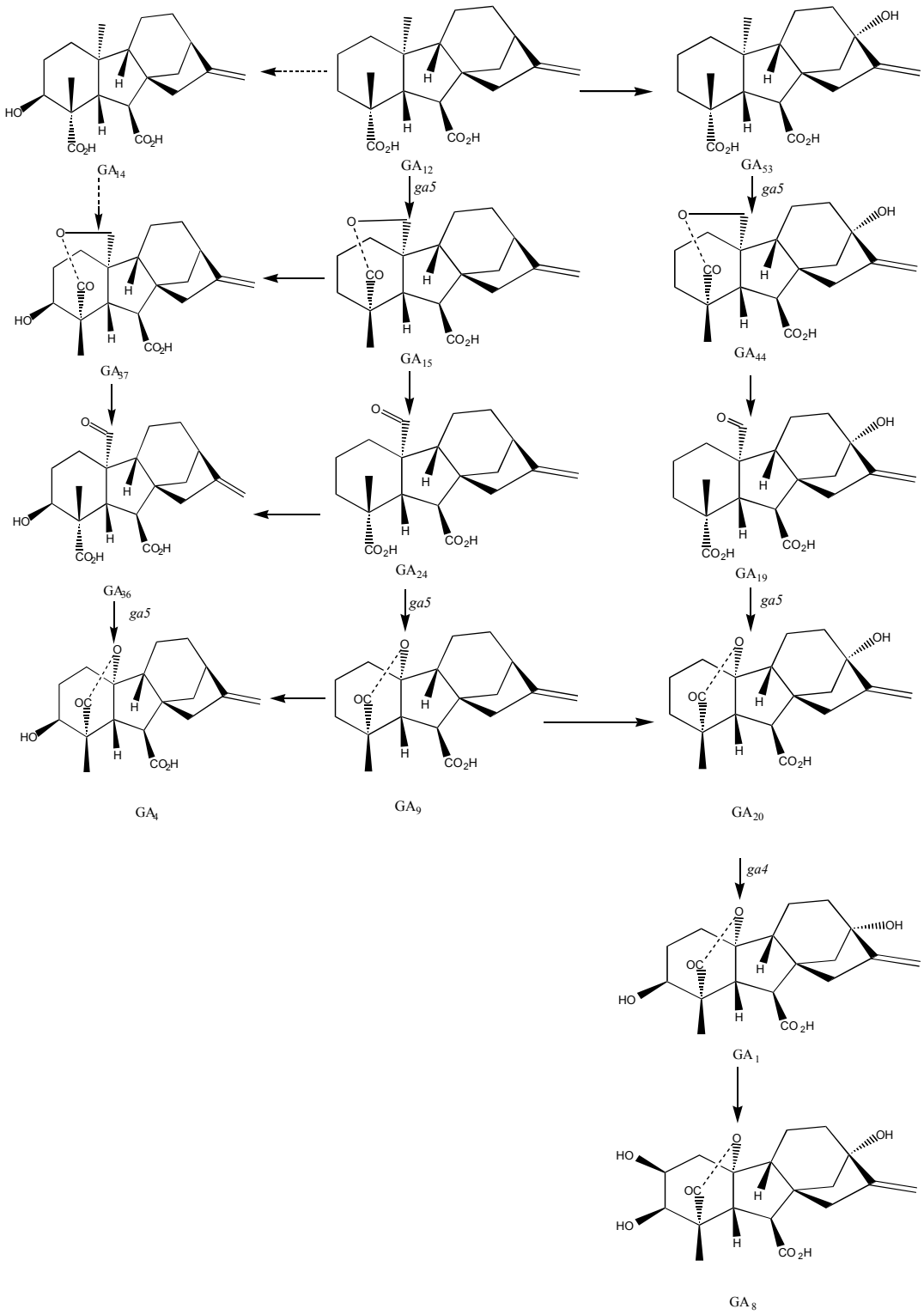


Figure 1.4 Illustration of the GA biosynthesis pathway in *Arabidopsis.* The GA 20-oxidase carries the oxidation reaction in three pathways.

and non-3, 13-hydroxy-GAs (GA_{15}, GA_{24}, GA_{25}, GA_9, and GA_{51}). This indicated that the *ga4* mutation might block the 3ß-hydroxylation in all three pathways (Graebe, 1987).

The Action of GA on General Plant Growth and Development

Plant growth and development is tightly regulated by various kinds of plant hormones and GA plays a major role in this regulation. It has been demonstrated that GA has effects on many stages of the plant life cycle from seed germination, stem elongation, flower formation, and male sex expression, to fruit set and growth (Metzger, 1987; Moore and Ecklund, 1975; Sponsel, 1985; Zeevaart, 1983; Pharis and King, 1985).

In the past, most of the studies on GA biosynthesis and metabolism have used seeds and fruit as research materials since those parts of the plant contain higher concentrations of GAs than vegetative organs. Most of the information on seed development as related to GA content was based on experiments employing cell-free extracts. High synthetic activity for kaurene was detected in early stage seed development in cell -free extract studies in barley and pea (Coolbaugh and Moore, 1969; Graebe, 1968). Nonetheless, the concentration of kaurene declined sharply in mature seeds (Coolbaugh and Moore, 1969; Graebe, 1968).

Extensive research focused on the association of fruit growth and GA content indicated a high level of GA-like substances in rapidly growing seeds and in cell suspension cultures of mesocarp and endocarp of peach and apricot (Jackson and Coombe, 1966; Jackson, 1968). Evidence gathered from studies performed on

different types of grapes also gave strong support to the hypothesis that seed-bearing berries have more GA-like activity than those without seeds (Iwahori et al., 1968). Application of GA has been shown to promote enlargement of seedless but not seeded berries in support of the role that GAs has in fruit development. This also indicated the close association of GA with seed development.

Plants with different dwarf mutations have been used as resources to elucidate the relationship between GA metabolism and stem growth. The first case indicating a clear link between dwarfism and GA deficiency was found in dwarf-5 (*d5*) maize (Hedden and Phinney, 1976). A single gene mutation of *d5* reduced stem length to only 20% of that of normal plants. However, normal growth of *d5* plants was rescued by applying GAs and GA precursors such as *ent*-kaurene, *ent*-kaurenol, and *ent*-kaurenoic acid. Molecular genetic characterizations of these mutants showed that the *d5* gene might be responsible for controlling the conversion of copalyl pyrophosphate to kaurene (Hedden and Phinney, 1976). Since the data indicated that GA content is associated with stem growth, it was important to investigate whether or not the level of GAs in stem tissues changed during plant development.

Stem heights of Alaska pea of different ages were measured after applying GA to shoot tips (Ecklund and Moore, 1968). It was observed that young seedlings tended to have a stronger response to exogenous GA than the older ones. Fourteen-day old seedlings showed no response in stem growth to GA applications. These results indicate that various levels of sensitivity of these seedlings to exogenous GA may account for the variations in the stem growth.

Further investigations conducted by the same group also found that the application of either GA or 2'isopropyl-4'-(trimethylammonium chloride)-5'-methylphenylpiperidine-1-carboxylate (Amo-1618), a growth inhibitor that inhibits the formation of *ent*-kaurene, to plants during the exponential phase of growth promoted or reduced stem elongation, respectively. A much smaller response from either one of these treatments was observed when plants were in the linear phase of growth. The changes in sensitivity to both exogenous and endogenous GAs were correlated with kaurene synthesis. Kaurene synthesis in cell-free extracts was detected in 3-day-old seedlings and reached its maximum level at day 9 in shoot tips of pea. The productivity of kaurene remained stable until the twenty-fourth day and the growth rate was increasing during the same period of time (Moore and Ecklund, 1974). This is strong evidence linking the control of stem elongation in Alaska pea to the rate of endogenous GA biosynthesis.

GA_1 has been recognized as the biologically active form of GA in many plants such as pea, pumpkin, spinach and maize (Ross et al., 1992; Swain and Reid, 1992). Quantitative analysis of the endogenous content of GA in the dwarf mutant of maize, *d1*, revealed a decline in GA_1 and GA_8 levels but an increase in GA_{20} and GA_{29} levels. Only the application of GA_1 can restore *d1* plants to a normal phenotype. In pea, an average wild type plant contains 10-20 times more GA_1 in shoot tips than the dwarf *le* plants (Ross et al. 1992). Ross et al. (1992) also found that more GA_{20}, GA_1 precursor, accumulated in the *le* plants than the wild types.

There has been some debate on which is the biologically active form of GA in *Arabidopsis*. Results from some studies indicated *ga4* mutants treated with GA_4 regained wild-type phenotype while GA_1-treated plants showed only intermediate response in restoring to their normal phenotype (Talon et al. 1990b). Moreover, wild type plants treated with calcium 3,5-dioxo-4-propionylcyclohexanecarboxylate (BX-112), a growth inhibitor that inhibits 3β-hydroxylation reaction in GA biosynthesis, phenocopied *ga4* plants. Only application of GA_4 or GA_{36} (products of 3β-hydroxylation) reversed the inhibition in growth (Zeevaart and Talon, 1992). Studies on the metabolism of exogenous GAs in *Arabidopsis* showed GA_4 can be converted to GA_1 following 13-hydroxylation, which is known to be the most bioactive form in many plant species (Talon and Zeevaart, 1992; Kobayashi et al., 1993).

The mechanism of the stem elongation at the cellular level had been studied long before the discovery of GA. The conclusions from those studies can be summarized as following: 1) the subapical meristem is the fundamental region for stem elongation; 2) cell division usually occurs prior to cell elongation during stem growth; and 3) the maximum length of internodes is pre-determined by the number of cells and the duration of cell division in the subapical region (Sachs, 1965).

After the isolation and structural determination of GAs, more scientists focused on the relationship between stem growth and GA biosynthesis *in vitro*. Sachs and Lang (1957) found that the induction of the stem elongation in rosette plants by GA application is associated with growth activity in the subapical meristem region. Many other reports also suggested GAs regulate stem elongation in various rosette plants

(Lang, 1956). However, the most important findings to demonstrate GA cause bolting were conducted by Sachs and Lang in 1957.

The mitotic figure of rosette plants was examined after plants treated with GA (Sachs and Lang, 1957). Within 24 hours after the treatment, a significant increase in mitotic activity in nonlignified tissues was detected below the eumeristem. The mitotic activity continued for several days and was accompanied by stem elongation. There was little or no effect of GA treatment on the eumeristem.

The cellular mechanism for thermoinduced stem growth had been reported in *Thlaspi arvense* (Metzger and Dusbabek, 1991). The rosette form of *Thlaspi arvense* is a cold-requiring plant, which means that flower formation accompanied with stem elongation occurs after exposure to low temperatures. Studies showed that bolting of these plants is associated with stem elongation as a result of increased cell division in *Thlaspi*. An increase in cell division was observed in the pith and cortex, a region about 0.5 and 5.0 mm below the stem apex during the cold treatment. Moreover, the increase in cell division induced by vernalization could be mimicked by exogenous GA_3. The effect of GA_3 on cell division was similar to that of 4 weeks of vernalization. Fewer cell divisions were found in the pith and cortex in GA-deficient dwarf mutant plants that were vernalized (Metzger and Dusbabek, 1991).

The promotion of stem elongation following cell division in the subapical region is found not only in rosette plants but also in caulescent plants. Sachs and Krofranek (1963) conducted a similar experiment using *Dendranthema grandiflora* to determine the relationship between GA and mitotic activity in shoot region. Plants

treated with GA inhibitors such as Amo-1618, 2-chlorocholine chloride (CCC), or Phosfon had fewer mitotic figures in the subapical region than the control plants. However, the number of mitotic figures reverted to normal or greater numbers when GA was applied at the same time. This demonstrated that GA regulates stem elongation in both rosette and caulescent plants via cell division in the subapical meristematic regions.

Environmental Factors and Plant Development

Seasonal changes in environmental factors such as temperature, light intensity and water supply during the growing season have a strong impact on plant growth and development. Using environmental factors as inductive signals during plant development is advantageous in several ways: 1) to increase the possibility of outcrossing; 2) to produce progeny under certain environmental conditions; and 3) to reduce or avoid reproduction during extended stress periods. Not only plants benefit from the response of environmental factors, plant breeders and horticulturists often optimized their agricultural and horticultural practices by manipulating environmental factor such as changing day length to synchronize flowering production. Photoperiodism and vernalization are the two major mechanisms of seasonal effects. Photoperiodism is the effect of daylength, while vernalization is a response of flowering brought about by exposure to cold, non-freezing temperatures.

The magnitude in the change of daylength is associated with latitude, or more specifically the distance from the equator (Thomas and Vice-Pruce, 1984). For

example, the average daylength at 60° latitude is 6 to 19 h while at 30º; it is 10 to 14 h. The rate of change in daylength is also dependent on the month of the year. From April to August, the average rate of daylength change is 40 min per week at 60° and 12 min per week at 30°. Since the length of daylight is constantly changing, it is essential for plants to develop a very sensitive system to respond to these changes. For some tropical plants flowering is strongly regulated by daylength, even though the seasonal daylength changes are small (Thomas and Vice-Prue, 1984).

Vernalization serves an additional role in seasonal flowering. It requires plants to start with seed germination in late summer or early autumn. Most of the plants can survive winter in rosette form and initiate flowering in the following spring. This mechanism helps ensure the onset of reproduction during the correct season.

Some plant species require both vernalization and photoperiodism for flowering (Lang, 1965). Most plants with this dual vernalization-photoperiodic requirement are categorized as long-day plants, which I will discuss later in this chapter. To understand the importance of these two environmental factors and their role in plant development, I will dedicate the following sections to cover studies emphasizing both photoperiodism and vernalization on stem growth, especially in rosette plants.

Photoperiodism and Reproductive Development

Definition of Photoperiodism

The terms of photoperiod and photoperiodism were first introduced by Garner and Allard in1920. It is the response to the length of day, the factor which from year to year gives the most reliable information about the passage of the seasons.

Photoperiodic responses have been commonly found in both plant and animal kingdoms (Vince-Prue, 1975). In plants, the most significant responses include flower initiation, the formation of storage organs, leaf development, stem elongation, bud dormancy and germination.

Diversity of Photoperiodic Response Types

In general, plants are classified into three photoperiodic types based on their flowering responses to daylength (Salisbury, 1982; Vince-Prue, 1994). They are: 1) short-day plants (SDP) that flower when the daylength is less than a specific critical value; 2) long-day plants (LDP) that flower only when the day length exceeds a critical value; and 3) day-neutral plants (DNP) in which photoperiod has no effect on flowering.

In most cases, the daylength required to promote flower development varies from one species to another. A SDP such as chrysanthemum requires a daylength less than 15-16 hours to flower, while *Hyoscyamus niger*, a LDP, needs for the days to be longer than 12.5 hours. Therefore, both plants flower when daylength is between 12.5 and 15 h.

Both SDP and LDP plants can be further divided based on the stringency of the requirement. Some plants have as an obligate or qualitative photoperiodic requirement. For example, *H. niger* is an obligate LDP plant, which will flower only when daylength exceeds 12.5 h. However, other plants are not so strict and have a facultative requirement or quantitative response for a specific daylength to promote flowering. *Arabidopsis* is a good example of a facultative LDP. It will eventually flower under non-inductive conditions. There are plants that show dual daylength requirement for flowering. Plants that flower when LD are preceded by SD are called short long day plants (SLDP). Other species have the opposite sequence requirement and are defined as long short day plants (LSDP).

Where is Daylength Perceived?

The site of perception of photoperiod was determined by Knott in 1934. He noticed that flowering only occurred when leaves of the LDP spinach were exposed to inductive conditions. No flowering was observed when only the apex was exposed to long photoperiods. The same result was observed in other LDP such as *Anethum graveolens*, *Brassica crenata*, *Hordeum vulgare* and *Lolium temulentum*; and in SDP such as *Xanthium strumarium*, *Glycine max*, *Kalanchoe blossfeldiana*, *Dendranthema grandiflora*, *Pharbitis nil* (cotyledons) and *Perilla* (Lang, 1965; Vince-Prue, 1975; Halevy, 1985).

Most of the photoperiodic sensitive plants require two systems for photoinduction to occur. The first one is a biological clock (circadian rhythm) to

measure the time and the other one is a photoreceptor (phytochrome) to distinguish between light and darkness. In general, plants' response to light is under the control of the phytochrome system (Thomas and Vince-Prue, 1997). Two photointerconvertible forms of each phytochrome exist: the Pr form which absorbs maximally at 660 nm, and the Pfr form which has an absorption maximum at 730 nm. Phytochrome has been demonstrated to be interconvertible by sequentially red and far-red light treatments. Under red light conditions, Pr is converted to Pfr and the Pfr is converted back to Pr only with far-red light. It appears that Pfr is the bioactive form that induces physiological response, while Pr is synthesized in dark.

There are at least five different phytochrome genes (*PHYA*, *B*, *C*, *D* and *E*) in *Arabidopsis* (Sharrock and Quail, 1989; Clack et al., 1994). Isolation and sequencing of phytochrome genes have been reported in more than fifteen plant species (Thomas and Vince-Prue, 1997). Characterization of these genes showed each phytochrome has its own distinct function. For example, PhyB is needed for continuous red light perception, while PhyA is necessary for continuous far-red light perception (Hartmann, 1966; Quail et al, 1995). Also, PhyA is associated with seedling development, while PhyB is believed to be involved with every stage of plant growth and development (Smith, 2000). Various physiological responses such as flower induction, light-stimulated germination, and de-etiolation are believed to be associated with phytochrome. Nevertheless, only a few studies had shown that the physiological processes under phytochrome control are mediated through GA biosynthesis and metabolism.

The germination response of lettuce was the first to give the idea that phytochrome affects germination via GAs. Recent studies of the GA biosynthesis pathway have also provided good resources to further understand the relationship of phytochrome and GA biosynthesis (Jackson et al., 2000). Two GA biosynthesis enzyme genes, 3ß-hydroxylase and GA 20-oxidase, have been cloned from lettuce by Toyomasu et al., (1998). An increase in the expression of Ls3h1 gene, which presumably is responsible for the production of 3β-hydroxylase, was observed following red light treatment. The effect was reversed by a far-red light treatment. Although the expression of both Ls20ox1 and Ls20ox2 genes, which are believed to encode GA 20-oxidase, was induced by seed imbibition in the dark, only Ls20ox2 expression was influenced by red-light treatment. This indicated that only the expression of Ls3h1 was induced by red-light via phytochrome (Toyomasu et al., 1998).

In 1998, Yamaguchi and his group successfully cloned *GA4H*, a homolog of the *GA4* gene from *Arabidopsis*. It was shown that *GA4* encodes an enzyme that functions as 3ß-hydroxylase. Both *GA4* and *GA4H* genes in imbibed seeds were induced by red light treatment. However only *GA4* was induced by red light treatment in *phyB-1* mutants, indicating that the expression of those two genes was regulated by one or more other phytochromes. The concentration of endogenous GA was analyzed in lettuce seeds. The GA_1 level was increased in red light treatment and high concentration of GA_{20} was also detected (Toyomasu et al., 1993). Together,

phytochrome might play a role in controlling GA biosynthesis during light-induction of seed germination.

To date there is little evidence that phytochrome affects GA biosynthesis during seedling growth. An increase in GA_8 levels was observed when young cowpea seedlings were transferred to light (Martinez-Garcia and Martinez-Garcia, 1995). Metabolic studies showed an elevation in GA_1 levels but a decline in $[^3H]GA_1$ in the elongating region of epicotyl when cowpea was treated with end-of-day far red-light (Kamiya and Martinez-Garcia, 1999). However, these effects were reversed following red light treatment. This indicated that stem elongation in light-grown seedlings might be controlled by phytochromes via regulation of the GA 2ß-hydroxylation.

Transgenic tobacco plants that overexpress the oat *PHYA* gene showed a decrease in endogenous GA level and had a short phenotype (Jordan et al., 1995). The phenotype was rescued by the applications of GA. Other studies showed contradictory results. For example, *phyB* mutants of pea (*lv*), cucumber (*lh*), and *Arabidopsis* with an elongated phenotype showed little change in the endogenous GA level when compared with that of the wild types plants (Weller et al., 1994; Lopez-Juez et al., 1995; Reed et al., 1996). In summary, more research is needed to better understand the relationship between GA biosynthesis and phytochrome during seedling development. Whether or not phytochrome-regulated GA biosynthesis is involved in stem elongation remains to be determined. There is more evidence that supports the idea that stem elongation, especially in LDP, is associated with GA biosynthesis.

The Relationship of Stem Elongation and GA Biosynthesis

It has long been proposed that the process of the stem elongation in rosette LDP is strongly regulated by GA biosynthesis. For most rosette LDP, stem elongation is also accompanied by floral initiation. The relationship between these two events varies from one plant species to another. For example, some rosette plants showed stem elongation prior to the formation of flower primoridia and never produced flowers (Cline and Agatep, 1970). Some other LDP include *Matricaria parthenoides*, *Centaurea cyanus*, *Coreopsis tinctoria*, barley, wheat, and millet showed a more obligate relationship between flowering and stem elongation (Greulach, 1942; Downs et al., 1958).

In some cases, stem elongation and flowering are two separate events. *Hyoscyamus*, a LDP, belongs to this category. Stem elongation occurs without any flower induction with a period of 11 h (critical daylength =12.5 h) light treatment at 20°C (Lang and Melchers, 1943). However, plants will flower under non-inductive (SD) conditions if leaves are removed. In *Rudbecia bicolor*, flower induction could be initiated without stem elongation when plants were grown at high temperatures in SD (Murneek, 1940). Studies showed that the application of GA to the LDP *Silene armeria* under SD promoted stem elongation without flowering (Wellensiek, 1973; Talon et al., 1991). Stem elongation in *Silene armeria* plants was induced by changes in endogenous GA levels in shoot tips when mature leaves were subjected to LD (Talon and Zeevaart, 1992). Moreover, flowering under LD without stem elongation was observed when plants were treated with a GA inhibitor at the same time (Talon et

al., 1991). These findings also suggest that stem elongation and floral initiation should be considered as two independent events but with a common perception mechanism (Thomas and Vince-Prue, 1997).

Photoperiodic after-effect is a phenomenon in which an induced response continues when the plant is returned to non-inductive conditions. There are three types of photoperiodic after-effect based on their effect on stem growth and flowering. The first type is when the physiological responses to both stem elongation and flower development are fulfilled regardless of transfer to noninductive conditions. *Silene armeria* is a perfect example in this category. The second type (eg. spinach, a LDP) is demonstrated by an immediate termination of flower development when returned from LD to SD. The last group of the photoperiodic after-affect response exhibits an intermediate response. In *Agrostemma*, stem elongation continues for some period of time under LD and ceases when plants are returned to SD. These plants generally are intermediate in height compared to those with continuous LD treatment. It is interesting to note that stem elongation in LDP is associated with GA biosynthesis regardless of the type of photoperiodic after-effect to which they exhibit (Cleland and Zeevaart, 1970; Wellensiek, 1985; Talon and Zeevaart, 1990).

It is well established that GA plays a crucial role in the daylength mediated regulation of stem elongation in rosette LDP. In *Silene armeria*, the concentration of GA_{19}, GA_{20} and GA_1 increased when transferred from SD to LD (Talon and Zeevaart, 1992). In spinach, stem and petiole elongation were promoted when plants were subjected to LD. The same effect can be achieved by treating plants with GA_3 under

SD. In contrast, the stem elongation response was reversible under LD when plants were treated with growth retardant such as AMO-1618. High levels of GA_{19} accumulated under SD while endogenous GA_{20} and GA_{29} levels increased seven-fold and GA_{19} declined five-fold under LD (Metzger and Zeevaart, 1980; 1982).

Metabolism studies showed the conversion of [^{14}C] GA_{19} to GA_{20} and GA_{29} only took place under continuous light (Gilmour et al., 1986). Plants treated with [^{14}C] GA_{44} showed no limitation in converting GA_{44} to GA_{19}. However, further conversion of GA_{19} to GA_{20} and GA_{29} was blocked when plants were grown in dark prior to the treatment. Plants grown under continuous light exhibited continuous conversion of GA_{19} to GA_{20}.

Cell free extracts from spinach leaves also have been used to investigate the regulation of GA metabolism in relation to daylength. The activity of GA 20-oxidase was high in the cell free extracts under LD, whereas lower activity was observed in leaves maintained under SD. Moreover, the rate of conversion of GA_{53} to GA_{44} and of GA_{19} to GA_{20} decreased rapidly when plants were returned to darkness, and enzyme activity was eliminated after 16h of darkness. This suggested that the early-13-hydroxylation pathway is regulated by daylength in spinach.

Similar results were also found in different LD rosette plants such as *Agrostemma githago* (Zeevaart and Gage, 1993). The endogenous content of *ent*-kaurene is at least 2.5 times higher in both *Agrostemma* and spinach plants under LD than under SD. The rate of *ent*-kaurene accumulation increased when they were moved from SD to LD (Zeevaart and Gage, 1993). Endogenous *ent*-kaurene levels in

mature leaves declined when plants were returned to SD (Zeevaart and Gage, 1993). In summary, the higher GA content under LD is associated with greater rates of GA biosynthesis in many rosette LDP. Presumably the higher rate of GA metabolism leads to the production of a bioactive GA that ultimately promotes stem elongation.

GA_1 has been suggested to be the GA responsible for biological activity in most LDP. In spinach, stem elongation was suppressed by BX-112 treatment in LD and the effect was reversible with the application of GA_1 but not with GA_{20}. Quantitative analysis of endogenous GAs in BX-112-treated plants showed high concentrations of GA_{53}, GA_{44} and GA_{20} but low levels of GA_1 and GA_8 (Zeevaart et al., 1993). The promotion of stem elongation through regulation of GA biosynthesis in different daylengths is also observed in woody plants. In *Salix pentandra* and birch, stem elongation is regulated by photoperiod. Stem growth is promoted by LD, but ceases when plants are grown under SD. This response to changing photoperiodic conditions is one of many associated with SD-induced dormancy (Junttila, 1993; Olsen et al., 1995). Application of either GA_1 or GA_{20} promotes stem growth under SD (Junttila, 1993). Moreover, the concentration of GA_1 and GA_{20} declined sharply within 48 h after plants were transferred from LD to SD (Olsen et al., 1995). These findings suggested GA_1 might be the primary active GA for LD-induced stem elongation.

Cellular basis of LD-induced stem elongation was also determined in *Silene armeria*. The cell numbers increased in the region of meristematic tissues 1.0-3.0 mm below the shoot apex, which led to stem elongation following LD treatment (Talon et

al., 1991). Further investigation found that the endogenous GA_1 level increased 30-fold in the region 0.5-3.5 mm below the shoot apex indicating an increase in GA_1 levels in the subapical region is associated with stem growth. Taken together, GA_1 is believed to be responsible for inducing stem growth in LD plants.

Evidence gathered from studies using crucifer plants such as *Thlaspi* and *Arabidopsis* contradicts the idea that GA_1 is the most bioactive from of GA as observed in other species. GA_9 is the most bioactive form of GA in *Thlaspi* grown under non-thermoinductive conditions (Metzger, 1990). Results from GA studies in *Arabidopsis* suggested that GA_4 is more bioactive than GA_1 (Zeevaart and Talon, 1990; Kobayashi et al., 1993). Thus, it is worthwhile to elucidate which GA is the bioactive form in *Arabidopsis*.

Vernalization

Characteristics of the Vernalization Response

Temperature is another common environmental factor that can change the process of plant development. In general, initiation of flowering in many plant species, especially those native to temperate regions, requires low temperatures. This phenomenon is known as vernalization. Thermoinduced treatment, a terminology often used in GA studies, refers to a kind of treatment in which plants are subjected to low, non-freezing temperatures (usually between 2-10°C).

Based on how plants respond to low temperatures for flower development, the vernalization response can be classified into two categories: annuals and biennials.

The winter annual plants have a facultative cold requirement which means this type of plants will flower eventually without any cold treatment. Moreover, winter annuals are sensitive to vernalizing temperatures at all stages of plant development. In contrast, biennial plants have an obligate cold requirement and require a full season of vegetative growth before acquiring sensitivity to vernalizing temperatures. Thus, in contrast to winter annuals, biennials require two growing seasons to flower and produce seed.

Regardless of differences in the two vernalization response types, winter annuals and biennials share common physiological characteristics (Metzger, 1995). In general, maximum vernalization is achieved at the temperature range of 0° to 10°C. Furthermore, vernalization is a quantitative process in that an increase in the duration of the cold treatment can accelerate the flowering response. Unlike the photoperiodic responses discussed earlier, there are no examples in which one inductive cycle (day) of vernalizing temperatures is sufficient for flowering. Most cold-requiring plants require four or more weeks of cold treatment to reach maximum induction (Metzger, 1995).

There has been much evidence indicating that shoot apex is the site of the perception of vernalizing temperatures (Curtis and Chang, 1930; Chroboczek, 1934; Purvis, 1940; Schwabe, 1954; Metzger, 1988; Wellensiek, 1964). However, other tissues can also be thermoinduced so long as cell division is occurring during vernalization (Wellensiek, 1964; Metzger, 1988; Burn et al., 1993a). Thus the thermoinductive state is stably propagated through mitosis requiring a direct effect on

an individual cell rather than a transmissible stimulus, as is the case for photoinduction. For example, vegetative growth was observed when non-vernalized shoot tips were grafted to cold-treated donor in *Thlaspi*, whereas flower induction was seen on non-vernalized shoot tips grafted to LDP *Sinapis alba* under LD (Metzger, 1988). Together, one may think of vernalization as an epigenetic cell-autonomous process.

Molecular Mechanism of Vernalization

More research is needed to elucidate the fundamental mechanism of vernalization at the molecular level. However, most of the hypotheses of the mechanism of vernalization were proposed based on physiological observations (Burn et al., 1993b). Burn et al. suggest that thermoinduction results from a decrease in DNA methylation during vernalization (Burn et al., 1993b). Much evidence from both animal and plant studies suggests that the epigenetic control of gene expression is related to the pattern of DNA methylation (Holliday, 1990).

It has been found that the cytosine residue of a GC dinucleotide in both plants and animals can be methylated at the C-5 position. It is believed that a new strand of DNA is duplicated by a methyltransferase enzyme, which uses hemimethylated double-stranded DNA as substrate during DNA replication. From this point on, the new strand of DNA with methylated cytosine is passed on to both daughter cells. Transcription is then inhibited if the methylation of cytosine residues occurred within the promoter region of a gene (Schwartz and Dennis, 1987). However, transcriptional

activity is increased in both plant and animal systems when a chemical demethylating agent, 5-azacytidine (5-azaC), is applied during the process of DNA replication (Klaas et al., 1989; Jones and Buckley, 1990).

Observations of the regulation of gene expression and the physiological characteristics in thermoinduced plants provided a good explanation of the mechanism of vernalization at molecular level (Metzger, 1996). Cold-requring plants remain vegetative under non-inductive condition because of suppression of one or more flowering related gene(s) in which one or more of the cytosines in their promoter regions are methylated. Activation of specific gene(s) during vernalization occurs because of cold-induced demethylation.

Flowering of the two cold-requiring ecotypes of *Thlaspi arvense* and *Arabidopsis thaliana* plants under non-inductive condition with 5-azaC treatment also provided strong supporting evidence to this theory (Burn et al., 1993a). Moreover, the levels of DNA methylation decreased for both vernalized plants and plants treated with 5-azaC. This indicates that the 5-azaC treatment on flower induction has the same epigenetic effects as vernalization. The effects of vernalization and 5-azaC treatment on flowering induction share several common features including: 1) they are meristem specific; 2) both processes require cell division and 3) the effects are not passed through to the progeny. These results are consistent with the hypothesis that the regulation of the DNA methylation plays a key role in thermoinduced flower initiation.

Currently in *Arabidopsis* two genes, *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*), that are involved in the regulation of the vernalization response have been identified by different research groups (Clarke and Dean, 1994; Sheldon et al., 1999; 2000; Michael and Amasino, 1999a; 1999b; 2000; Johanson et al., 2000). The expression of the *FLC* gene is strongly associated with vernalization. Two vernalization-sensitive late-flowering ecotypes of *Arabidopsis*, C24 and Pitztal, contain high levels of the *FLC* transcript under non-vernalized conditions. The levels of the *FLC* mRNA declined significantly after the vernalization treatment. As discussed earlier, flowering induction of the vernalized plants is associated with the duration of the cold treatment. There was also progressive reduction in the expression of *FLC* as the duration of the cold treatment increased. This suggests that *FLC* plays a key role in the vernalization response.

Johanson et al. (2000) proposed that the variation of flowering time in different ecotypes of *Arabidopsis* was determined by the alleles of the *FRI* gene. Early flowering ecotypes such as Columbia and *Landsberg erecta* carry a mutation at the *FRI* locus whereas late flowering ecotypes such as Stockholm, whose late-flowering phenotype can be eliminated by cold treatment, do not contain this mutation in *FRI*. Therefore, one may conclude that the loss-of-function of the *FRI* gene caused early flowering of some ecotypes of *Arabidopsis*.

The interactions between *FLC* and *FRI* have been investigated in a number of recent studies in *Arabidopsis*. The expression of the *FLC* gene was not detectable in early flowering plants of the Columbia ecotype, which has recessive *FRI* alleles

(Michaels and Amasino, 1999a; Sheldon et al., 1999). However, strong expression of *FLC* was detected in late-flowering plants such as *ld*, which has a dominant allele of *FRI*. Based on the interactions found between *FRI* and *FLC*, it has been suggested that the late-flowering phenotype can be eliminated by suppression of *FLC* through cold treatment or by introducing null alleles of *FRI*. It is clear both *FLC* and *FRI* play an important role in the vernalization process.

Effects of Vernalization and Gibberellin on Flowering Development

With the exception of GA, most plant hormones such as auxins, cytokinins and abscisic acid have little or no effects on flowering. In a number of LDP and cold-requiring plants exogenous GA can substitute for the inductive environmental cue (Lang, 1965; Bagnall, 1992; 1993; Metzger, 1988).

Thlaspi arvense has been chosen as a model to investigate stem growth and regulation of the GA biosynthesis pathway at low temperatures (Metzger, 1985). In *T. arvense,* exogenous GA can substitute for vernalization in the induction of bolting. Also, thermoinduced bolting can be inhibited with CCC and this inhibition is reversed when GA is applied.

Further research showed that the perception site of thermoinductive treatment was localized in the shoot apex (Metzger, 1988). To identify the GA responsible for thermoinduced stem elongation in *Thlaspi*, the activities of various exogenous GAs and GA precursors were compared in both vernalized and non-vernalized plants (Metzger, 1990). Surprisingly, the results showed that GA_9 is the most bioactive form

in non-vernalized plants. This was contrary to the widely accepted theory that GA_1 is the bioactive form in most plant species. Moreover, GA precursors, such as kaurene, kaurenol, and KA can only induce stem elongation in thermoinduced plants. These results indicated that the metabolism of *ent*-kaurenoic acid (KA) to GAs was blocked prior to vernalization. More supporting data came from KA metabolism studies under both non-inductive and inductive conditions (Hazebroek and Metzger, 1990). The rate of metabolism of exogenous [^{2} H]-KA in the shoot tips of the thermoinduced plants was 19 times higher than in non-thermoinduced plants. However, the rate of conversion of [^{2} H]-KA to GA_{20} was about the same in leaves of both induced and non-induced plants.

Changes in the endogenous content of KA following vernalization were also analyzed by the gas chromatography-mass spectrometry (GC-MS). The concentration of KA in the shoot tips of non-vernalized plants was 47 times higher than the thermoinduced ones, while no differences in KA levels in the leaves of thermo- and non-induced plants were observed. KA levels dropped by 10-fold just two days after plants were returned from the cold to warm temperatures. Furthermore, cell extracts from shoot tips of *Thlaspi* were used to measure the enzyme activity in the conversion of KA to 7-OH KA. Only microsomal extracts from shoot tips showed an increase in the enzyme activity, while KA-hydroxylase activity was similar in leaves from induced and non-induced plants. Together, it is reasonable to think that bolting in *Thlaspi* is initiated by a thermoinduced increase in the conversion of KA to GA.

Results from studies conducted by different research groups also support the idea that GAs are involved in the regulation of bolting after vernalization. For instance, high concentrations of GA_1 and GA_3 were detected in the shoot tips of the thermoinduced plants comparing to non-thermoinduced plants in *Brassica napus*. Metabolic experiments showed that the conversion of $[^3H]$-GA_{20} to GA_1 was increased in vernalized plants (Zanewich and Rood, 1995). However, data obtained from *Raphanus sativus* studies failed to demonstrate that thermoinduced flowering was mediated by the regulation of GA biosynthesis (Nakayama et al., 1995).

Research Objectives and Strategies

The purpose of this study is to illustrate how environmental factors promote stem elongation in *Arabidopsis* via the GA biosynthesis pathway. Two different late flowering lines of *Arabidopsis* were used in this study. A late flowering mutant line, *FLOWERING LOCUS F* (*FLF*) was created by the insertion of two adjacent T-DNA copies (Burn et al., 1999a). This late flowering phenotype is overcome by either an eight-week vernalization treatment, or by the continuous application of GA. The *FLF* gene has been renamed as *FLC* (*FLOWERING LOCUS C*), also referred to as cold requiring (CR) line in this dissertation, since both *FLF* and *FLC* are at the same locus in *Arabidopsis*. The second late flowering line, CS933, has a quantitative requirement for long-days. Flowering under SD was delayed for 10 weeks (Metzger and Zheng, 1998). However most plants flowered when transferred from SD to LD within 7-9 days. Since CR and CS 933 lines would flower under inductive conditions, they

provide a suitable material to investigate the regulation of GA biosynthesis by environmental factors in *Arabidopsis*. To study which GA is a key factor in regulating stem elongation and to identify the block(s) in the GA biosynthesis, five different kinds of experiments were conducted and are presented in this dissertation.

Identification of the Block(s) in the GA Biosynthesis under Non-inductive Conditions

Based on the information gathered in the literature review, it is possible that more than one step of GA biosynthesis is under environmental control. To identify one or more block(s) in the GA biosynthesis, two experiments were conducted to determine if the conversion of KA to 7-OH KA is the regulation step in GA biosynthesis under environmental control. First, conduct quantitative analysis of the endogenous KA levels in plants grown under different growth conditions using GC-MS. Second, examine effects of KA on plants grown under non-inductive and inductive conditions. Presumably, high levels of KA and little stem growth are expected in plants grown under non-inductive conditions.

To further investigate other potential rate-limiting step(s) in GA biosynthesis under environmental control, a third experiment to see the effect of various GA on stem growth of plants grown under non-inductive (SD and non-cold treatment) and inductive (LD and cold treatment) conditions was conducted.

Which GA is Responsible for Inducing Stem Growth

To determine whether more than one form of GA is biologically active in *Arabidopsis*, candidate GAs were selected from previous experiments for dose-response studies. It is expected that the more bioactive a GA is, the stronger effect it has on stem elongation. Stem growth was also expected to increase when a higher dose of more bioactive GA was applied to plants.

CHAPTER 2

REGULATION OF GIBBERELLIN METABOLISM IN RELATION TO LONG-DAY PLANTS

Introduction

Environmental cues such as day-length and temperature are known to trigger regulatory mechanisms in plants that alter growth and development. Stem elongation of rosette plants is induced as a result of elevated GA levels under LD. For example, in *Silene armeria* concentrations of GA_1 and GA_{20} were elevated after plants were moved from SD to LD (Talon and Zeevaart, 1992). Greater stem elongation was found in spinach, another long day plant, when grown in LD or with the application of GA_3 to plants in SD. Quantitative analysis of the endogenous contents of GAs in spinach showed that GA_{19} levels were decreased dramatically when transferred from SD to LD. The content of GA_{20} and GA_{29} increased significantly with the same treatment. Results of further studies indicated the oxidation of GA_{53} and GA_{19} only took place when plants were grown under continuous light (Gilmour et al., 1986). The metabolic experiments showed the conversions of GA_{53} to GA_{44} and GA_{19} to GA_{20} decreased rapidly when plants were returned to complete darkness. These data suggest that the early 13-hydroxylation pathway is regulated by day length in spinach.

On the other hand, *Thlaspi arvense* has been used as a model to investigate stem elongation via regulation of GA biosynthesis in low temperature (Metzger, 1985). Results of these studies gave strong support to the hypothesis that GA biosynthesis may be blocked in non-vernalized plants (Metzger, 1985). It was proposed that one of the consequences of vernalization is to lift one or more blocks in the biosynthetic pathway leading to the GA responsible for controlling stem growth.

Stem elongation of cold requiring plants was promoted with the application of GA under non-vernalized conditions in *Thlaspi*. Application of KA under non-vernalized condition failed to promote stem growth while thermoinduction resulted in the lift of one or more blocks in KA metabolism and elicited stem growth (Metzger, 1988). Further quantitative analysis of the content of endogenous KA showed that the KA concentration was 47 times higher in the shoot tips of non-thermoinduced plants than in the thermoinduced ones. Moreover, the endogenous KA level was decreased by 10-fold 48 hours after the cold treatment (Hazebroek et al., 1993). These results suggested that cold-induced stem elongation might be regulated in the process of converting KA to 7-OH KA in *Thlaspi* shoot tips.

The major regulatory step(s) before and after the formation of GA_{12} in GA biosynthesis pathway in the photoperiodic plant remains unclear. In my studies, I chose to use *Arabidopsis thaliana* as a model to delineate the photoperiodic regulation of GA metabolism and to identify the step(s) in GA biosynthesis under photoperiodic control.

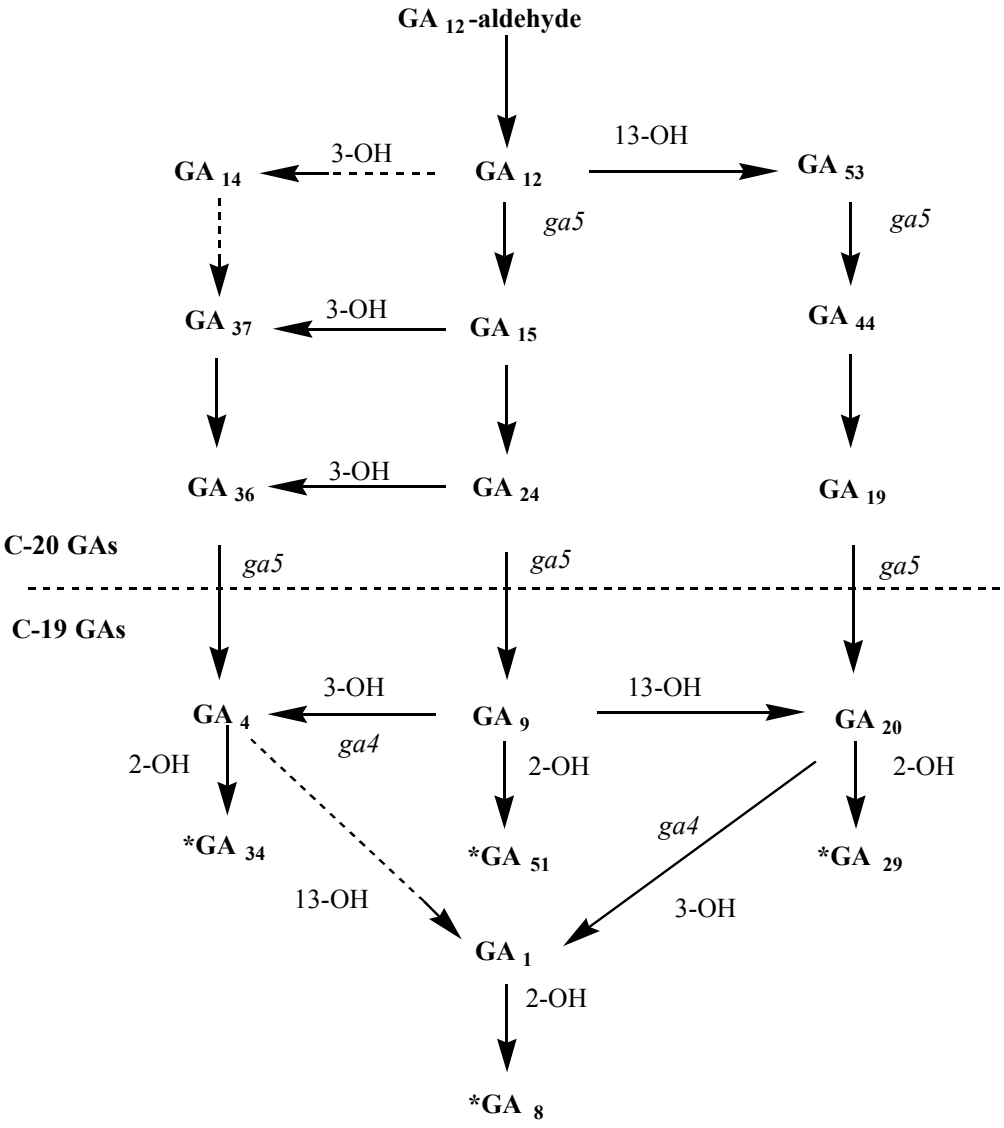


Fig 2.1 GA metabolism pathway in *Arabidopsis thaliana.* Three parallel pathways of GA metabolism after the synthesis of GA_{12} have been identified in *Arabidopsis*. Solid lines represent pathways that have been confirmed by other researchers. Dotted lines represent possible reactions. GA_{14} has not been identified in the feeding experiment in *Arabidopsis*. All biologically inactive forms of GAs are indicated with the aster on the upper corner.

Three parallel pathways of GA metabolism after the synthesis of GA_{12} have been found in *Arabidopsis*: the early 13-hydroxylation pathway, the non-3,13-hydroxylation pathway and the early 3-hydroxylation pathway (Fig 2.1). The early 13-

hydroxylation pathway begins with the conversion of GA_{12} to GA_{53}. As a result, a C-19 GA (GA_{20}) is generated at the end of this pathway. The non-3,13-hydroxylation pathway starts with the conversion of GA_{12} to GA_{15} followed by a series of oxidation which leads to the formation of GA_9. The third pathway, the early 3-hydroxylation pathway, first converts GA_{15} to GA_{37} and then to GA_4 through a series of continuous oxidation reactions. GA 20-oxidase, a single enzyme encoded by the *GA5* gene, is responsible for carrying out a series of oxidation steps of carbon 20 in *Arabidopsis*.

The expression of the GA 20-oxidase gene, *GA5*, has been studied at the molecular level in *Arabidopsis*. The increase in the expression of *GA5* was observed only when *Arabidopsis* plants were transferred from SD to LD (Phillips et al., 1995; Xu et al., 1995; 1997; 1999). The expression of *GA5* decreased when plants were treated with a bioactive GA such as GA_4 (Phillips et al., 1995; Xu et al., 1995). Furthermore, over-expression of *GA5* in *Arabidopsis* showed in earlier flowering plants with longer hypocotyls and elongated stems (Huang et al., 1998). The endogenous content of GA_1, GA_9 and GA_{20} were also significantly higher. These results suggest that the GA 20-oxidase gene may play a critical role in the regulation of GA biosynthesis in *Arabidopsis*.

Only a few GAs have been demonstrated to possess intrinsic biological activity in plants, and GA_1 is generally believed to be the one responsible for biological activity. However, no studies to date have been able to demonstrate which form of GAs is responsible for biological activities in *Arabidopsis*. Studies had shown that application of GA_4 on *ga4* plants, in which the 3ß-hydroxylation is impaired, restored

the wild-type phenotype while plants treated with GA_1 showed only moderate response in terms of stem growth. Results from other experiment also demonstrated no stem growth on plants treated simultaneously with GA_9 and BX-112, a GA biosynthesis inhibitor that inhibits a 3β-hydroxylation reaction required to generate biological active form of GAs (Zeevaart and Talon, 1992). There has been some evidence indicating that GA_4 may be converted to GA_1 via the C13-hydroxylation pathway (Zeevaart and Talon, 1992; Kobayashi et al., 1993). Clearly, more research is needed to identify the bioactive form of GA in *Arabidopsis*.

The main objectives of the research described in this chapter were to: 1) illustrate how regulation of the GA biosynthesis is altered by a change in the prevailing photoperiod, and 2) identify the most bioactive forms of GAs using *Arabidopsis*. The following approaches were taken to better address and elucidate the objectives as described above. First, the application of KA under different photoperiodic conditions would help to determine if KA hydroxylation is the regulatory step in GA biosynthesis in *Arabidopsis*. Quantitative analysis of endogenous KA content in plants transferred from SD to LD condition will provide further evidence of its involvement in the regulation of GA biosynthesis in *Arabidopsis.* Second, application of various GA precursors and GAs under both SD and LD conditions would help to identify the possible regulatory step(s) in the GA biosynthesis pathway. Stem height of each individual plant was used as a major criterion to evaluate the responses of *Arabidopsis* plants to different GA/KA treatments.

Materials and Methods

Plant Material and Growing Conditions

A line of *Arabidopsis* was selected from a population of the ecotype Columbia (CS933; Arabidopsis Resource Center, Ohio State University, Columbus OH) in which a large delay in flowering of plants grown under SD is eliminated by extending a 9h SD with 6h of low intensity light from incandescent lamps (Metzger and Zheng, 1998). Two to three seeds were germinated and grown in 7 cm diameter pots containing Metro-Mix 360 (Scotts-Sierra Horticultural Products Company, Marysville, OH). After two weeks one seedling was selected per pot. All experiments were conducted in a growth chamber with the temperature at 25°C. Light for the non-inductive (SD) treatment was supplied by fluorescent and incandescent lamps; the inductive (LD) treatment was generated by supplying the same light source as SD followed by an additional 6 h incandescent light. In most cases, experiments were conducted after plants were grown in the growth chamber for four to five weeks. Each treatment contained seven plants. Five different elements including rosette leaf number, cauline leaf number, total leaf number, stem height and petiole length were collected to evaluate the biological activity of GA.

Application of Various GAs and Kaurenoic Acid (KA) to Plants Grown under SD and LD

Three different experiments were conducted including application of various GAs and KA under non-inductive and inductive conditions, application of KA under

non-inductive and inductive conditions, and application of C-20 GAs under non-inductive conditions. All plants were grown under SD for 5 to 6 weeks before treatments began. Prior to being placed in LD for the inductive treatment, each plant was watered every other day with 2mM 2-chlorocholine chloride (CCC) for one week to inhibit the production of the endogenous GA (Metzger, 1990). No CCC was applied in the C-20 GAs application experiment since plants were maintained under non-inductive conditions. Plants were either transferred to LD or maintained under SD and both were treated with 10 μl of solution containing 0.1μg of the test compound (GA_1, GA_4, GA_9, GA_{12}, GA_{19} or KA), 50% (v/v) acetone and 0.05% polyoxyethylene-20-sorbitan monolaurate (Tween 20) to the apex in one experiment on alternate days for a total of six applications. Plants in the control group were treated with the same solution as described above except that no GA was added. Stem heights were measured two weeks after the last application. The number of rosette and cauline leaves on each plant was counted before and after the experiment. Since the application of 1 μg of KA or various GAs showed weak stem growth in the first experiment, I decided to increase the total amount of KA and various GAs in the subsequent experiments to 10 μg per treatment in order to obtain a stronger response and to understand their effect on stem growth by comparing data obtained in these two experiments.

Quantitative Analysis of Endogenous KA

The relationship between endogenous KA levels and LD-induced stem elongation was investigated. Plants were grown in SD for four to five weeks before being transferred to LD. After 0 (SD control), 1, 2, 5, 9 and 12 days in LD, plants were harvested into two parts (leaves and stems), frozen in liquid N_2, and lyophilized. Freeze dried samples were then stored at –80^{o}C until extraction.

Prior to extraction, samples were warmed to room temperature and weighed. Each sample was mixed with 90 ml of 100% methanol and ground with a homogenizer (Biospec Products, Bartesville, OK) for 5 min. The homogenized mixture was filtered through two layers of filter paper (Whatman International Ltd, Maidstone, England). The filter cakes were re-extracted with 50ml of 100% methanol and homogenized for 5 min. The two filtrates were combined and 100-200 ng of [^{2}H]-KA (a gift from Dr. L. Mander, Australian National University, Canberra, Australia) was added as an internal standard. Its volume was further reduced to about 90 ml by using a rotary evaporator (Büchi, Switzerland) at 30ºC. Distilled water was added to bring the total volume to 100 ml.

At least 15g of material from a C_{18} bulk solid-phase extraction packing (Alltech Associates, Deerfield, IL) was packed into a 60 ml syringe barrel that was connected to a vacuum manifold (Alltech Associates, Deerfield, IL). The filtrate was forced through the syringe at a pressure of 20 kPa. KA was eluted from the column with 10ml of 90% methanol. The eluate containing KA was then transferred to a 50 ml boiling flask and the methanol removed under reduced pressure. The remaining

aqueous solution was adjusted to pH 2.5 and extracted three times with equal volumes of hexane. The hexane phases were combined and dried under reduced pressure. The remaining residue was re-dissolved in 50 µl of methanol and subjected to reverse phase HPLC. Reverse-phase HPLC was conducted with an 805 Manometric Module (Gilson Medical Electronics Inc, Middleton, WI) and a 250x9.5 mm i.d. Partisil 10 ODS-3M column (Whatman, Inc Clifton, NJ). The solvent program began with 30% aqueous methanol for one min at a flow rate of 5 $ml.min^{-1}$. The concentration of methanol was linearly increased to 100% within 25 min and remained at 100% for 5 min. The fraction containing KA was collected between 26-27 min and dried using a Speed Vac Concentrator (Labconco, Inc, Kansas City, MO). Residues were then dissolved in 5 µl of acetonitrile and 10 µl N-methyl-N-trimethysilyl-trifluoroacetamide (MSTFA) to generate the trimethylsilyl ester of KA (TMSKA).

Purified samples containing TMSKA were analyzed by gas chromatography-mass spectrometry (GC-MS). The gas chromatograph was a Hewlett-Packard 5890 series II equipped with a split/splitless injector operating at 250ºC in the splitless mode and was connected to a HP 5927 series Mass Selective Detector. A 30mm x 0.25mm i.d. HP 5MS capillary column (Hewlett-Packard Co. USA) with a 0.2µm thickfilm of 5% phenylmethylsiloxane was used for the gas chromatography. Two µl of the samples were introduced into the capillary column and the temperature program was set to an initial 150º C for 2 min, followed by a 10ºC min^{-1} increase until reaching 300ºC and maintained at that temperature for an additional 2.5 min. Helium gas was used as the carrier gas with a head pressure of 100 kPa. The positive ions of the

endogenous methylated TMSKA and the TMS[^{2}H]-KA internal standard were measured by selected ion monitoring (SIM). The amount of endogenous KA was calculated using the equation below.

$$Y= [(Ci/Cf)-1]*X$$

Where Y is the amount of the free KA in the tissue in ng; Ci is the initial percentage of the internal standard; Cf is the final percentage of [^{2}H] KA, which was obtained by the ratio of the areas of m/z 374 over 374+376; X is the amount of [^{2}H] KA added in ng.

Application of C-19 GAs and GA Precursors under Non-inductive Conditions

The biological activities of C-19 GAs and GA precursors on the stem growth of CS933 plants were compared under SD. Seven different C-19 GAs, (GA_1, GA_3, GA_4, GA_5, GA_8, GA_9, GA_{20}) as well as the GA precursors kaurene, kaurenol, kaurenoic acid (KA) and steviol (13-C hydroxylated KA) were used in these studies. Ten µl of a solution containing 0.1µg of the test compound, 50% (v/v) acetone, and 0.05% Tween 20 were applied to the apex on alternate days for a total of six applications. Plants in the control group were treated with the same solution as described above except that no GA was added. Plants were maintained under SD after the last treatment for an additional 14 days before stem height was measured. The number of rosette and cauline leaves on each plant was also counted as described before. Since exogenous GA is known to increase petiole growth in *Thlaspi arvense*, a single young leaf of each plant was randomly selected and the lengths of that petiole before and after the treatment were recorded (Metzger, 1988)

Dose-Response Experiment

The relative biological activities of GA_1, GA_4 and GA_9 were compared by conducting a dose-response experiment. Plants were grown as described before and maintained under SD for the duration of the experiment. GA_1, GA_4 or GA_9 were used in this study. 0.1, 1, or 10 µg of the GAs were applied to the shoot tips three times a week for two weeks. The two lower concentrations of GAs were made by dissolving 1µg of each GA in 100µl of an aqueous solution containing 50%(v/v) acetone and 0.05%(v/v) Tween-20. A solution containing 70%(v/v) acetone and 0.05%(v/v) Tween-20 was used to dissolve 1 µg/µl of GAs. The height of the stem was measured from the soil level to the top of the inflorescence. The number of rosette and cauline leaves on each plant was counted as described before.

Results

2.1 Determination of the Rate-limiting Step in GA Biosynthesis Pathway

2.1.1 Application of KA under SD and LD

The effects of exogenous KA on stem growth between plants grown under non-inductive (SD) and inductive (LD) conditions were compared. Figure 2.2 showed KA had no effect on the number of rosette leaves of plants grown under SD or LD (Fig. 2.2). However, the number of rosette leaves (52) was greater on SD-grown plants than those grown under LD (41), indicating that LD significantly promoted flowering.

The number of cauline leaves was recorded on plants grown under both daylength conditions two weeks after KA treatment. The average number of cauline leaves was 7.5 and 12 for plants grown under SD and LD, respectively; however these two values were not significantly different (Fig 2.2).

Finally, plants grown under SD with or without KA treatment produced no significant difference in the total number of leaves. Moreover, exogenous KA had no effect on stem growth under SD. In contrast, application of KA in LD increased stem growth by nearly nine fold (Fig. 2.3). These results are consistent with the hypothesis that the conversion of KA to 7-OH KA represents a rate-limiting step under SD, and more KA was converted to some bioactive GA that resulted in stem growth upon transfer of plants to LD.

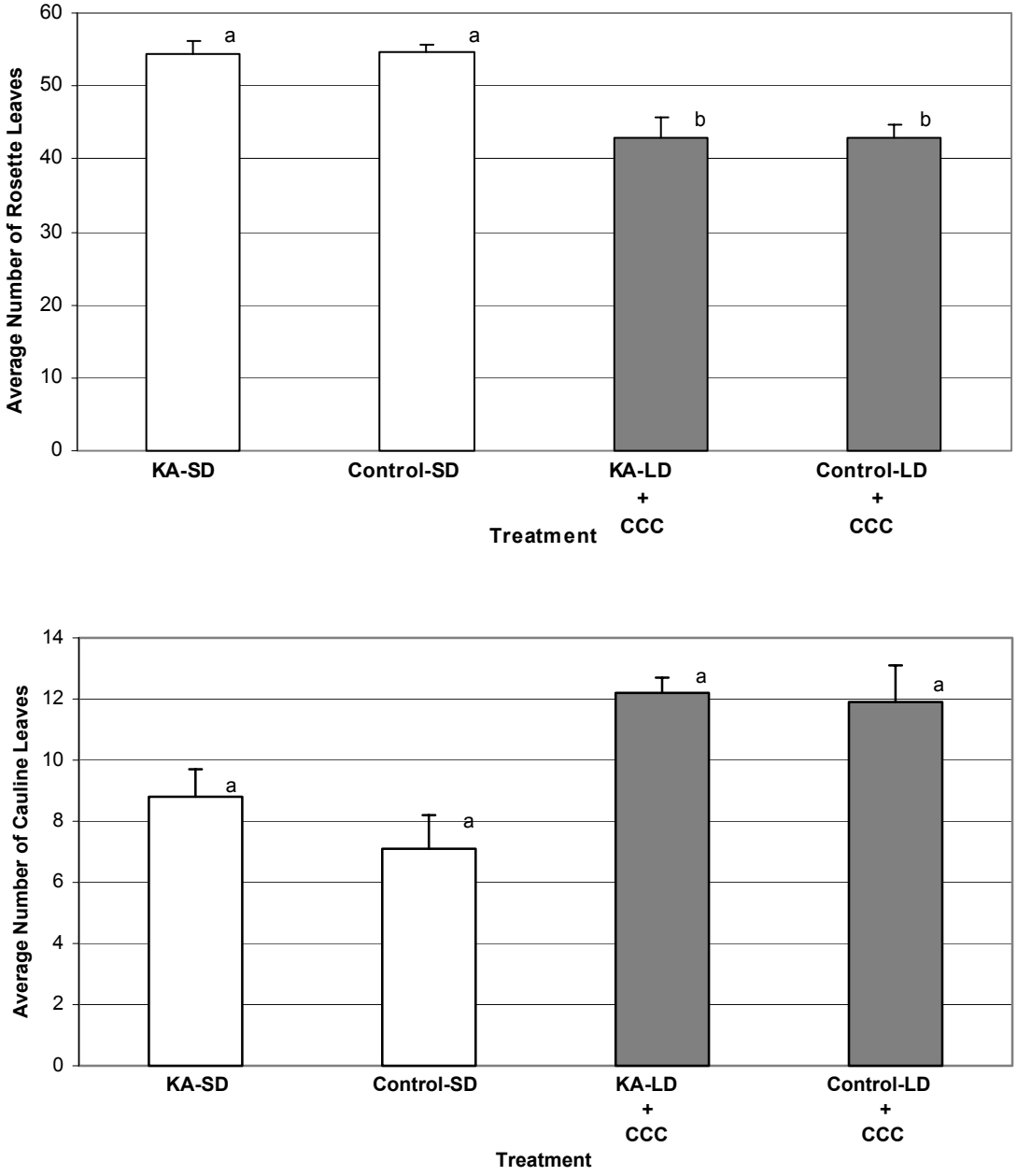


Figure 2.2 Effects of KA on the number of rosette and cauline leaves under non-inductive (SD) and inductive (LD) conditions. Plants were treated with 10 μg of KA three times a week for two weeks. Plants grown under LD were irrigated with 2 mM CCC every alternate day for two weeks. The number of rosette leaves was recorded two weeks after the last application. Values represent the means of 7 plants in each experiment for three individual experiments and the vertical bars represent standard errors of the means. Columns with different letters are significantly different (P=0.01) as determined by Student's t-test.

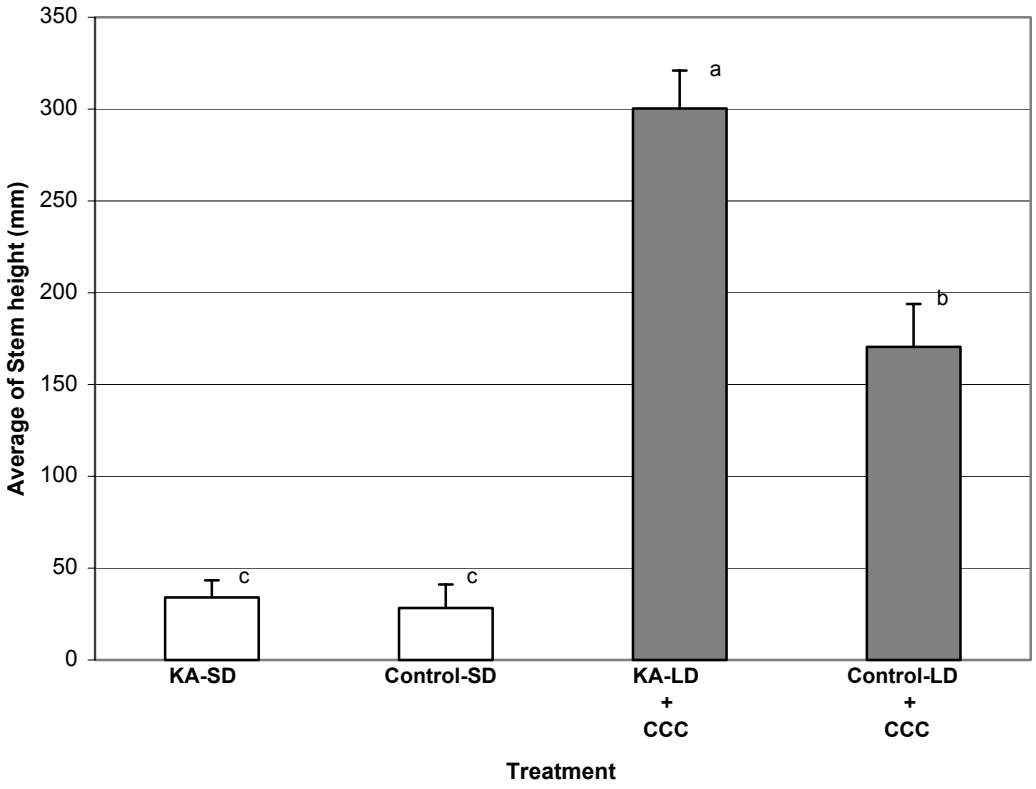


Figure 2.3 Effects of KA on stem growth under SD and LD. Plants were treated with 10 µg of KA three times a week for two weeks. Plants grown under LD were irrigated with 2 mM CCC every alternate day for two weeks. Stem height of each plant was measured two weeks after the last application. Values represent the means of 7 plants in each experiment for three individual experiments and the vertical bars represent standard errors of the means. Columns with different letters are significantly different (P=0.01) as determined by Student's t-test.

2.1.2 Quantitative Analysis of the Endogenous KA

Changes in endogenous KA levels in plants following transfer from SD to LD were monitored by GC-MS. Average endogenous KA levels declined from 23.6 ng per shoot to 9.3 ng per shoot 24 hours following the transfer from SD to LD where the levels remained relatively stable for the next 3 days (Fig 2.4). Five days after the initiation of the LD treatment, KA levels began to rise. This increase in KA levels coincided with the initiation of stem growth. However, KA levels appeared to level off at 14 days (Fig 2.4).

2.2 Determination of Other Steps in GA Biosynthesis Potentially under Photoperiodic Regulation

2.2.1 Application of KA and Various GAs under SD and LD

To locate other potential step(s) in GA biosynthesis that may be under photoperiodic control, the biological activities of six different GAs (GA_1, GA_4, GA_9, GA_{12}, GA_{19} and GA_{20}) were compared under SD and LD. GA_1, GA_4, GA_9 and GA_{20} are C-19 GAs whereas GA_{12}, and GA_{19} are C-20 GAs (Fig 2.5). Five different criteria were used to assess the biological activity of each GA. They include: number of rosette leaves, stem height, number of cauline leaves, petiole length and total number of leaves. Stem elongation was expected on plants treated with a more bioactive form of GA.

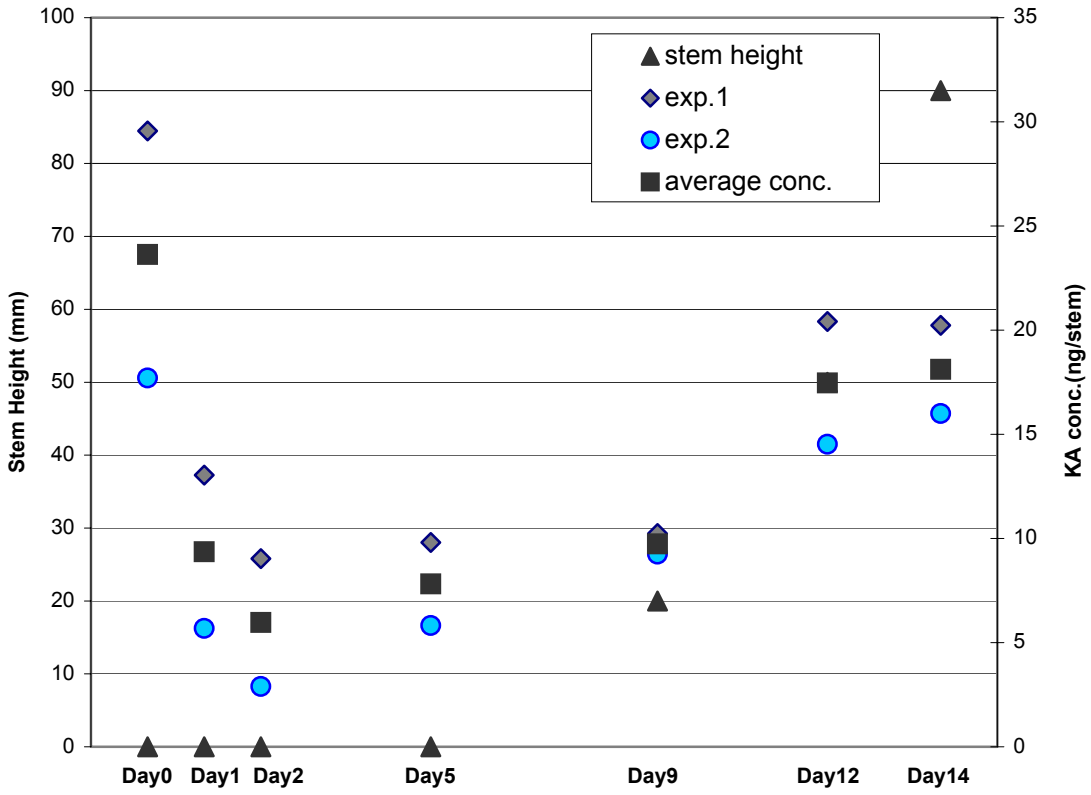


Figure 2.4 Effects of LD on stem growth and endogenous KA levels of *Arabidopsis* plants. Plants were grown under SD for six weeks before transferring to LD. Stems were harvested for the KA extraction in differential photoperiodic treatments. Day 0 represents the day plants were moved from short day to long day. Values indicated by ■ represent the average of two individual experiments. Values indicated by ◆ and ● represent actual individual data point obtained from the first and second experiment, respectively. Values indicated by ▲ represent the average of stem height.

None of the treatments had a significant effect on total leaf number (Fig 2.6). However, there were differences in the proportion of rosette and cauline leaves (Fig 2.7). In general, plants treated with C-19 GAs had fewer rosette leaves and proportionally greater number of cauline leaves than those treated with C-20 GAs (Fig.2.7). However, this shift in proportion of cauline leaves was much less pronounced with GAs containing a 13-OH (e.g. GA_{20} and GA_1) compared to non-13 hydroxylated GAs such as GA_9 or GA_4 (Fig 2.7). There was no apparent effect on the timing of flower initiation since plants from all treatments, including control plants, had visible flower buds (data not shown) at the end of the experiment and the same total leaf number (Fig 2.6).

These conclusions are corroborated by the data presented in Fig. 2.8. Although GA_4 and GA_9 elicited the greatest amount of stem growth, all of the C-19 GAs had, in varying degrees, some effect on stem growth in plants under SD. C-20 GAs and KA, were biologically inactive (Fig 2.8). C-19 GAs with a 13- OH (GA_1 and GA_{20}) had significantly less biological activity than GA_4 and GA_9.

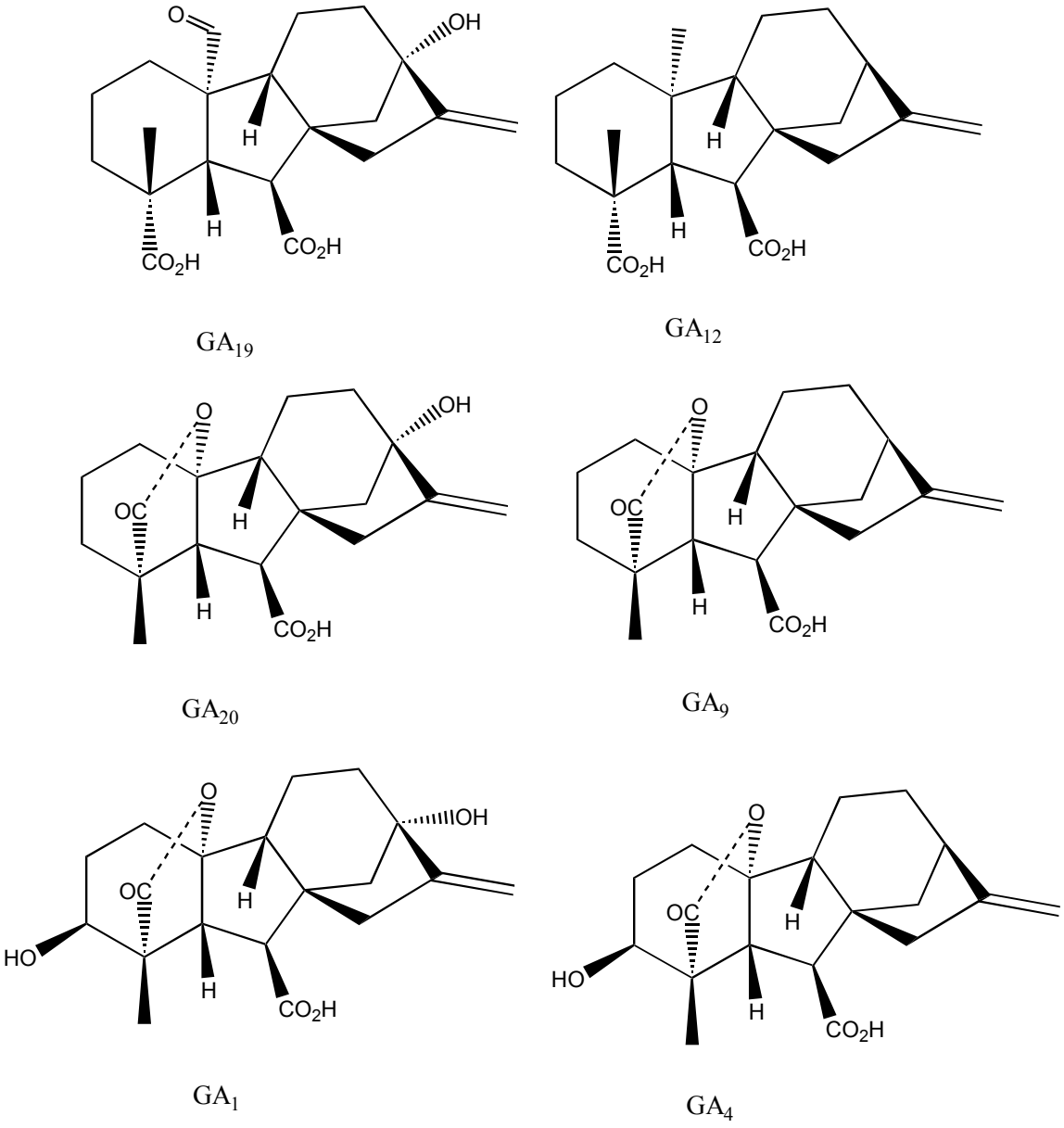


Figure 2.5 Structure of GAs used in the experiments described in 2.2.1. GA_{12} and GA_{19} are C-20 GAs whereas GA_1, GA_4, GA_9 and GA_{20} are C-19 GAs. A hydroxyl group is linked to GA_1 and GA_{20} at C-13 whereas no 13-hydroxyl group is present in GA_4 and GA_9.

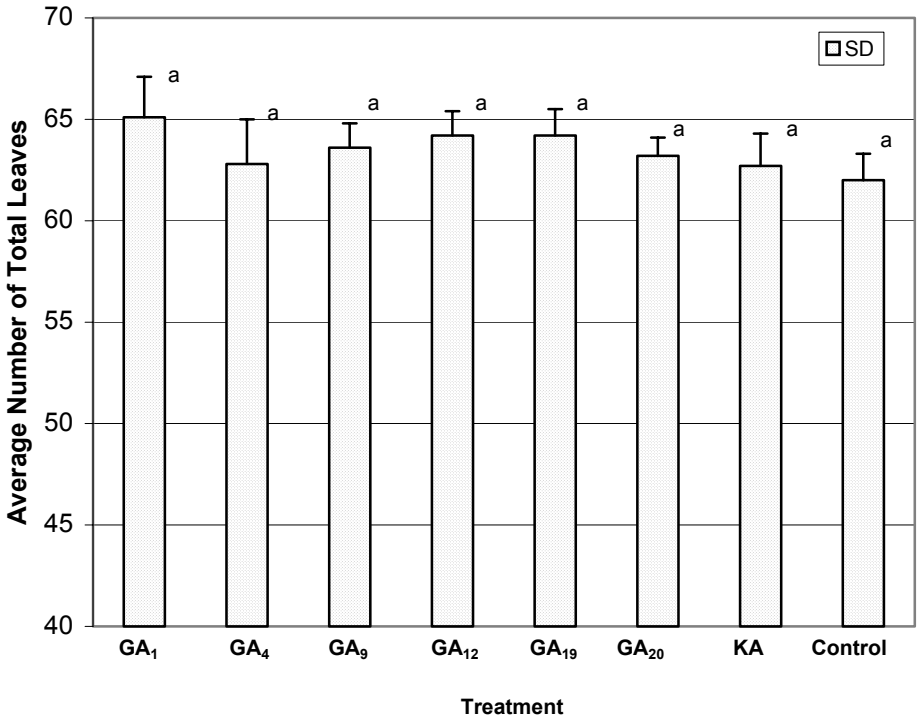


Figure 2.6 Effects of KA and various GAs on the total number of leaves under SD. Plants were treated with 1 μg of KA or various GAs three times a week for two weeks. The total number of leaves is the sum of both rosette and cauline leaves. The number of rosette and cauline leaves of each plant was recorded two weeks after the last application. Values represent the means of 7 plants in each experiment for three individual experiments and the vertical bars represent standard errors of means. Columns with different letters are significantly different (P=0.01) as determined by Student's t-test.

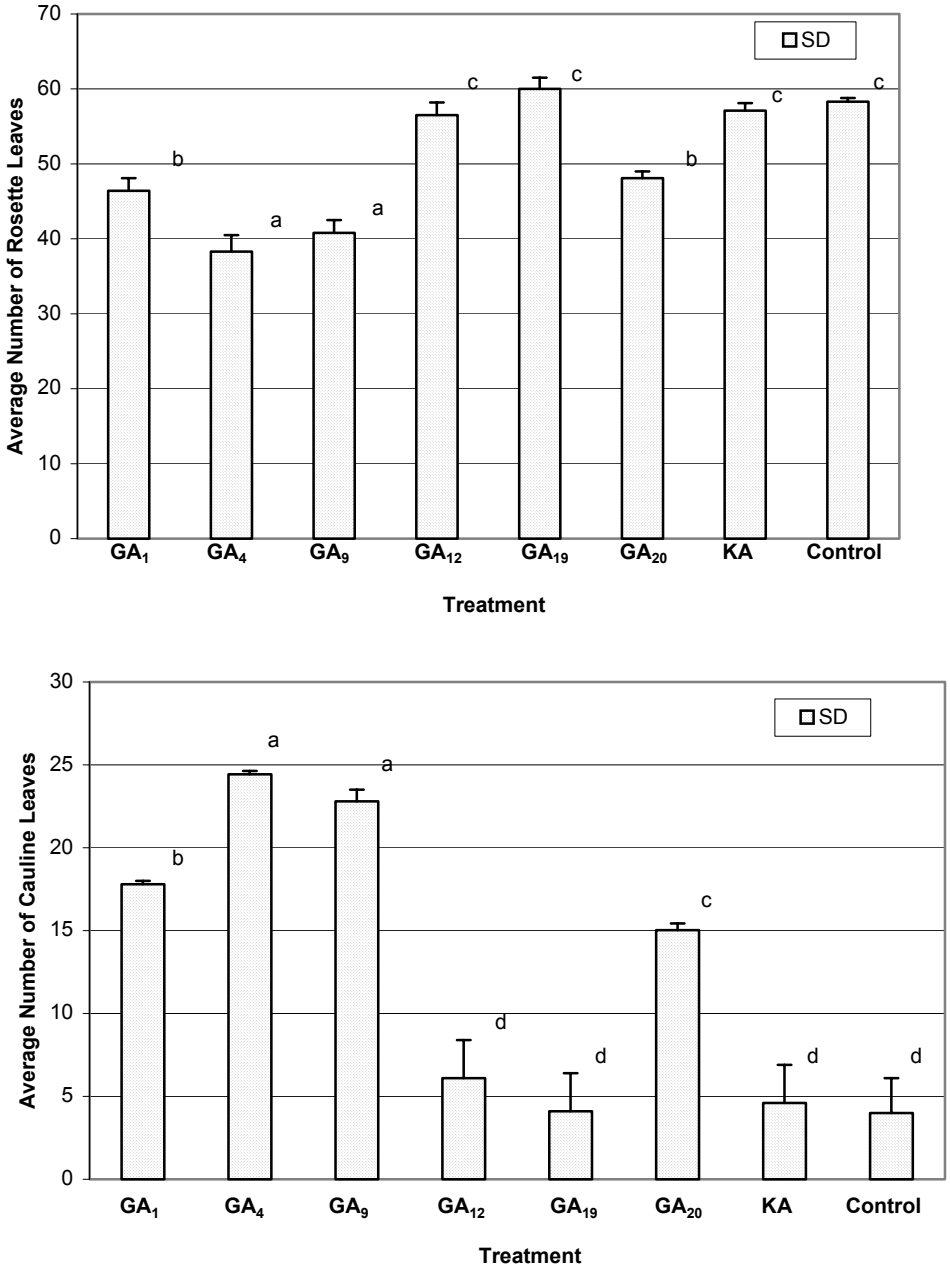


Figure 2.7 Effects of KA and various GAs on the number of rosette and cauline leaves under SD. Plants were treated with 1 µg of KA or various GAs three times a week for two weeks. The number of rosette and cauline leaves of each plant was recorded two weeks after the last application. Values represent the means of 7 plants in each experiment for three individual experiments and the vertical bars represent standard errors of means. Columns with different letters are significantly different (P=0.01) as determined by Student's t-test.

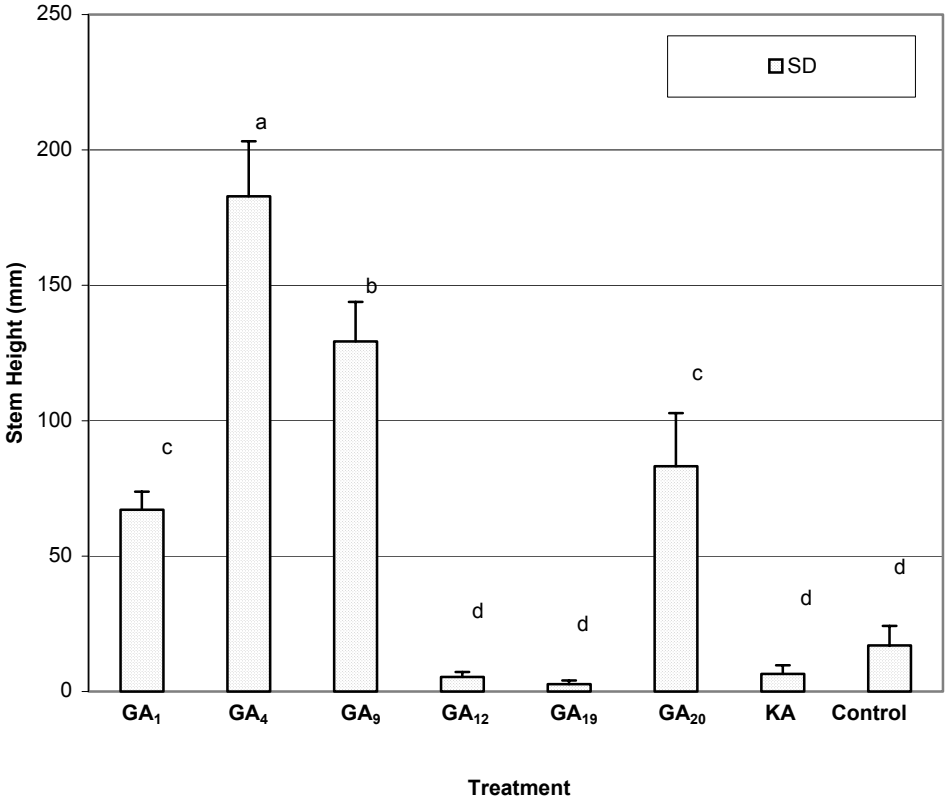


Figure 2.8 Effects of KA and various GAs on stem growth of rosette *Arabidopsis* plants under SD. Plants were treated with 1 μg of KA or various GAs three times a week for two weeks. Stem height of each plant was recorded two weeks after the last application. Values represent the means of 7 plants in each experiment for three individual experiments and the vertical bars represent standard errors of means. Columns with different letters are significantly different (P=0.01) as determined by Student's t-test.

The biological activities of the GAs were compared when plants were subjected to LD. No significant differences in the total number of leaves produced by plants were observed among the various treatments (Fig 2.9). However, there were significant differences in the proportion of rosette and cauline leaves among the

various treatments (Fig 2.10). As was observed when the same GAs were applied to plants under SD, those treated with C-19 GAs produced a greater proportion of cauline leaves (Fig 2.10). The two 13-hydroxylated C-20 GAs, GA_1 and $GA_{20,}$ also caused a shift in the proportion of cauline leaves, but the effect was less pronounced (Fig 2.10). The difference in biological activities between C-19 GAs based on 13-hydroxylation was also observed under SD (Fig 2.7).

As was observed in SD, there was no increase in the proportion of cauline leaves on plants with KA or C-20 GA treatment with the exception of GA_{12} (Fig 2.10). Thus, apparently transfer to LD resulted in an increased conversion of GA_{12} to the biological active form of GA that led to stem growth.

Flowering was promoted by LD as indicated by the reduction in total leaf number of the controls compared to SD (Fig 2.6 and 2.9). Thus, there was no apparent promotion of flowering by GA since application of none of the GAs resulted in further reduction in total leaf number (Fig 2.9).

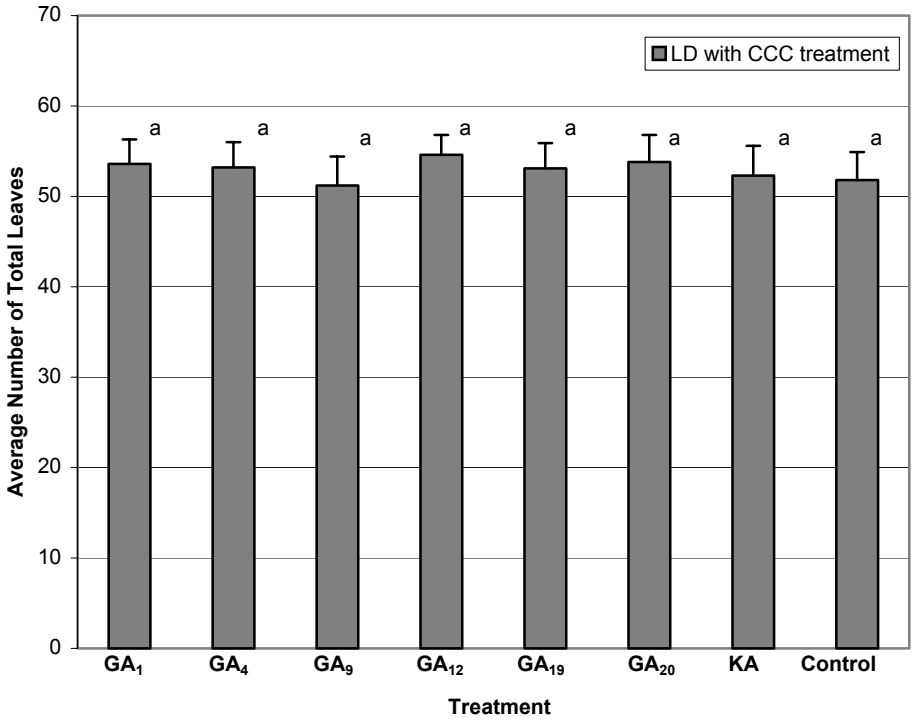


Fig.2.9 Effects of KA and various GAs on the total number leaves when applied to plants under LD. Plants were irrigated with 2 mM CCC on alternate days in addition to the applications of 1 µg of KA or various GAs three times a week for two weeks. The number of total leaves of each plant was recorded two weeks after the last application. Values represent the means of 7 plants in each experiment for three individual experiments and the vertical bars represent standard errors of means. Columns with different letters are significantly different (P=0.01) as determined by Student's t-test.

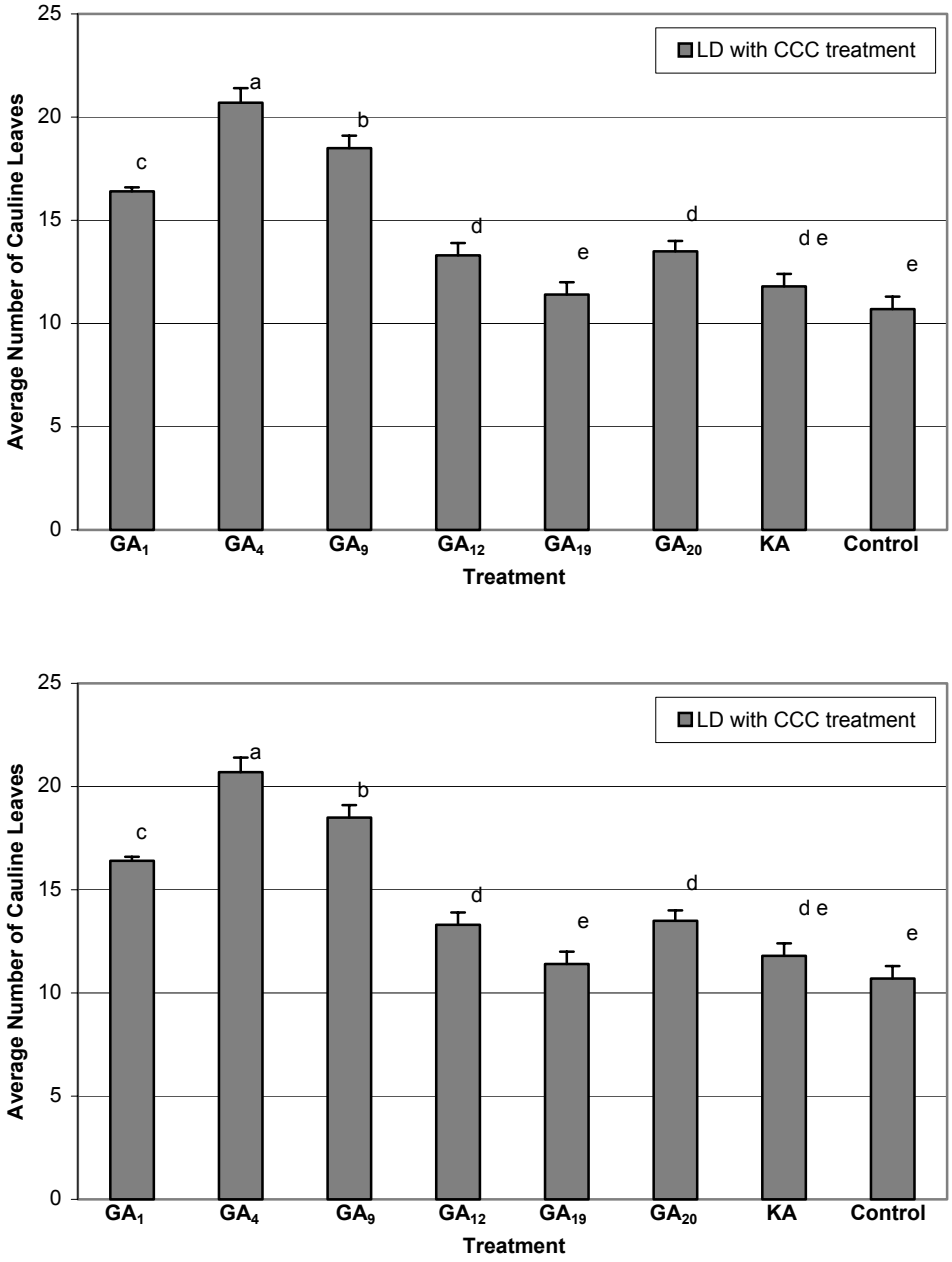


Figure 2.10 Effects of KA and various GAs on the number of rosette and cauline leaves when applied to plants under LD. Plants were irrigated with 2 mM CCC on alternate days in addition to the applications of 1 µg of KA or various GAs three times a week for two weeks. The number of rosette leaves of each plant was recorded two weeks after the last application. Values represent the means of 7 plants in each experiment for three individual experiments and the vertical bars represent standard errors of means. Columns with different letters are significantly different (P=0.01) as determined by Student's t-test.

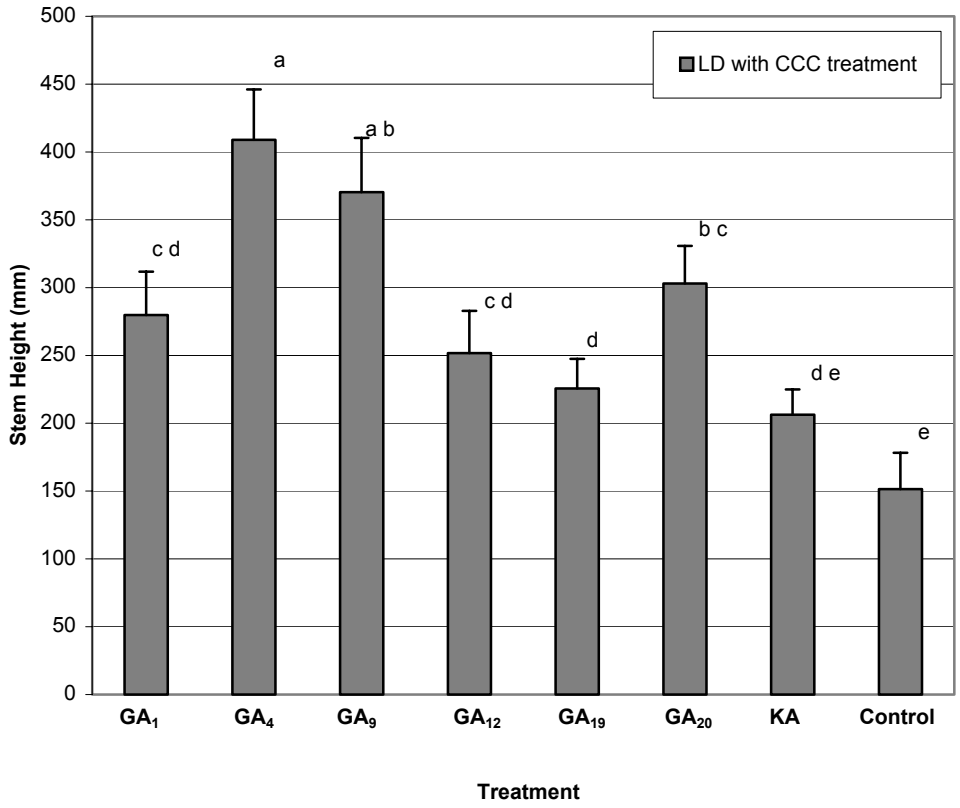
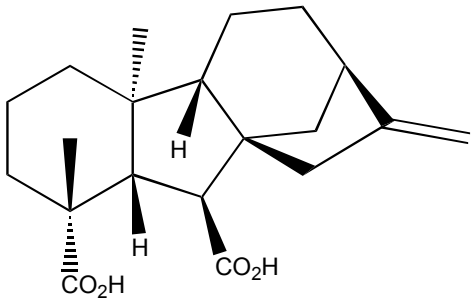
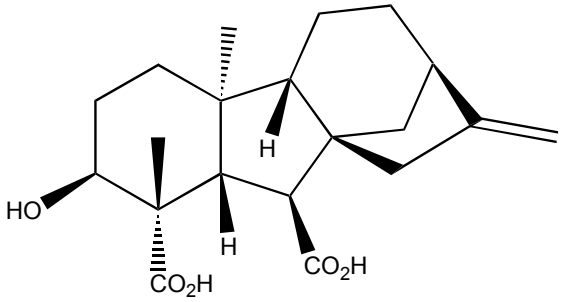


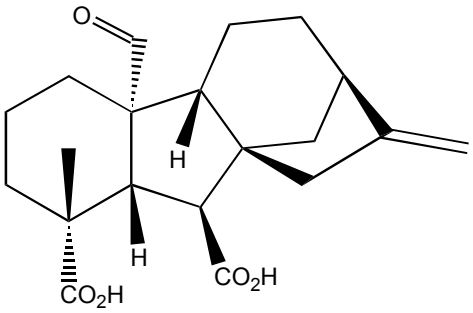
Figure 2.11 Effects of KA and various GAs on stem growth when applied to plants under LD. Plants were irrigated with 2 mM CCC on alternate days in addition to the applications of 1 µg of KA or various GAs three times a week for two weeks. Stem height of each plant was recorded two weeks after the last application. Values represent the means of 7 plants in each experiment for three individual experiments and the vertical bars represent standard errors of the means. Columns with different letters are significantly different (P=0.01) as determined by Student's t-test.



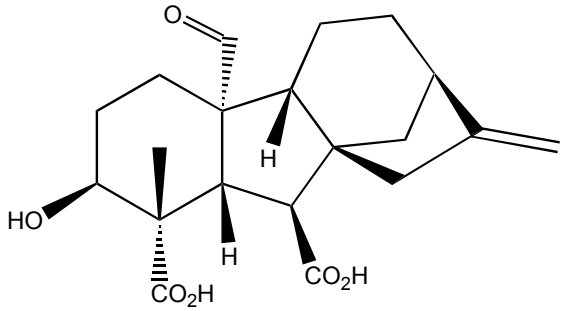
GA_{12}



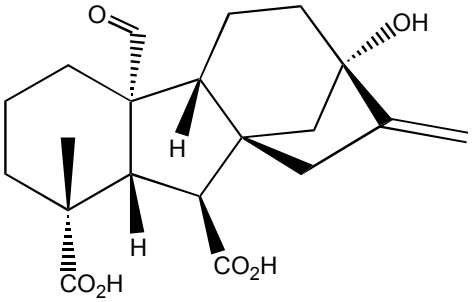
GA_{14}



GA_{24}



GA_{36}



GA_{19}

Figure 2.12 Structure of various C-20 GAs described in 2.2.2. The only structural difference between GA_{12} and GA_{14} is the additional hydroxyl group at carbon 3. The same variation also exits between GA_{24} and GA_{36} whereas GA_{19} has a hydroxyl group on carbon 13.

2.2.2 Effect of Various C-20 GAs on Stem Growth under SD

To further test if GA 20-oxidase is a rate-limiting step under SD, the effects of various C-20 GAs on stem elongation under SD were compared. The structures of the GAs are shown in Fig. 2.13. Two of the GAs contain a methyl group at carbon 20 (GA_{12} and GA_{14}) while an aldehyde is present at carbon 20 in GA_{19}, GA_{24} and GA_{36}. The GAs also vary by number or position of hydroxyl groups. Both GA_{12} and GA_{24} have no hydroxyl group while GA_{19}, GA_{14} and GA_{36} have one hydroxyl group located on position 3 or 13, respectively (Fig 2.12). Since the structures of GA_{14} and GA_{36} are very similar to GA_4, a strong growth response on plants with these two GA treatments was expected. On the other hand, plants treated with GA_{24}, a GA_9 precursor, were expected to exhibit only an intermediate response.

Plants treated with GA_{14} (a carbon 3 hydroxylated homolog of GA_{12}) had fewer rosette leaves but many more cauline leaves (Fig 2.13). The effects of the biological activity of GA_{24}, GA_{36} and GA_{19} on the number of rosette leaves numbers were compared. These three C-20 GAs contain an aldehyde at carbon 20, but differ in the number and location of hydroxyl groups. GA_{24} contains no hydroxyl groups, while GA_{19} and GA_{36} contain a hydroxyl group at carbon 13 and 3, respectively (Fig 2.12). Under SD, plants treated with GA_{36} had fewer numbers of rosette leaves but more cauline leaves than those treated with GA_{24} or GA_{19}

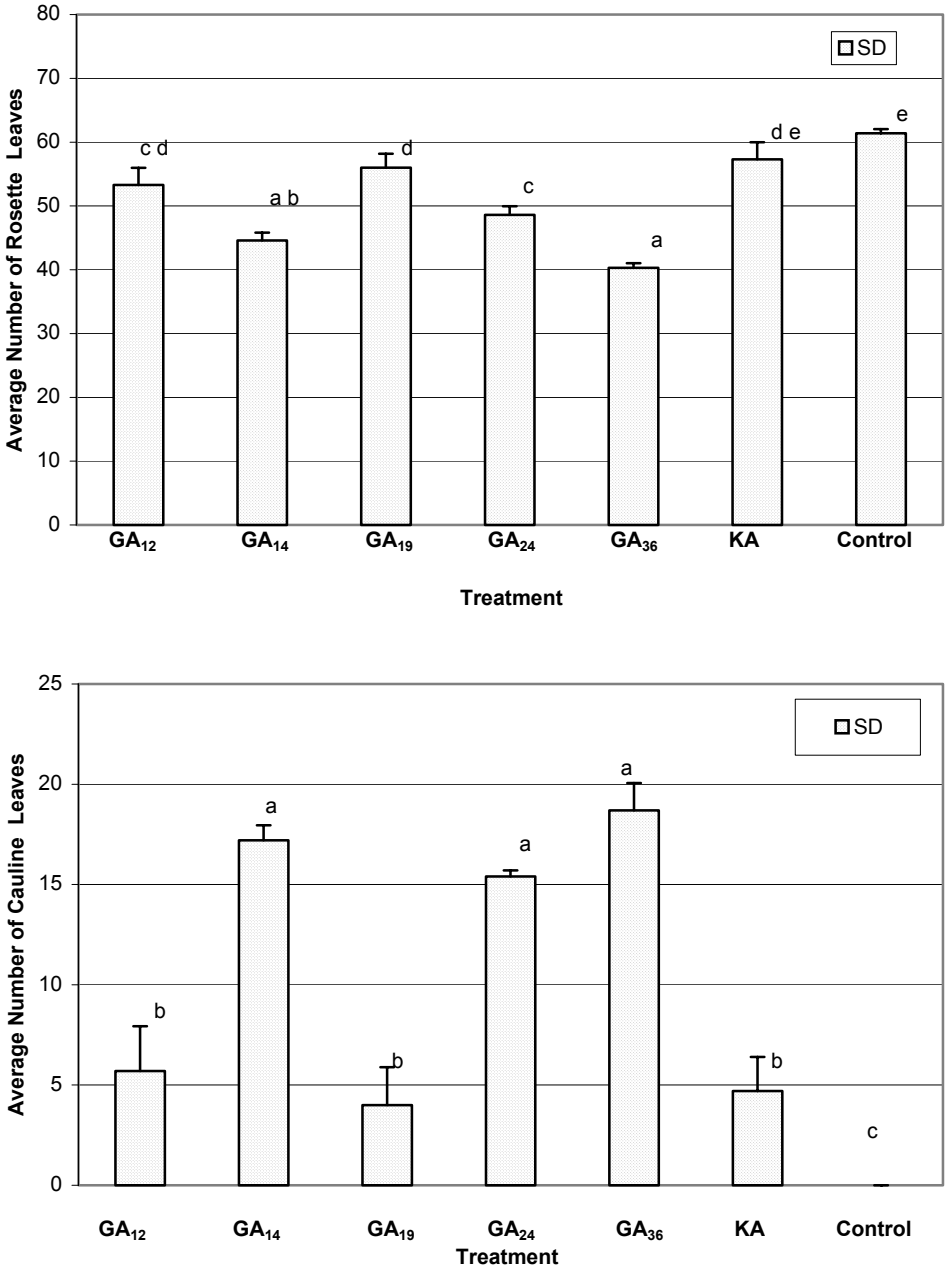


Figure 2.13 Effects of KA and C-20 GAs on the number of rosette and cauline leaves under SD. All plants were treated with 10 μg of KA or various GAs three times a week for two weeks. The number of rosette and cauline leaves of each plant was recorded two weeks after the last application. Values represent the means of 7 plants in one experiment and the vertical bars represent standard errors of means. Columns with different letters are significantly different (P=0.01) as determined by Student's t-test.

No statistical differences in rosette and cauline leaf numbers were observed between KA-treated plants and plants with GA_{19} application (Fig 2.13). When comparing the effects of GAs from both non-3, 13-hydroxylation and 3ß-hydroxylation pathways on the growth of *Arabidopsis* plants, both GA_{14} and GA_{36} treated plants produced fewer rosette leaves than those with GA_{12} and GA_{24} application (Fig 2.13). No effect on the number of rosette leaves was observed on plants treated with GA_{12} and GA_{24}. Similar results were observed on plants treated with GA_{14} and GA_{36} treatment (Fig 2.13).

Maximum stem growth was elicited with GA_{24} and GA_{36}; the presence of a hydroxyl group on carbon 13 (GA_{19}) resulted in a greater reduction in biological activity (Fig 2.14). Hydroxylation at position 3 had some positive effects on stem elongation only when carbon 20 was a methyl group (GA_{12} vs. GA_{14}) (Fig 2.14).

2.3 Determination of Biologically Active Form of GA

2.3.1 Application of GA Precursors and C-19 GAs under SD

To further investigate structure /function relationship, I examined the effects of additional C-19 GAs and GA precursors by applying them to *Arabidopsis* plants under SD using the same application methods as described before. While GA_1 and GA_3 are hydroxylated at carbon 3 and 13, respectively, GA_3 contains a double bond between carbon 1 and 2 (Fig 2.15). GA_9 and GA_{20} have one hydroxyl group located at carbon 3 or 13 respectively. GA_5 also has one hydroxyl group at carbon 13; but, in addition it

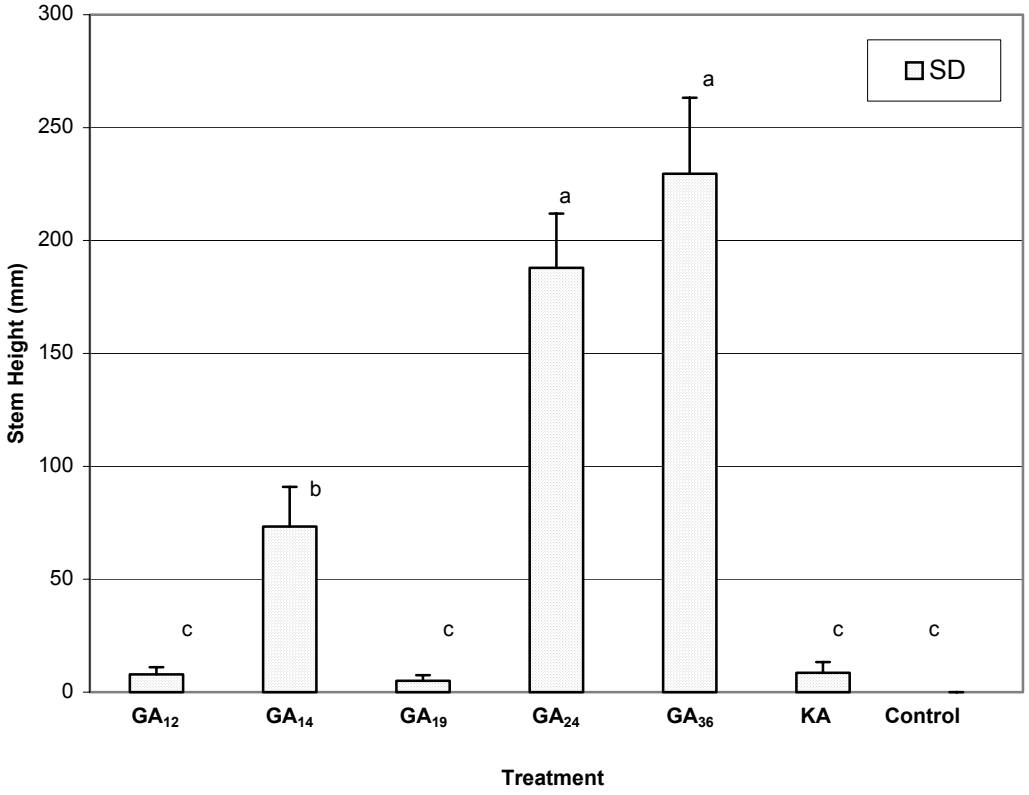
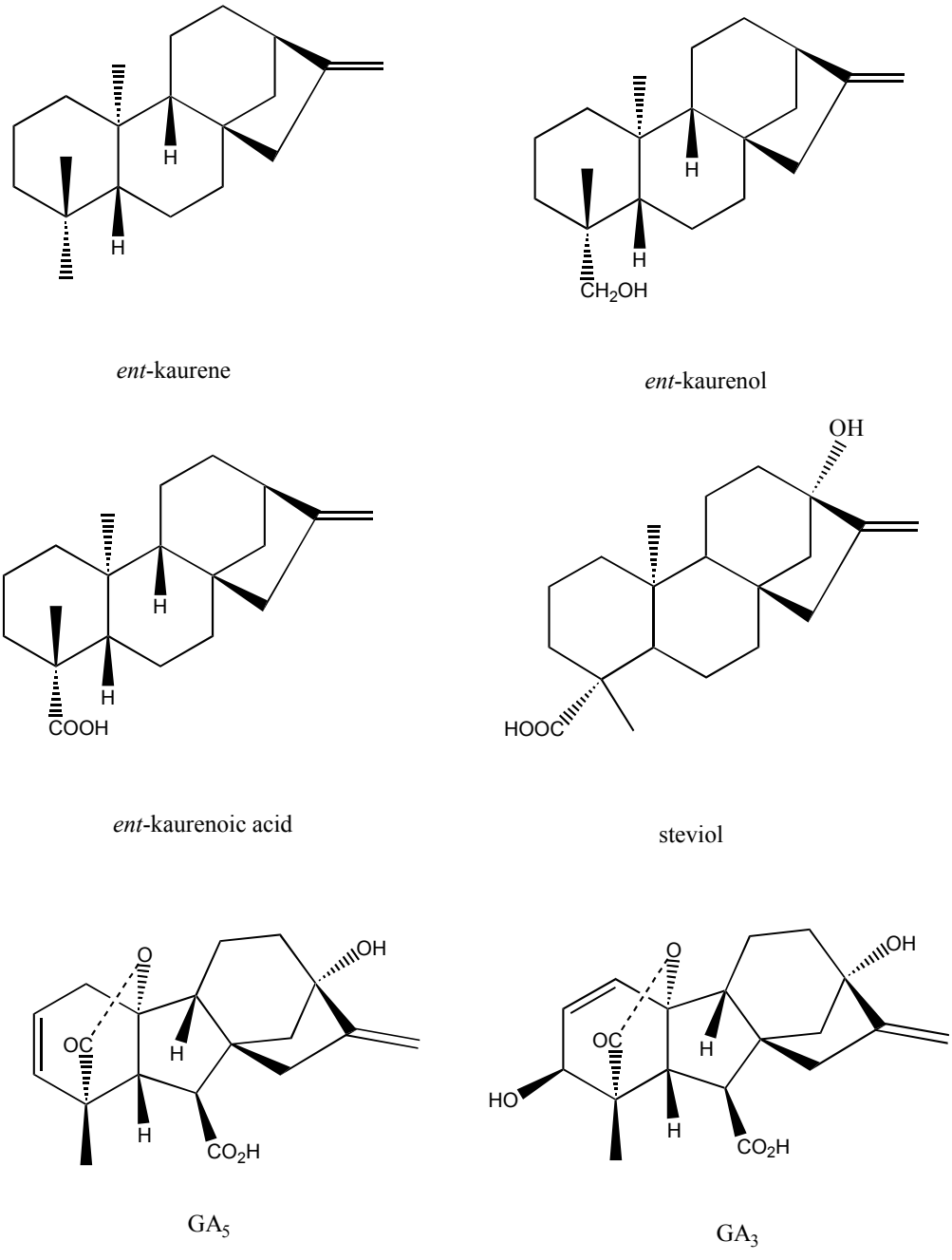


Figure 2.14 Effects of KA and C-20 GAs on stem growth under SD. All plants were treated with 10 µg of KA or various GAs three times a week for two weeks. Stem height of each plant was recorded two weeks after the last application. Values represent the means of 7 plants in one experiment and the vertical bars represent standard errors of means. Columns with different letters are significantly different (P=0.01) as determined by Student's t-test.

contains a double bond located between carbon 2 and 3. GA_8, which has three hydroxyl groups on carbons 2, 3 and 13, is believed to be inactivated product of GA_1. In *Arabidopsis* and maize, GA_{20} serves as the immediate precursor to GA_5, which is converted directly to GA_3 (Fujioka et al., 1990; Phinney, 1984). Steviol, an analog of kaurenoic acid containing an additional hydroxyl group at carbon 13 was also used in this study.

No significant difference was found in the number of rosette and cauline leaves between plants treated with GA precursors (kaurene, kaurenol and KA) and steviol in SD (data not shown). A smaller number of rosette leaves and more cauline leaves were observed on plants with GA_4 and GA_9 treatment. The ability to reduce rosette leaf number was significantly reduced when plants were treated with GAs with a hydroxyl group at carbon 13 (GA_1, GA_5 and GA_{20}) (Fig 2.16). Only plants treated with GA_3 showed the same response as GA_4 and GA_9 in SD (Fig 2.16). This indicates that the biological activity of GA in *Arabidopsis* is determined by the degree and variation of hydroxylation. While petiole growth was well correlated with GA bioactivity in *Thlaspi arvense* (Metzger, 1988), no significant difference in petiole growth was found among GA treatments in *Arabidopsis* (data not shown).



ent-kaurene

ent-kaurenol

ent-kaurenoic acid

steviol

GA_5

GA_3

Figure 2.15 Structure of steviol, C-19 GAs and GA precursors. Kaurene, kaurenol and kaurenoic acid (KA) are the precursors in the GA biosynthesis pathway. Steviol, a molecule with its structure similar to KA, has an additional hydroxyl group on carbon 13. GA_5 is the precursor of GA_3 in the GA biosynthesis pathway in maize (Fujioka et al., 1990).

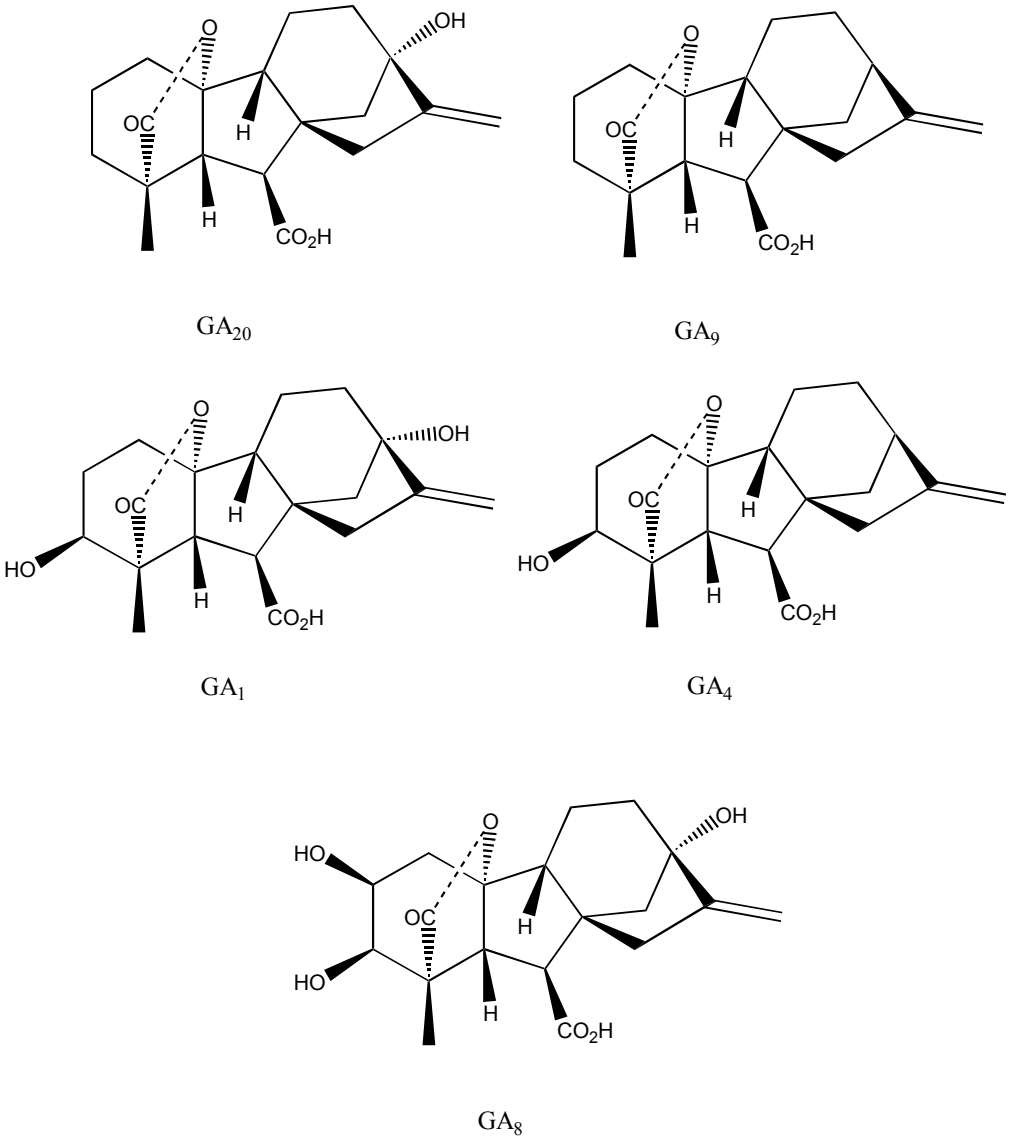


Figure 2.15 (Cont.) Structures of C-19 GAs used in the experiments. GA_{20} is one of the products in the early C13-hydroxylation pathway and is converted into GA_1. Further conversion takes place to generate an inactive form of GA_8. GA_9, a non 3,13hydroxy-GA, is converted to GA_4 by a 3ß-hydroxylation reaction (Phinney, 1984).

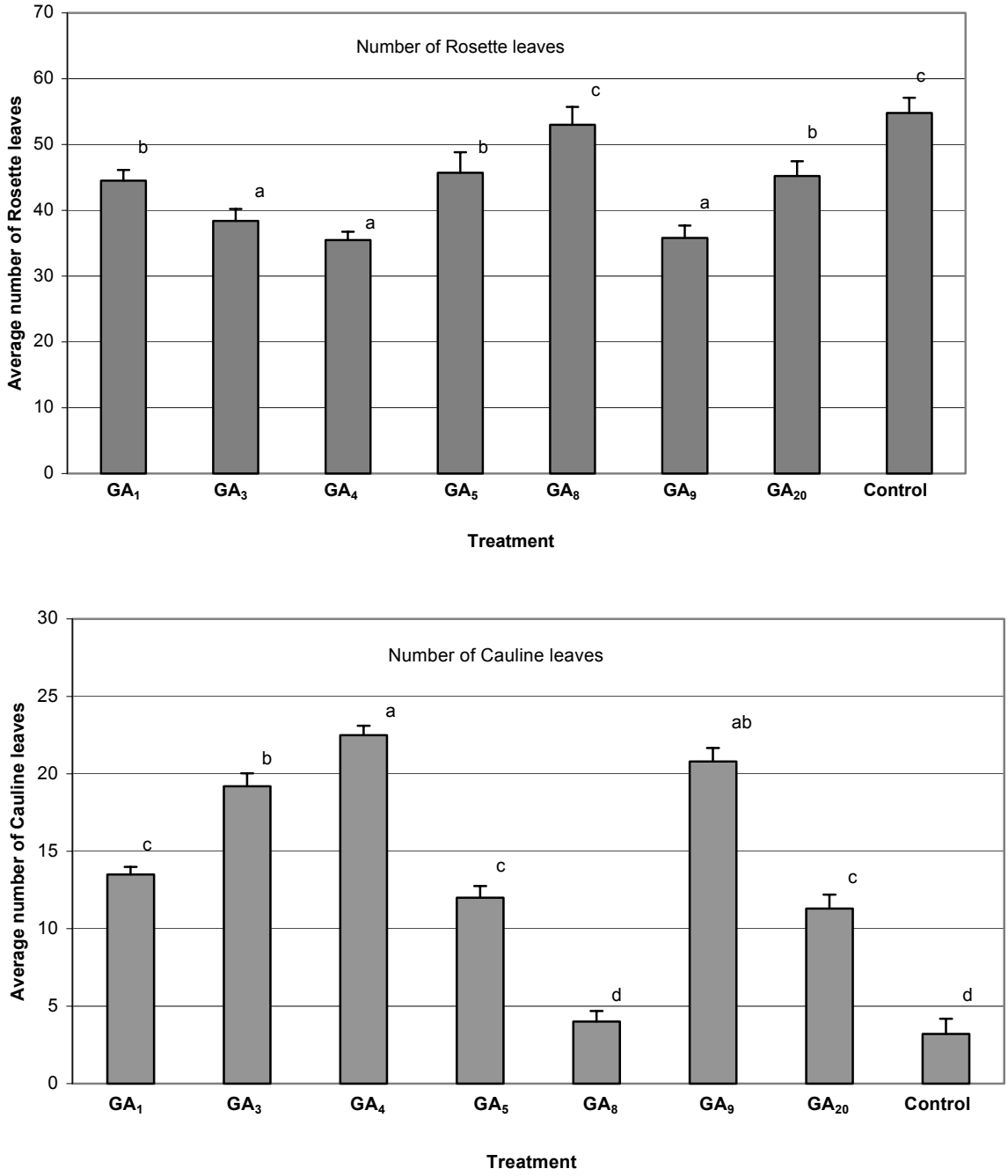


Figure 2.16 Effects of C-19 GAs and GA precursors on the number of rosette and cauline leaves under SD. Plants were treated with 1 μg of GAs or GA precursors three times a week for two weeks. The number of rosette and cauline leaves in each plant was recorded two weeks after the last application. Values represent the means of 7 plants in each experiment for three individual experiments and the vertical bars represent the standard errors of the means. Columns with different letters are significantly different (P=0.01) as determined by Student's t-test.

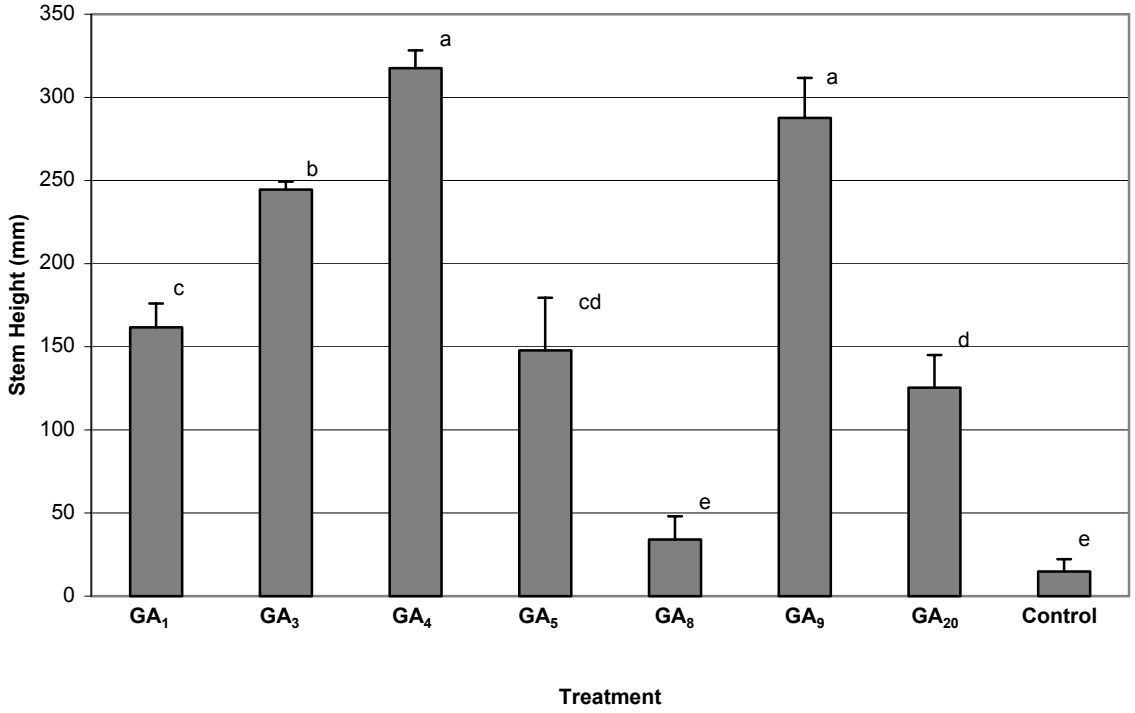


Figure 2.17 Effects of C-19 GAs and GA precursors on stem growth under SD. Plants were treated with 1 μg of GAs or GA precursors three times a week for two weeks. Stem height of each plant was measured two weeks after the last application. Values represent the means of 7 plants in each experiment for three individual experiments and the vertical bars represent the standard errors of the means. Columns with different letters are significantly different (P=0.01) as determined by Student's t-test.

2.3.2 Dose Response Experiment

Previous results suggested that GA_4 and GA_9, the products of the 3ß-hydroxylation pathway promote stem growth more than the 13-hydroxylation products such as GA_1 and GA_{20}. To further compare the biological activities of the endogenous

GAs in *Arabidopsis*, three different concentrations (0.1, 1, and 10 µg) of GA_1, GA_4 and GA_9 were applied to plants in SD.

Plants treated with 0.1 µg of GA_4 had fewer rosette leaves than those treated with GA_9 or GA_1 (Fig.2.18). There was a slight difference in the number of rosette leaves between controls and those treated with GA_1. Significant differences in the number of rosette leaves were found among three GA treatments at 0.1µg per plant (Fig 2.18). Although higher rosette leaf numbers were exhibited by plants treated with 1µg of GA_1 compared to those treated with GA_4 and GA_9, no difference in rosette leaf numbers was observed between 1 µg of GA_4 and GA_9. However, this trend did not continue on plants treated with 10 µg.

Significant differences in rosette leaf numbers were observed on plants treated with various concentrations of GA_1. While differences in rosette leaf numbers were observed in plants treated with 0.1 µg of GA_4 and GA_9, there was no statistical significant difference in rosette leaf numbers with either 1 or 10 µg of GA_4 and GA_9.

In terms of the proportion of total number of leaves that were cauline, the relative order of biological activity was $GA_4 \geq GA_9 > GA_1$. This is shown in Figure 2.18. First, the order of effectiveness at the lowest dose (0.1µg) was $GA_4 \geq GA_9 > GA_1$. Second, the maximum cauline leaf number was obtained with 1µg GA_4 and GA_9 while 10µg of GA_1 was required to achieve the same result (Fig. 2.18).

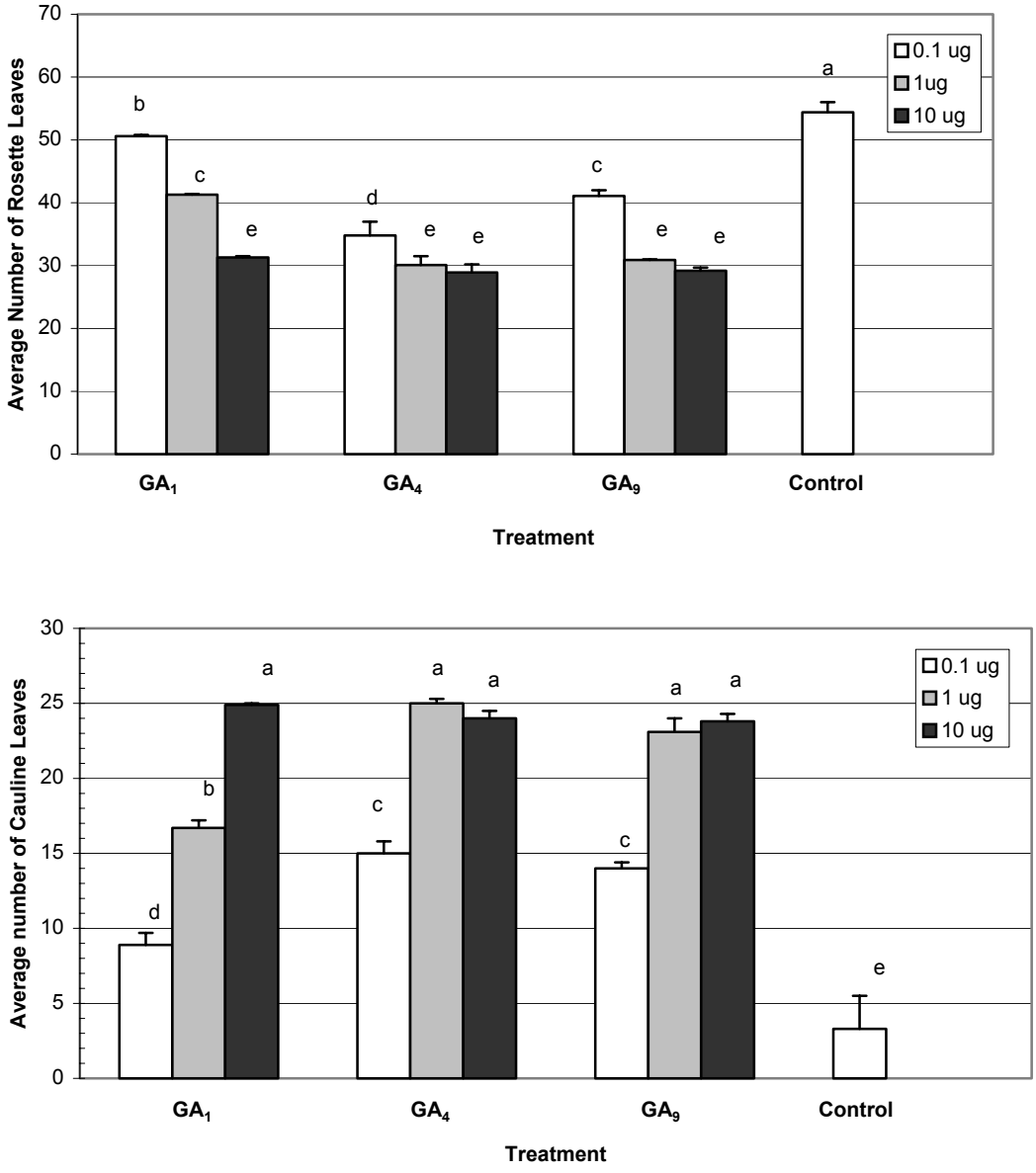


Figure 2.18 Comparison of the effects of different doses of GA_1, GA_4 and GA_9 on the relative number of rosette and cauline leaves when applied in SD. Plants were treated with 0.1, 1 or 10 µg of the three GAs three times a week for two weeks. The number of rosette and cauline leaves was recorded two weeks after the last application. Values represent the means of 7 plants in each experiment for three individual experiments and the vertical bars represent the standard errors of the means. Different letters above individual columns indicate significant differences (P=0.01) as determined by Student's t-test.

The dose-response relationships were clarified further by analyzing the effects of different doses of each GA on stem growth. Stem growth was plotted as a function of log amount of the applied GA (Fig. 2.19). Stem heights of plants treated with 0.1 μg of GA_1 were distinctly lower than those treated with GA_4 or GA_9 (Fig 2.19). However, no difference in stem height was found between the 0.1 μg and 1μg treatments (Fig 2.19). Plants responded the most to 10 μg of GA_4, while stem growth was the same in plants treated with 10 μg of GA_1 or GA_9 (Fig 2.19).

Statistical analysis showed non-parallel regression lines between GA_1, GA_4 and GA_9 suggesting the effect of each GA on stem growth is significantly different. Another way to compare the biological activity of each GA is by looking at the minimum amount of each GA required to promote stem growth. The x-intercept value of the regression line of GA_1 indicates that more than 0.106 μg of GA_1 is needed to promote stem elongation. In contrast, only 0.018 of GA_4 and 0.019 μg of GA_9 were needed to stimulate stem elongation based on the x-intercept value of their regression line (Fig 2.19). In other words, GA_4 and GA_9 were 5 times more effective in promoting stem growth than GA_1.

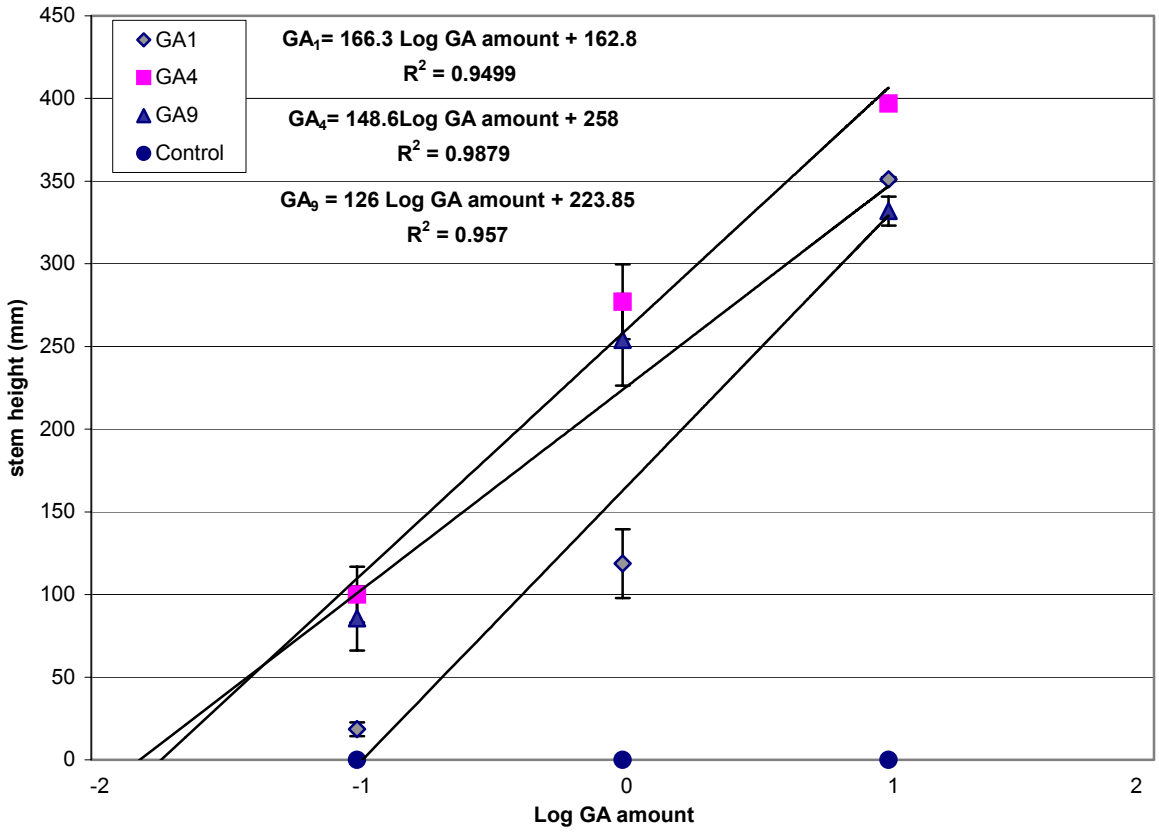


Figure 2.19 Comparison of the effects of different doses of GA_1, GA_4 and GA_9 on stem growth in SD. Plants were treated with 0.1, 1 or 10 µg of the three GAs three times a week for two weeks. Stem height of each plant was recorded two weeks after the last application. Each regression line was created based on the average of 21 plants in each concentration in the experiment. The experiment was repeated once with similar results. Values indicated by different symbols represent the means of two repeats experiments.

Discussion

KA Hydroxylase May not be under Photoperiodic Control in *Arabidopsis*

Experimental results with *Thlaspi* led to the hypothesis that the conversion of KA to 7-OH KA is the rate-limiting step in GA biosynthesis (Hazebroek and Metzger, 1990). Since *Arabidopsis,* a member of the same family as *Thlaspi* (Brassicaceae), was used in my studies to determine regulated step(s) in GA biosynthesis, one would predict similar results. Thus, the use of *Arabidopsis* in my studies serves as a convenient test of the KA hydroxylase hypothesis, or at least of its general applicability to other species. To confirm if KA is also the rate-limiting step in GA biosynthesis in *Arabidopsis*, experiments were conducted to compare the biological activity of exogenous of KA on Ar*abidopsis* plants grown under both inductive (LD) and non-inductive (SD) conditions.

The results clearly showed that the application of KA promoted stem growth and reduced the production of rosette leaves only under inductive conditions (Figs 2.2 and 2.3). This suggests that: 1) the metabolism of KA is an important step in the GA biosynthesis pathway and 2) the rate-limiting step in the GA biosynthesis pathway exists following KA synthesis. This also suggests that LD lifts one or more blocks in the pathway from KA to the GA responsible for biological activity. If the block is at the conversion of KA to 7-OH KA, there should be more endogenous KA in SD and much reduced endogenous KA levels following transfer to LD.

Quantitative analysis of endogenous KA levels using GC-MS showed the endogenous KA levels dropped by one-half 24 hours after transferring plants to LD (Fig 2.4). This indicates that the metabolism of KA is accelerated under LD. The endogenous KA level reached bottom 48 hours into the entire course of the study before gradually coming back to a level near that at the beginning of the experiment.

Overall, data obtained from this research is not consistent with the results obtained using *T. arvense*. The endogenous KA levels in *T. arvense* shoot tips decreased by 50-fold just a few days after the end of the cold treatment, and remained low (Hazebroek and Metzger, 1990). This inconsistency may have resulted from the different ways of collecting plant materials. Only shoot tips were collected for the analysis of the endogenous KA levels in the *Thlaspi* research while both shoot tips and elongated stems were used to analyze the content of KA in my research. It is possible that elevation of the endogenous KA levels in the later stage is contributed from the elongated part of plants. Plants need to synthesize more bioactive form of GA during stem elongation. The enzyme responsible for KA synthesis is activated under LD causing the endogenous KA levels to rise. This is consistent with the findings from Zeevaart's lab that the endogenous content of GA_1, GA_{19} and GA_{20} increases when *Silene armeria*, a LDP, is transferred from SD to LD (Talon and Zeevaart, 1990). Overall, the evidence from this study did not fully support the hypothesis that KA hydroxylase is the rate limiting step in GA biosynthesis under SD.

Quantitative analysis of GA content in *Silene armeria* indicates that the highest concentration of GA_{53} is observed in SD while the lowest level is seen in LD (Talon

and Zeevaart, 1990). This result indicates that GA biosynthesis may be regulated downstream of GA_{53} metabolism in *Silene*. Therefore further experiments were performed to investigate whether similar steps in GA biosynthesis are under photoperiodic regulation in *Arabidopsis*.

Gibberellin 20-oxidase is the Important Regulation Step in the GA Biosynthesis Pathway

Applications of CCC substantially reduced stem elongation induced by transfer from SD to LD (Fig 2.11), indicating a significant but not complete depletion in endogenous GA levels. Nevertheless, applications of the two C-19 GAs lacking a 13-hydroxyl group (GA_4 and GA_9) were able to completely reverse the inhibition of stem growth caused by CCC applications (Fig 2.11). Both GA_1 and GA_{20} elicited much less stem growth, which is similar to the relative responses to the various C-19 GAs when applied in SD (Figs 2.8 and 2.11).

However, in contrast to a lack of promotion of stem growth when applied in SD, the two C-20 GAs and KA, elicited an increase in stem growth over the CCC control (Fig 2.11). One interpretation of these results is that under non-inductive (SD) conditions, there is a block in the conversion of C-20 GAs to C-19 GAs. Upon transfer to LD, this block is lifted and the C-20 GAs can be converted to biologically active C-19 GAs such as GA_1 or GA_4.

The application of high concentrations of various GAs and their precursors under SD and LD conditions has provided a greater understanding of the

photoperiodic regulation of the GA biosynthesis in *Arabidopsis*. First, in comparison of the stem height and the number of cauline leaves, plants showed no difference with the C-20 GAs (GA_{12}, GA_{19}) and KA treatment in SD whereas stem height and the number of cauline leaves increased in various levels with C-19 GAs (GA_1, GA_4 and GA_9) treatments (Fig 2.7 and 2.8). This suggests that the conversion from GA_{12} (a C-20 GA) to GA_9 (a C-19 GA) is blocked in the GA biosynthesis pathway under SD.

Second, stem growth in plants treated with C-20 GAs or KA was not induced under SD (Figs 2.7, 2.8, 2.10 and 2.11). When the regulatory step in the GA biosynthesis is eliminated under LD, GAs including GA_{19}, GA_{12} and KA were able to be metabolized into a biologically active form of GA which promoted stem elongation. These results led to my conclusion that GA 20-oxidase is blocked under SD. This conclusion is also consistent with the hypothesis that the GA20-oxidase plays a crucial regulatory role in the GA biosynthesis pathway (Phillips et al., 1995).

Applications of various C-20 GAs, which are the intermediate forms of GAs in the GA biosynthesis pathway, under SD provided further evidence supporting my hypothesis that the GA metabolic pathway is blocked in the conversion of C-20 GAs to C-19 GAs. However, there are three pathways involved in the conversion of C-20 GAs into C-19 GAs. Furthermore, three different kinds of enzymes (3ß-hydroxylation, 13ß-hydroxylation and GA 20-oxidation) are known to catalyze different reactions in the final stage of GA biosynthesis pathway to generate biological active from of GA (Fig 2.1). Based on the results gathered thus far, it is reasonable to suggest that under

SD GA biosynthesis is blocked at one or more steps in the conversion of GA_{12} to C-19 GAs. The argument to support this idea is as follows.

First, no significant differences in stem growth were observed in plants treated with GA_{19} (13-hydroxy-GA) and KA in SD (Fig. 2.8 and 2.14). Interestingly, although greater stem growth was induced by another 13-hydroxy GA, GA_{20}, than by GA_{19}, its effect was not as great as with the non-hydroxylated-GA (GA_9) or the 3-hydroxy-GA (GA_4). Likewise, when comparing the effect of GAs with carbon-20 aldehyde (GA_{36}, GA_{24} and GA_{19}) on stem growth, it is clear that the 13-hydroxy-GA (GA_{19}) was the least active (Fig2.14). These results suggest that the 13-hydroxylation process is not necessary for biological activity, and in fact, diminishes biological activity, in the GA biosynthesis. Thus the early C 13 hydroxylation pathway ($GA_{12} \rightarrow GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20} \rightarrow GA_1$) may not play an important role in the regulation of stem growth as in other species such as maize (Fujioka et al., 1988; Kamiya and Graebe, 1983).

Second, when comparing the effects of GA_{12} and GA_{19} with those of GA_{14}, GA_{24} and GA_{36} on stem growth (Fig. 2.14), it is reasonable to suggest that 3ß-hydroxylation may not be the rate-limiting factor in the GA biosynthesis pathway under SD. Although GA_{14} is somewhat more active than GA_{12}, it is still not clear whether or not the 3ß-hydroxylation reaction converting GA_{12} to GA_{14} is an endogenous reaction since GA_{14} has not been detected in *Arabidopsis* (Talon et al., 1990b). On the other hand, the 3ß-hydroxylation reaction that converts GA_{24} to GA_{36} has been demonstrated to occur (Phillips, 1998). If a 3β-hydroxylation is blocked in the GA biosynthesis pathway under SD, one may assume that plants treated with GA_{24}

will have less stem growth than with GA_{36}. However, there was no difference in stem height between GA_{24} and GA_{36} treatments under SD (Fig 2.14). Therefore, it is likely that 3ß-hydroxylation pathway is not limiting under SD in *Arabidopsis* (Fig 2.14).

Third, significant differences in stem growth were observed when plants were treated with various C-20 GAs in SD (Fig 2.14). Stem elongation was strongly promoted by GA_{24} and GA_{36} treatment whereas no or much weaker responses were found in plants treated with GA_{12} or GA_{14} in SD (Fig 2.14). This indicates that under SD, GA metabolism might be blocked at one or more steps between GA_{12} and the GAs containing a carbon 20 aldehyde, GA_{12} and GA_{14}. These GAs are upstream components in the conversion of C-20 GAs into C-19 GAs, whereas GA_{24} and GA_{36} are the downstream products in that pathway. However, it is still unclear whether or not the block specially lies in the conversion from carbon 20 methyl to alcohol, from alcohol to aldehyde, or both.

LDP, *Silene armeria*, has been used to study the relationship between GA biosynthesis and stem growth (Talon and Zeevaart, 1990; Talon et al., 1991). Quantitative analysis of GA content under SD shows high accumulation of GA_{53} in *S. armeria*. GA_{53} levels declined sharply following transfer from SD to LD. This suggests that photoperiod regulates GA biosynthesis in *S. armeria* by controlling the conversion of GA_{53} (13 hydroxylated GA_{12}) to GA_{44}. This conclusion is consistent with findings in my research that plants treated with either GA_{12} or GA_{14} in SD have less stem growth than with the downstream products such as GA_{24} and GA_{36} because GA_{53} is structurally similar to GA_{12} and GA_{14}.

In spinach, long day treatment not only results in changes in the endogenous level of GA_{53} but also that of GA_{19} (Talon et al., 1991). GA_{19}, an intermediate product of GA 20-oxidase, is further converted into GA_{20}. Results from experiments with cell-free extracts in spinach show that the enzyme activity to oxidize GA_{19} increased in LD and decreased in SD. This indicates that the final reaction carried by GA 20-oxidase in which the carbon 20 aldehyde is removed and replaced with a lactone bridge may also play a crucial role in GA biosynthesis in spinach. No experiments involving application of equal amounts of GA_{36} and GA_4 to plants in SD was conducted in this study. Therefore, it is unclear whether GA_{36} has the same biological activity as GA_4 under SD in *Arabidopsis*. Studies using the same amount of GA_4 and GA_{36} in SD may be conducted to determine if the conversion of GA_{36} to GA_4 is also blocked in GA biosynthesis.

Quantitative analysis of the endogenous GAs in *Arabidopsis* has been conducted in *ga4* and *ga5*, the two semi-dwarf mutant lines (Talon et al 1990b). High concentrations of 13-hydroxy-GAs were detected by gas chromatography whereas low levels of 3-hydroxy- and 3,13-dihydroxy-GAs were found in the *ga4* mutant line. This indicated that the product of the *GA4* gene encodes the 3ß-hydroxylase. Moreover, the content of C-20 GAs was relatively high compared to that of C-19 GAs in the *ga5* mutant line. This led to the conclusion that the *GA5* gene encodes a protein responsible for the elimination of C-20 aldehyde.

Applications of GA_9, a non 3,13 hydroxylated GA, to *ga4* and *ga5* plants showed differences in stem growth. GA_9 had the ability to promote stem growth in

ga5 but had no effects in *ga4* plants (Talon et al 1990b). These findings suggest that 3ß-hydroxylation plays a crucial role in determining the biological activity of certain GAs. This is consistent with my observation that 3ß-hydroxylation GAs such as GA_{36} promotes stem growth strongly and that GA_4 is the most biological active GA (Figs 2.8 and 2.11).

GA_4 is Responsible for LD-induced Bolting

Results from the application of various GAs under SD suggest that stem growth was greatest when either GA_4 or GA_9 was applied (Fig. 2.17). The response was substantially less when plants were treated with the 13 hydroxylated analogs such as GA_1 and GA_{20}. It has been suggested that structure alterations on the C/D ring including the addition of a hydroxyl group on carbon 13 affects its interactions with the GA receptor (Sponsel, 1995). This alteration, which is seen in the C-13 hydroxylated GAs, may have been one of the reasons why GA_1 and GA_{20} have less biologically activity than carbon-3 hydroxylated GAs (GA_4) in shoots. Greater stem growth was found in plants with GA_3 treatment than GA_1 treatment. The structure of GA_1 is identical to that of GA_3 except that the single bond between carbon 2 and 3 of GA_1 is replaced with a double bond in GA_3. The possible explanation is that GA_3 may be metabolized into an inactive form at a slower rate than GA_1 allowing more GA_3 to remain in plants and promote stem growth. It is possible that adding a double bond on the A ring of GA_3 may alter the three-dimensional structure of GA facilitating interaction with the GA receptor and increase stem growth.

The dose response experiments have provided further evidence which endogenous GAs have intrinsic biological activity in *Arabidopsis*. Although GA_1 is believed to have intrinsic biological activity responsible for stem growth in most plants including maize, pea, spinach and *Silene armeria* (Fujioka et al., 1988; Ross et al., 1992; Swain and Reid, 1992; Talon and Zeevaart, 1990; Zeevaart et al., 1993), my study results indicated that both GA_1 and GA_4 have biological activity in *Arabidopsis*. In *Arabidopsis*, GA_4 is more active than GA_1 under SD. Thus, GA_4 is not likely to be converted to GA_1 for activity. In addition, different slopes of dose response curve further demonstrated that GA_4 is much more active than GA_1 since the value of the x-intercept from GA_1 is 5 times greater than that from GA_4 (Fig 2.19).

Since the slope of the regression line for GA_1 was significantly greater than either GA_4 or GA_9 (Fig 2.19), it is possible that at high doses, GA_1 may have the greatest biological activity. This prediction is consistent with the results published by the Kobayashi group (1993), which showed that plants treated with a high level of GA_1 were taller than those treated with GA_4. There are at least two possible explanations for this observation. First, GA 2-hydroxylase can use GA_1, GA_4 or GA_9 as substrates and convert them to the 2β hydroxylated inactive forms of GA_8, GA_{34} and GA_{51}, respectively. The GA 2-oxidase activity is up-regulated by the bioactive GAs (Thomas et al., 1999). It may be that GA_4 is more effective at inducing the GA 2-hydroxylase than is GA_1. Thus, as application rates are increased, GA 2-oxidase activity is induced to higher levels with GA_4 than GA_1, thereby leading to greater rates of GA_4 deactivation. Consequently higher levels of GA_1 may persist resulting in

greater stem growth. Another possible explanation for the differences in slope is that the Km for the substrate of this enzyme is higher for GA_1 than for the other two GAs and therefore the rate of deactivation will be slower.

Statistical analysis showed no significant differences on the values of y-intercepts between GA_4 and GA_9 (Fig 2.19). Stem heights of plants treated with 0.1 μg of either GA_4 or GA_9 were not statistically different. However, stem growth began to diverge at 1 μg and was substantially different at 10 μg. It is worth noting that 3β hydroxylase catalyzes the conversion of GA_9 toGA_4 and this reaction is required for GA_9 to be biologically active. It is possible that 3β-hydroxylase is saturated and reaction becomes rate-limiting for activity when GA_9 level is above 1 μg. Perhaps the excessive GA_9 may increase the activity of GA 2-oxidase, which converts GA_9 to the inactive GA_{51}. Therefore, plants treated with 10 μg of GA_9 have less stem growth than those treated with GA_4. It is worthwhile to study changes in the endogenous levels of GA_{51} in plants treated with different doses of GA_9 under SD. Results from this experiment will help to understand if a decline in stem growth with higher doses of GA_9 might result from the conversion of GA_9 to GA_{51} by the increased GA 2-hydroxylase activity.

Effects of GA Treatment on Flowering

The most important change that flowering plants undergo is the transition from the vegetative to reproductive state. To accomplish this transition, plants integrate various environmental signals such as light and temperature as cues to initiate

appropriate response(s). Several experimental criteria have been used in the past to determine flowering responses to different kinds of treatments. Flowering responses can be measured by the time needed for: 1) the appearance of visible floral buds, 2) the appearance of the first open flower or 3) the number of nodes formed before the first flower (Metzger, 1995; Lang, 1965; Bernier et al., 1981). For determinate plants, flowering responses can be measured by counting the number of leaves after the treatment. The earlier the transition to flowering, the fewer the total number of leaves generated (Martinez-Zapater and Somerville, 1990; Reeves and Coupland, 2001; Wilson et al., 1992; Blázquez et al., 1998).

Rosette LDP have stem elongation with floral initiation. However, these two events might have an obligate relationship in plants including *Martricaria parthenoides*, barley, wheat and millet (Greulach, 1942; Downs et al., 1958). On the other hand, in many plants including *Silene armeria*, *Spinacia oleracea*, *Agrostemma githago* bolting (stem elongation) is not obligatorily tied to flower formation (Zeevaart, 1997; Cleland and Zeevaart, 1970; Jones and Zeevaart, 1980). In this research, *Arabidopsis* was used as a model to investigate the relationships between GA metabolism and stem growth under SD and LD. Since *Arabidopsis* is a rosette plant and flowering is usually accompanied by stem elongation, I decided to use the number of leaves as one of the criteria for measuring flowering response and determining the most bioactive GA. My results showed that, overall, flower initiation was not changed in *Arabidopsis* plants treated with GA.

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CHAPTER 3

REGULATION OF GIBBERELLIN METABOLISM IN RELATION TO VERNALIZATION PLANTS

Introduction

The transition from vegetative to reproductive phase plays a crucial role in the growth and development of higher plants. This transition is regulated, in part, by environmental and hormonal factors. During the past several decades, *Arabidopsis* has been used as a model to elucidate the mechanism of reproductive development in higher plants. At least four different pathways that control flowering time in *Arabidopsis* have been identified through genetic analysis (Levy and Dean, 1998). The autonomous and the GA pathways are related to the control by the endogenous developmental state of the plants, whereas the photoperiod and the vernalization pathways are environmentally determined.

Vernalization is defined as a phenomenon in which plants need to have a period of exposure to low, non-freezing temperatures to initiate flowering. There are numerous plant species that require vernalization to initiate reproductive development in winter and to complete the final stage of floral development in the following spring.

Plants that require vernalization can be categorized into two groups: winter annuals and biennials. Only winter annual plants are sensitive to low temperatures during their entire life cycle. It is well known that sensitivity to vernalization is regulated by the developmental stage of plants, the length of the treatment and the temperature employed (Napp-Zinn, 1969; Metzger, 1985). Unlike photoperiodism, in which the perception of photoperiodic response is located in the leaves, the site of the perception of vernalization is presumably at the shoot tip. It is also clear that the thermoinduction of flowering is an epigenetic processes, which means the effects would only last for a single generation and the progeny plants must be vernalized in order to flower.

The mechanism of vernalization has been investigated in the past few years using different approaches. Flowering in two related crucifer species *Thlaspi arvense* and *Arabidopsis* can be promoted under non-vernalized conditions with the application of 5-azacytidine, a DNA demethylation agent (Burn et al., 1993a). However, a late-flowering mutant line that is insensitive to vernalization did not respond to the same treatment (Burn et al., 1993a).

Further investigation also found that transgenic *Arabidopsis* plants with the presence of DNA methyltransferase (*METI*) antisense gene flowered earlier than non-transformed plants under SD (Finnegan et al., 1998). Analysis showed that the *METI* antisense plants contained a lower level of DNA methylation than that of control plants. Those results suggest that DNA methylation plays an important role in the process of the vernalization. Another *Arabidopsis* late flowering mutant line, *FLOWERING LOCUS F* (*FLF*), was created by the insertion of two adjacent T-DNA

copies (Sheldon et al., 1999). *FLF* plants show strong flowering responses to both vernalization and GA treatment.

The *FLF* gene has been renamed as *FLC* (*FLOWERING LOCUS C*) since both *FLF* and *FLC* are at the same locus in *Arabidopsis*. It was found that the T-DNA insertion occurred at about 2.3kb upstream of the start codon of the *FLC* gene. The insertion resulted in the overexpression of *FLC* mRNA suggesting that *FLC* serves as a flowering repressor (Sheldon et al., 2000). Only vernalization results in decreased levels of the *FLC* transcript. Studies have found that an increase in the duration of cold treatment causes the level of *FLC* mRNA to drop rapidly. However, stem growth was promoted by continuous application of GA_3 under SD. This suggests that the reproductive development for the vernalization requirement plants is via the regulation of GA biosynthesis. Additional evidence gathered from *Thlaspi* research supports this hypothesis.

Thlaspi arvense, a winter annual weed with a vernalization requirement, has been used as a model to elucidate the relationship between flower induction and GA biosynthesis (Metzger, 1985; 1988; 1990; Hazebroek and Metzger, 1990; Metzger and Dusbabek, 1991; Metzger and Hassebrock, 1990). Flowering was promoted when plants were given a localized cold temperature treatment in the shoot tip. Plants whose non-induced shoot tips were grafted onto induced donors remained vegetative rosettes.

Results from further studies also showed an increase in cell division occurred in the pith and cortex region below the stem apex after a four-week cold treatment (Metzger and Dusbabek, 1991). The production of new pith and cortex cells and the

stem growth rates were also correlated with the length of the cold treatment. The same reports also showed that cell division in the pith and cortex region occurred on GA_3-treated plants without a vernalization. This was consistent with previous observation that exogenous GA_3 treatment can substitute for thermoinduction. On the other hand, a GA-deficient dwarf mutant showed little mitotic activity in the pith and cortex when plants were subjected to a cold treatment. This finding supports the idea that gibberellin may mediate thermoinduced stem growth in *Thlaspi arvense*.

Quantitative analysis in the endogenous level of kaurenoic acid (KA) found that the shoot tips of non-induced plants have 47 times more endogenous KA than in thermoinduced shoot tips (Hazebroek and Metzger, 1990). Moreover, the content of KA in the apex declined 10-fold two days after the thermoinduced plants were returned to 21ºC, whereas no effect of vernalization on the endogenous level of KA in leaves was observed (Hazebroek et al., 1993). The enzyme activity of *ent*-kaurenoic acid oxidase, which catalyzes the oxidation reaction from KA to *ent*-7α- hydroxy kaurenoic acid (7-OH KA) increased in microsomal extracts from shoot tips after the end of the cold treatment. Together, it suggests that the metabolism of KA to GAs is blocked in non-induced shoot tips, resulting in the accumulation of KA. The conversion of KA to 7-OH KA is the primary step in GA metabolism regulation by vernalization in *Thlaspi* shoot tips.

Like *Thlaspi*, *Arabidopsis thaliana* is a member of the crucifer family with separate vegetative and reproductive phases. The relationship between stem elongation and GA biosynthesis at the molecular level needs to be clarified. As described in the

previous chapter (Fig 2.1), three different GA biosynthesis pathways originating from GA_{12} have been identified: early 13-hydroxylation pathway, early 3-hydroxylation pathway, and the early non 3,13-hydroxylation pathway (Kobayashi et al., 1993; Kobayashi et al., 1994; Talon et al., 1990a; 1990b; Zeevaart and Talon, 1992). Only products from the early 13-hydroxylation pathway leading to GA_1 and from non 3,13-hydroxylation pathway leading to GA_4 promote stem elongation, and yet it is not known which one is primarily responsible for regulating stem growth in *Arabidopsis*. The purpose of this study was to investigate how temperature via GA biosynthesis pathway promotes stem growth in *Arabidopsis*.

Material and Methods

Plant Material and Growing Conditions

A vernalization requiring line was selected from the T-DNA insertion in the *Arabidopsis* C24 ecotype and is referred to as the cold requiring (CR) line from here on. The insertion was located in the promoter region of the *FLC* gene, causing overexpression of the *FLC* gene. Overexpression of the *FLC* gene results in a late flowering phenotype (Sheldon et al., 1999). Seeds of CR were germinated and grown in 7 cm pots filled with a commercial soilless medium (Metro-Mix 360; Scott-Sierra Horticultural Products Company, Marysville, OH).

All of the experiments were conducted in the growth chamber with a temperature of 25ºC and under short-days as described in Chapter 2. Vernalization treatments were provided by a cold room at 6ºC and 8 h of light from fluorescent lamps for eight weeks. After vernalization, plants were returned to the growth chamber at 25ºC and LD conditions as described in Chapter 2.

Unless otherwise stated, all experimental methods used in this chapter are the same as described as in the previous chapter including those used in the application of various GAs and GA precursors to plants maintained under non-thermoinductive conditions, the application of various GAs and KA in both thermoinductive and non-thermoinductive conditions, the dose-response studies and the quantitative analysis of endogenous KA levels. Additional experiments that were conducted on CR plants are described as follows.

Plant Age Effect Experiment

The purpose of this experiment is to understand the effects of GA bioactivity on plant growth and development at various stages. Two batches of CR seeds were germinated two weeks apart. All plants were grown under SD in the same growth chamber for at least 4-6 weeks prior to the GA treatment. 10 µl of an aqueous solution containing 50%(v/v) acetone, 0.05%(v/v) Tween-20 and 1 µg of various GAs was applied to the shoot tips of the plants three times a week for two weeks. Plants were continuously grown under the non-thermoinductive conditions for two more weeks and the stem height was measured.

Results

3.1 Identification of Potential Steps in GA Biosynthesis under Thermoinductive Control

3.1.1 Comparative Effects of Exogenous GAs and GA Precursors on Plants

To determine which step(s) in GA biosynthesis may be under thermoinductive control, KA and four different GAs were applied to plants grown under vernalized and non-vernalized conditions.

Results showed GA_4 and GA_9 were the most active in non-vernalized plants (Fig. 3.2). In contrast, plants treated with GA_1 and GA_{20} showed modest increases in stem growth. There were no significant differences in stem growth between KA and the control treatments (Fig 3.2). None of the plants flowered at the end of the experiment regardless of treatment.

On the other hand, eight weeks of vernalization dramatically increased the response to exogenous GAs (Fig. 3.3). Unlike non-vernalized plants, vernalized plants flowered at the end of experiment. Plants treated with GA_4 or GA_9 had the greatest response. Vernalized plants were five times taller with GA_4 treatment than non-vernalized plants treated with the same GA (Fig 3.2 and 3.3). Stem height in vernalized plants treated with GA_1 and GA_{20} was 20-24 times taller than those without the cold treatment (Fig 3.2 and 3.3).

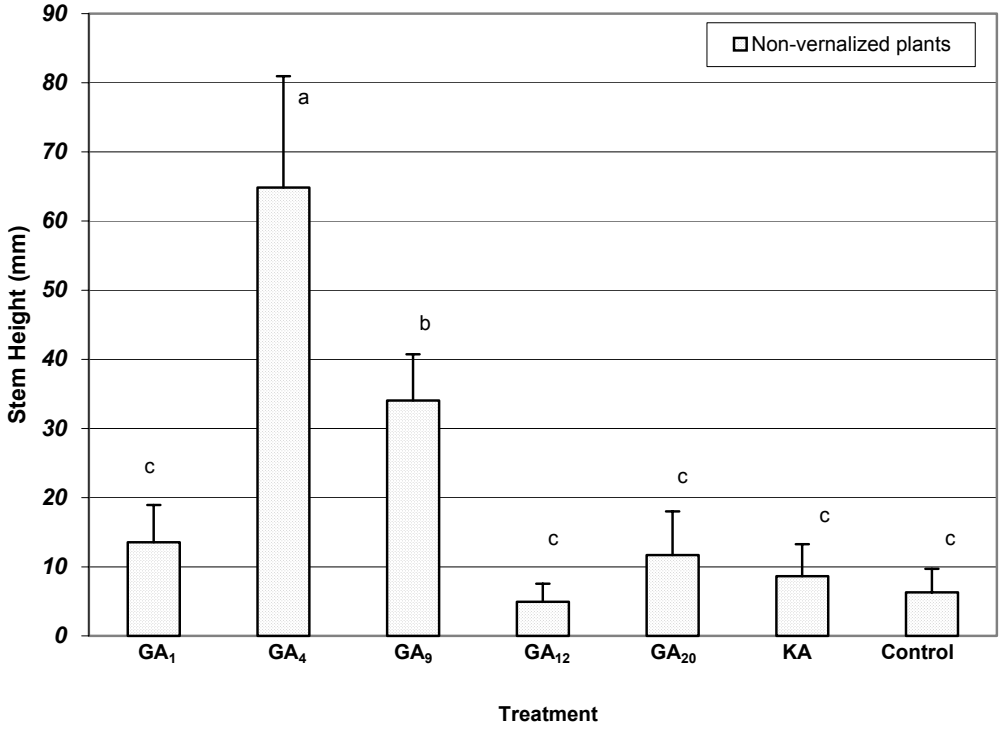


Figure 3.1 Comparison of biological activities of various GAs and KA on stem growth in non-vernalized CR plants. Plants were grown under SD in the growth chamber for 12 weeks before treatment. Plants were transferred from SD to LD. 10µl of an aqueous solution containing 50%(v/v) acetone, 0.05%(v/v) Tween-20, and 1 µg of various GAs or KA was applied to shoot tips three times a week for two weeks. Values represent the means of 7 plants in each experiment for two individual experiments. The vertical bars represent standard errors of the means. Columns followed by a different letter are significantly different (p=0.01) as determined by Student's t-test.

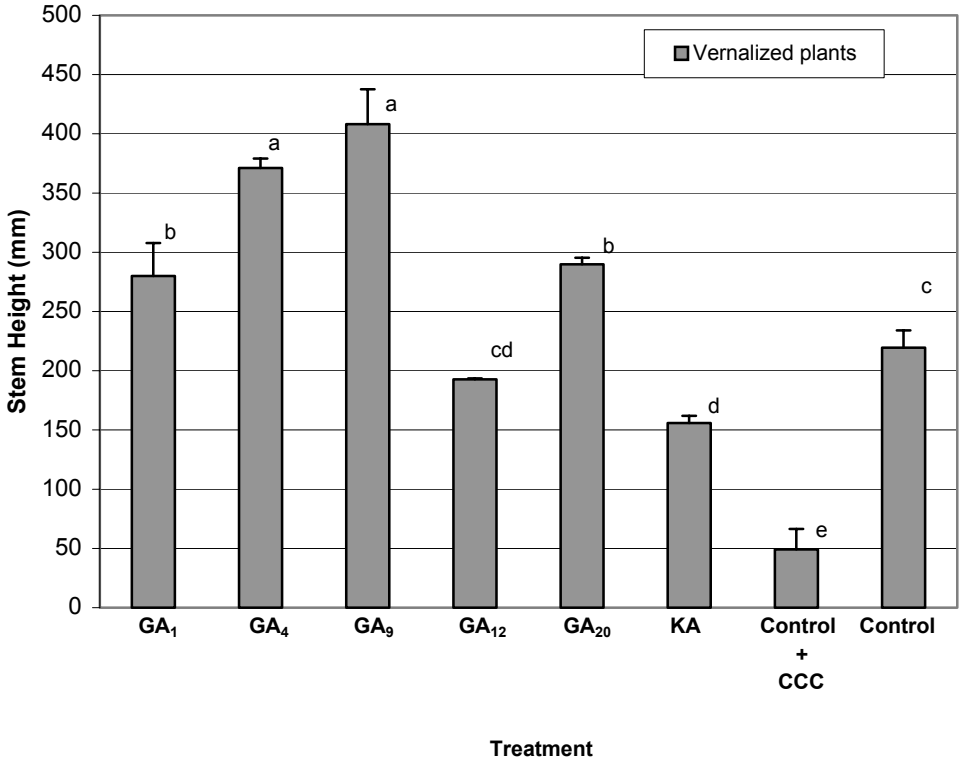


Figure 3.2 Comparison of biological activities of various GAs and KA on vernalized CR plants. Plants were grown in the growth chamber under SD for 4 weeks before subjected to an 8-week-long cold treatment. Starting one week prior to transferring plants to room temperature, endogenous GA levels were reduced by applying 2 mM CCC to plants every other day until the end of the experiment. Upon return to warm temperature, plants were grown under LD. 10µl of an aqueous solution containing 50%(v/v) acetone, 0.05%(v/v) Tween-20, and 1 µg of various GA or KA was treated to shoot tips three times a week for two weeks. Values represent the means of 7 plants in each experiment for two individual experiments. Two different controls were used in this experiment which are labeled as Control and Control + CCC in the figure above. The Control column represents plants with a 8-week cold treatment. The other control group (Control + CCC) consists of plants treated with continuous applications of CCC in addition to the cold treatment during the course of experiment. The vertical bars represent standard errors of the means. Columns followed a different letter are significantly different (p=0.01) as determined by Student's t-test.

Nevertheless, since the C-19 GAs (GA_1, GA_4 and GA_9) exhibited biological activity in non-vernalized condition (Fig 3.2), it is reasonable to predict that one or

more steps leading from KA to conversion of C-20 GAs to C-19 GAs are under thermoinductive control.

3.1.2 Quantitative Analysis of Endogenous KA Levels

The endogenous levels of KA on plants grown under vernalized and non-vernalized conditions were determined. Quantitative analysis of endogenous KA levels in *Thlaspi* suggested that the GA biosynthesis was blocked in non-vernalized plants at kaurenoic hydroxylase (Hazebroek et al., 1993). Hypothetically, A*rabidopsis*, also a member of the crucifer family, should have the same or similar regulation mechanism of GA biosynthesis.

CR plants were grown in the cold room for eight weeks before returning to room temperature. Stems and leaves of individual plant were harvested 0, 2, 5, 9, 12 and 14 days after termination of the cold treatment. The content of KA in the stems declined by 60% by day 2 and continued to drop before reaching its lowest level of 6.0 ng on day 5 (Fig. 3.1). At this time, flower buds were clearly visible, but the stem had not yet shown any sign of elongation.

The increase in endogenous KA levels was coupled with stem growth in *Arabidopsis*. The KA contents in the stems increased since day 9 as the stems started to elongate (Fig 3.1). Endogenous KA levels reached their highest levels (12.31 ng) on day14. On the other hand, leaf levels of KA remained the same regardless of the day of the harvest (data not shown).

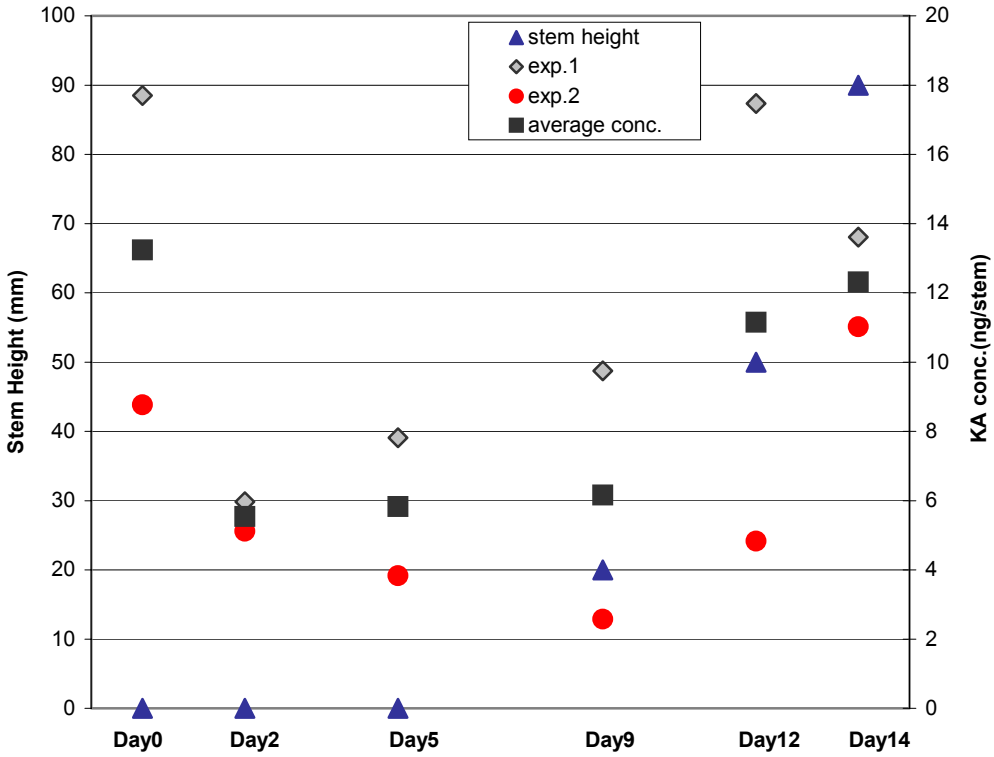


Figure 3.3 Effects of cold treatment on stem growth and endogenous KA levels of CR plants. All plants were first grown under SD for six weeks before vernalization. Stems and leaves of at least 30 plants were harvested for endogenous KA extraction 0, 2, 5, 9, 12 and 14 days after termination of the cold treatment. Day 0 represents the day plants were moved from the cold room to a growth chamber at 25ºC. Values indicated by ■ represent the means of two individual experiments. Values indicated by ◆ and ● represent actual individual data point obtained from the first and second experiment, respectively.

Results of the quantitative analysis in endogenous KA levels did not fully support the hypothesis that the conversion of KA to 7-OH KA is under thermoinductive control in CR. This led me to investigate if there are some other steps in GA biosynthesis that may be the limiting-step under thermoinductive control.

3.1.3 Effect of Various C-20 GAs on Stem Growth under Non-inductive Conditions

Three different pathways are involved in the conversion of GA_{12} to the bioactive forms of GAs such as GA_1 and GA_4 (Fig 2.1). To find out which pathway may be under thermoinductive control, GAs that were associated with these three pathways were used in the following experiment.

All GAs used in this study shared very similar chemical structures with slight variations (see Fig 2.12). For instance, GA_{12} contains a hydrogen atom on carbon 3 while GA_{14} has a hydroxyl group in the same position. In contrast, GA_{24}, a downstream product of GA_{12}, does not contain any hydroxyl group on carbon 3 and 13 while GA_{36}, presumably a downstream product of GA_{14}, contains a hydroxyl group on carbon 3. Nonetheless, only one hydroxyl group is attached on carbon 13 of GA_{19}, one of the products in the early 13-hydroxylation pathway (see Fig 2.12). GA_{24}, GA_{36} and GA_{19} serve as intermediate products in the GA 20-oxidation reactions and also have a common structure in which an aldehyde group is located on C-20.

Consistent with previous results (Fig 3.2), GA_{12} lacked biological activity in non-induced plants. However, GA_{14}, the 3ß-OH homolog of GA_{12}, did elicit a small, but significant amount of stem growth (Fig 3.4). One interpretation of this result is that the 3ß-hydroxylation step is limiting under non-vernalized conditions. However GA_{24}, which lacks a hydroxyl group, but in which carbon 20 is an aldehyde, had as much biological activity as GA_{36} (the 3ß OH homolog) and GA_{14}. In contrast, GA_{19}, the 13-OH homolog of GA_{24}, lacked biological activity (Fig 3.4). Taken in isolation, this latter result is consistent with the conclusions from the previous chapter namely that

the oxidation of carbon 20 from a methyl to an aldehyde is limiting under non-vernalized conditions.

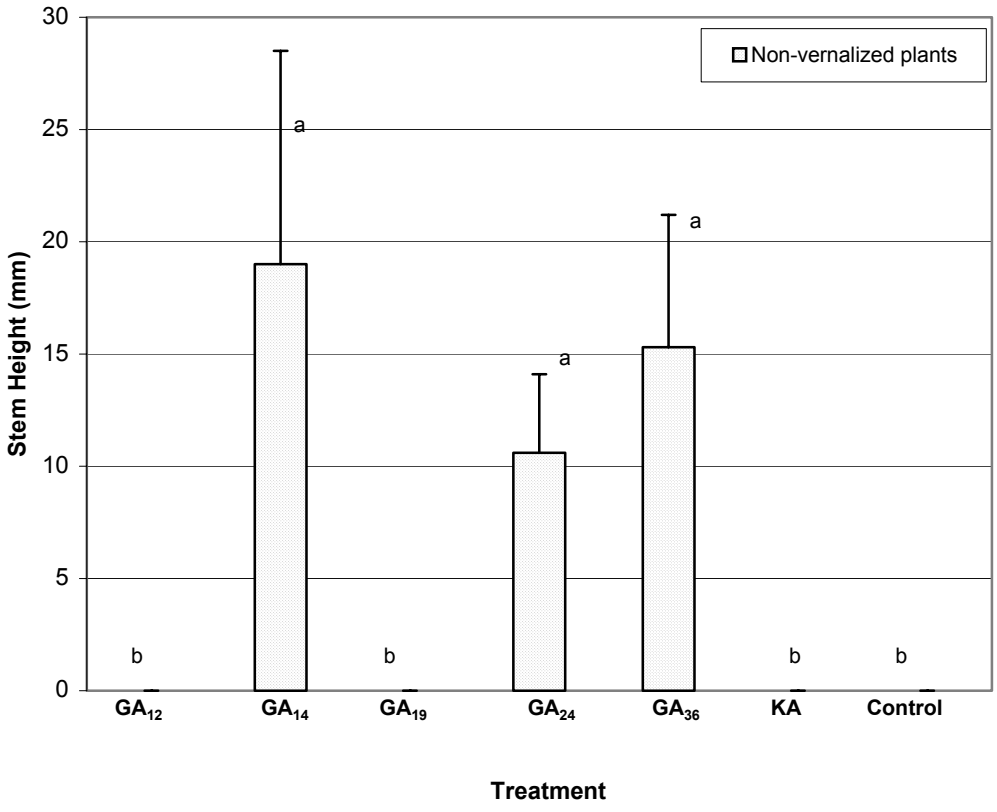


Figure 3.4 Effects of various GAs and KA treatments on stem growth on CR plants under non-vernalized conditions. Plants were grown under SD in the growth chamber for 12 weeks before treatment. 10 µl of an aqueous solution containing 50%(v/v) acetone, 0.05%(v/v) Tween-20, and 10 µg of a GA or KA were applied to shoot tips three times a week for two weeks. Values represent the means of 7 plants in each experiment for two individual experiments. The vertical bars represent standard errors of the means. Columns followed a different letter are significantly different (p=0.01) as determined by Student's t-test.

3.2 Which GAs Have Intrinsic Biological Activity

3.2.1 Comparison of Biological Activities of Various Exogenous GA Precursors and C-19 GAs under Non-vernalized Conditions

The biological activity of various GAs and their precursors were compared under non-inductive conditions. None of the plants flowered at the end of the experiment regardless of the type of GAs or GA precursor applied. Therefore, only

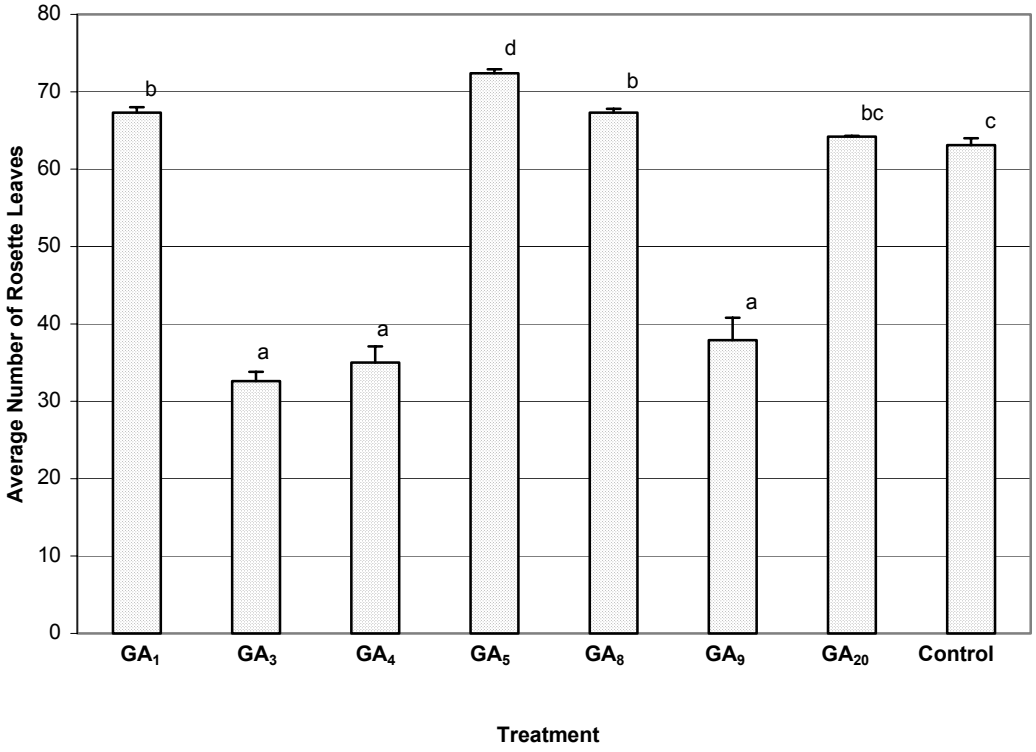


Figure 3.5 Effects of various GAs and GA precursors on the number of rosette leaves under non-vernalized conditions. 10 µl of an aqueous solution containing 70%(v/v) acetone, 0.05%(v/v) Tween-20, and 1 µg of a GA or GA precursor was applied to shoot tips three times a week for two weeks. Values represent the means of 7 plants in each experiment for two individual experiments. The vertical bars represent standard errors of the means. Columns followed a different letter are significantly different (p=0.01) as determined by Student's t-test.

data on the number of rosette leaves and stem height were collected and used as the criterion in determining the relative effectiveness for eliciting stem growth. The results showed plants treated with GA_4 and GA_9 had significantly fewer rosette leaves (Fig 3.5). On the other hand, no significant difference in the number of rosette leaves was found between plants treated with GA_1 or the GA precursors. Other GA precursors did not reduce the number of rosette leaves (data not shown). This indicates that *Arabidopsis* plants are more sensitive to GA_4 and GA_9 than any other GAs.

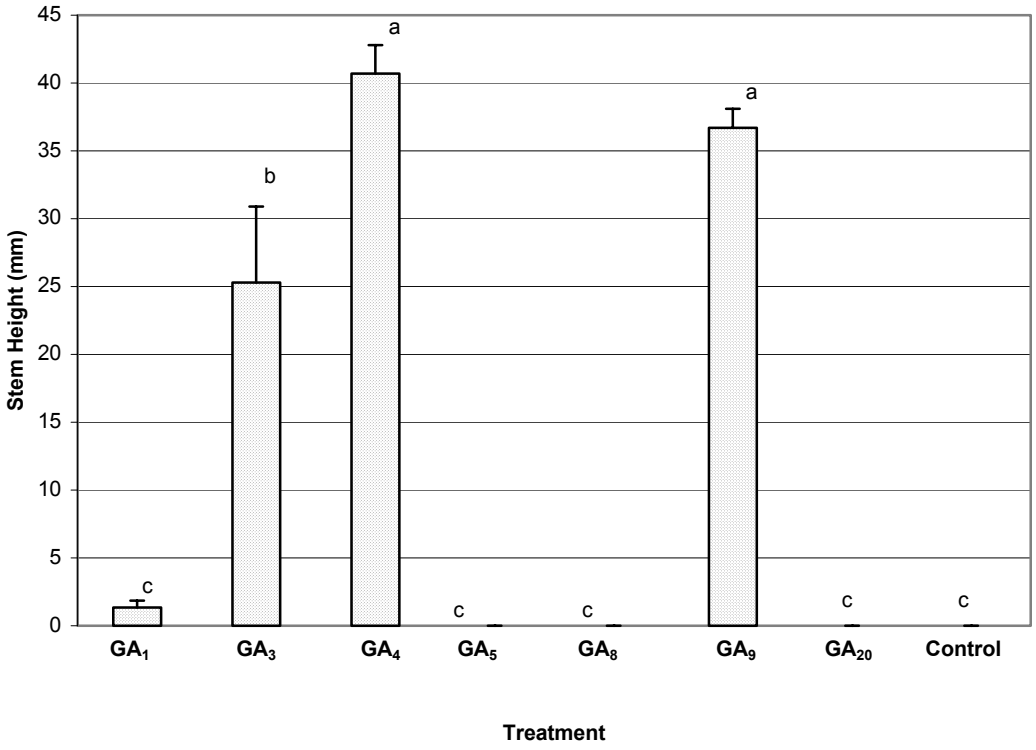


Figure 3.6 Effects of various GAs and KA treatments on stem growth under non-vernalized conditions. 10µl of an aqueous solution containing 70%(v/v) acetone, 0.05%(v/v) Tween-20, and 1 µg of a GA or KA was applied to shoot tips three times a week for two weeks. Values represent the means of 7 plants in each experiment for two individual experiments. The vertical bars represent standard errors of the means. Columns followed a different letter are significantly different (p=0.01) as determined by Student's t-test.

Stem elongation has served as one of the most important indicators for comparing the biological activity of various GAs. Plants treated with GA_4 or GA_9 exhibited more stem growth than with other GA treatment under non-thermoinductive conditions (Fig. 3.6). *Arabidopsis* plants were less sensitive to GA_3 than GA_4. Although non-vernalized plants treated with GA_1 showed some stem elongation, no growth was observed in plants treated with the various GA precursors (data not shown).

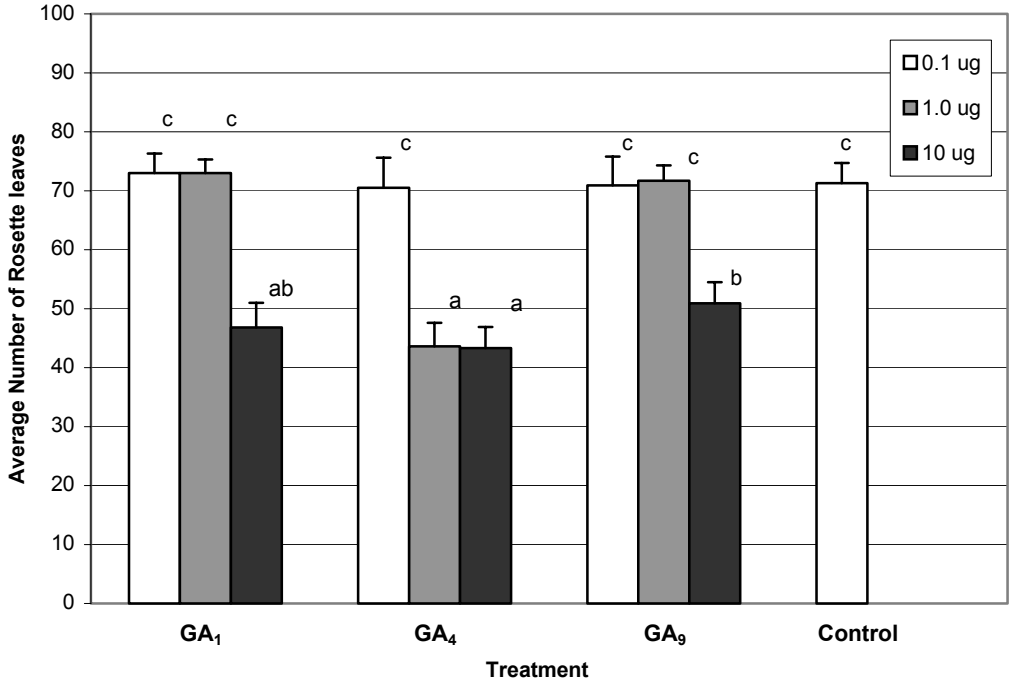
3.2.2 Dose Response Experiment

Arabidopsis plants were treated with different doses of GA_1, GA_4 or GA_9 under non-vernalized conditions. The resulting dose response relationships were used as a tool to help discern whether GA_4 and GA_9 have to be converted to GA_1 for biological activity or whether they have intrinsic biological activity. No difference in the number of rosette leaves was observed in all 0.1 µg GA treatments (Fig. 3.7). While similar rosette leaf numbers were observed on plants with 1 µg of GA_1 and GA_9 treatments, the number of rosette leaves was significantly less with GA_4. Plants had a similar number of rosette leaves when treated with 10 µg for all three GAs.

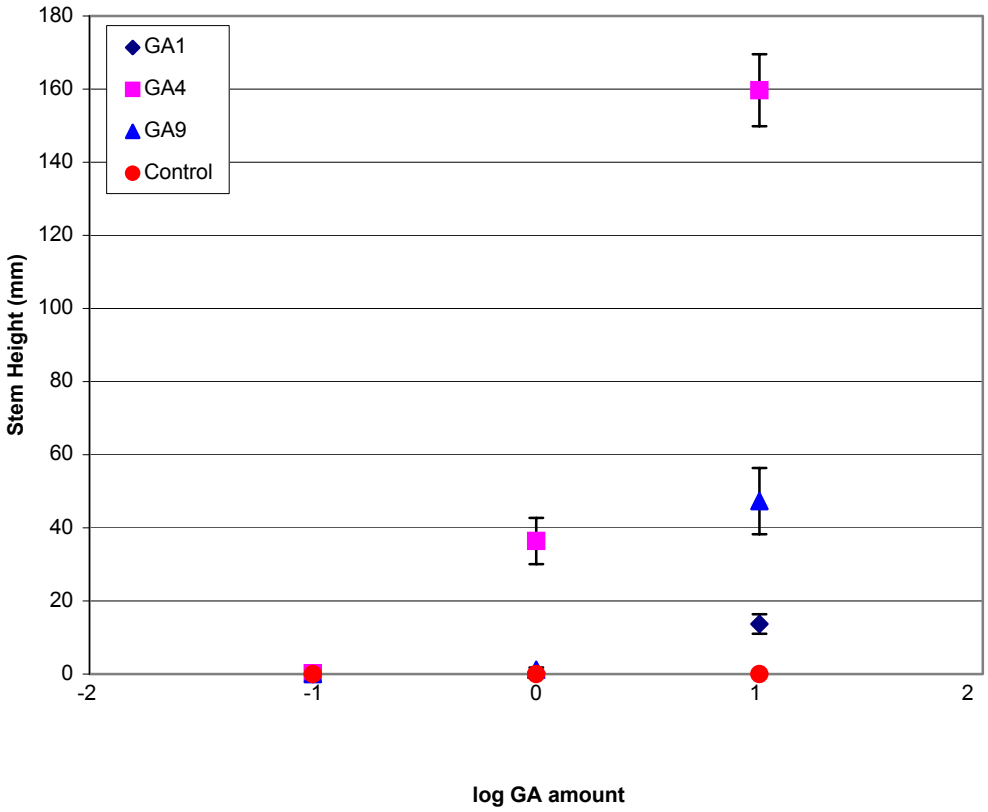
None of the GAs elicited a response at the 0.1µg dose (Fig.3.8). While the application of 1 µg of GA_4 stimulated stem growth significantly, no increase in stem height was observed on plants treated with GA_1 or GA_9 at that concentration. Plants treated with 10 µg GA exhibited further increases in stem growth. Stem heights were 5-fold higher in the plants treated with 10 µg of GA_4 than with the 1 µg of GA_4. At the

10μg dose, GA_4 was 50 and 30 times more active than GA_1 or GA_9, respectively.

Overall, the order of biological activity of the three GAs was GA_4>GA_9>GA_1.



Figures.3.7 Dose-response relationships of various GAs on the number of rosette leaves in non-vernalized plants. Plants were treated with 0.1, 1 or 10 μg of various GAs three times a week for two weeks. The number of rosette leaves of each plant was recorded two weeks after the treatment. Values represent the mean of 7 plants in each experiment for two individual experiments. The vertical bars represent standard errors of the means. Columns followed a different letter are significantly different (p=0.01) as determined by Student's t-test.



Figures 3.8 Dose-response relationships of various GAs on the stem growth in non-vernalized plants. Plants were treated with 0.1, 1 or 10 µg of various GAs three times a week for two weeks. Stem height of each plant was recorded two weeks after the start of treatments. Values represent the average of 7 plants in each experiment for two individual experiments. The vertical bars represent standard errors of the means. Columns followed a different letter are significantly different (p=0.01) as determined by Student's t-test.

3.2.3 Plant Age and Responsiveness to Exogenous GA

The results from experiments in which various GAs were applied to non-vernalized plants (Fig 3.2) were slightly different than those obtained in the later experiment (Fig3.6). In the first experiment, stem growth was promoted in all treatments regardless of GA (Fig 3.2 and 3.6). The average stem height of plants with

GA_1 treatment was higher than that of plants treated identically in the second experiment. In addition, little or no stem elongation was observed in plants treated with GA_1 and GA_{20} in the second experiment (Fig 3.6). One possible explanation could be that plants differ in their responsiveness to exogenous GAs depending on plant age.

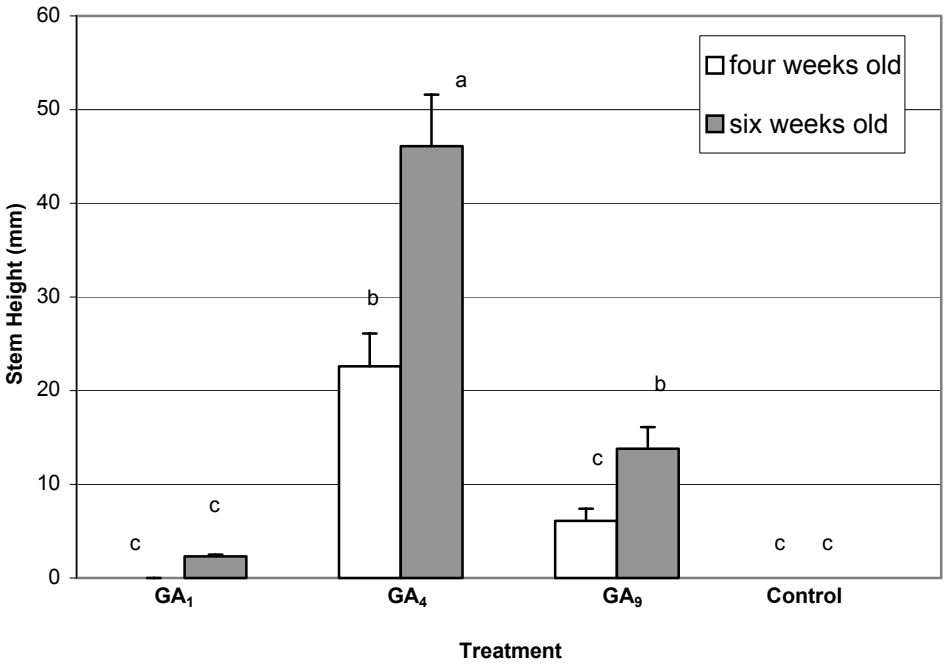


Figure 3.9 The effect of age on the sensitivity of non-vernalized CR plants to exogenous GAs. 10µl of an aqueous solution containing 70%(v/v) acetone, 0.05%(v/v) Tween-20, and 1 µg of a GA_1, GA_4 or GA_9 was applied to shoot tips three times a week for two weeks. Values represent the average of 7 plants in each experiment for two individual experiments. The vertical bars represent standard errors of the means. Columns followed different letter are significantly different (p=0.01) as determined by Student's t-test.

The effects of plant age on the response to exogenous GAs was determined by applying 1 µg of either GA_1, GA_4 or GA_9 to non-vernalized plants that were either

four or six weeks old. The results indicate that the sensitivity to GA is strongly associated with developmental age in *Arabidopsis*. For all GA treatments, six-week-old plants responded with greater stem growth than the four-week-old plants (Fig 3.9). GA_1 did not elicit stem elongation on four-week-old plants, while an average of 4 mm in stem height was observed on six-week-old plants with the same treatment (Fig 3.9). The bioactivity of GA_9, the precursor of GA_4, was significantly increased in the later part of the developmental stage in *Arabidopsis*.

Discussion

KA Hydroxylase May not be the Rate-limiting Step in GA Biosynthesis

Hazebroek and Metzger (1990) reported that the content of KA in *Thlaspi arvense* decreased by at least 50-fold by two days after the cold treatment. They also proposed that the GA metabolic pathway is blocked in the conversion of KA to 7-OH KA under thermoinductive control. However, quantitative analysis of KA levels in the *Arabidopsis* CR line under the same treatment showed only a mild decrease when plants were returned to room temperature. Moreover, unlike the results seen in *Thlaspi* in which endogenous KA levels remained low 10 days after the cold treatment, the content of endogenous KA in the *Arabidopsis* CR line increased again when the stem began to elongate at the end of experiment. The evidence did not support the idea that the conversion of KA to 7-OH KA is blocked in the GA biosynthesis pathway in *Arabidopsis* .

Results published from different groups suggest that GA 20-oxidase might be blocked in the GA biosynthesis pathway under SD (Wu et al., 1996; Coles et al., 1999). The objective of the following two experiments was to determine if the GA 20-oxidase is also the rate-limiting step in the GA biosynthesis in *Arabidopsis* as suggested in other plants.

GA 20-oxidase is the Rate-limiting Step in GA Biosynthesis under Non-vernalized Conditions

Treatment with either GA_{12} or KA resulted in an increase in stem growth in induced plants by 18 to 38-fold compared to non-induced plants (Fig. 3.1 and 3.2). All of the compounds except KA either restored growth of CCC treated plants to control levels or exceeded it (Fig 3.2). This suggests that cold treatment not only has an effect on lifting the block(s) in the GA biosynthesis, but also can increase GA responses by vernalized plants. Also, while KA did not fully restore growth to control levels, it did have some biological activity. This may indicate that KA, an upstream compound in GA biosynthesis, can be converted into different forms of GAs when GA_{12} is generated, but that only a small proportion of KA is actually converted to a biologically active GAs. Therefore, it would not be expected that KA exhibits the same effect as 19 carbon GAs such as GA_4 and GA_1.

Results of these studies have provided new information in the regulation of GA biosynthesis in *Arabidopsis*. Non-vernalized plants did not respond to applications of GA_{12} or GA_{20} (Fig 3.1). However, stem growth was promoted with GA_{12} or GA_{20} treatment of vernalized plants (Fig 3.2). This indicates that one or more steps in the GA biosynthetic pathway downstream from KA are activated as a result of thermoinduction. However, the fact that GA_{12} exhibited biological activity only after plants had been vernalized indicates that steps other than, or in additional to, KA hydroxylase are under thermoinductive control as proposed by other researchers (Hazebroek and Metzger, 1990; Hazebroek et al., 1993).

Stem growth was obtained by applying 10 μg of GA_{24} or GA_{36} in each treatment (Fig 3.4). Although applications of the intermediate products of GA 20-oxidase (GA_{24} and GA_{36}) promoted stem growth under non-vernalized condition, stem growth was significantly weaker than with GA_4 or GA_9 treatment (Fig 3.2 and 3.4). Stem growth of plants treated with 1μg of GA_9 or GA_4 was three to four times greater than those plants treated with 10 μg of GA_{24} or GA_{36} under non-vernalized conditions (Fig3.2 and 3.4). It is reasonable to predict that stem growth will be much less on plants treated with 1 μg of GA_{24} or GA_{36} under non-vernalized condition than with GA_4 or GA_9 treatment. If that is true, then the conversion of either GA_{24} or GA_{36} to GA_9 or GA_4, respectively might be regulated under non-vernalized condition. However, applying high amount of GAs that lacks a hydroxyl group on C-13 such as GA_{24} or GA_{36} may trigger GA 20-oxidation reaction to generate bioactive form of GA and promote stem growth in *Arabidopsis*. This hypothesis can be verified by analyzing GA 20-oxidase activity or by examining the expression of GA 20-oxidase gene after applying GA_{24} or GA_{36} to plants under non-vernalized condition.

Another possible explanation for the biological activities in both GA_{24} and GA_{36} under non-vernalized condition is that plants may constitutively express low amount of GA 20-oxidase to maintain physiological responses such as petiole or hypocotyl growth. This low amount of GA 20-oxidase can facilitate the conversion of either GA_{24} or GA_{36} to bioactive form of GA and promote stem growth. However, stem growth caused by the GA_{24} or GA_{36} treatment is less significant when compared to that caused by the GA_4 or GA_9 in non-vernalized plants (Fig 3.2 and 3.4).

At least three GA 20-oxidase genes (At2301, At2353 and YAP169) have been cloned in *Arabidopsis* (Phillips et al., 1995). Hybridization experiments showed the expression of these three genes are tissue-specific. The expression of At2301 has been found in stem and inflorescences whereas At2353 predominantly exists in the inflorescences and siliques. Finally, the expression of the YAP169 gene was only found in the siliques. This result indicates the expression of GA 20-oxidase is spatially and temporally regulated.

The report published by Phillips et al., (1995) also points out that GA 20-oxidase was less effective to oxidize GA_{53} than GA_{12} in the cell free extracts. Moreover, each individual GA 20-oxidase exhibited different ratio of the products when GA_{53} or GA_{12} was used as substrate. This evidence supports the idea that a low amount of GA 20-oxidase is constitutively expressed in plants, and that this type of GA 20-oxidase can then convert low amounts of GA_{14}, GA_{24} or GA_{36} to a C-19 GA (presumably GA_4) resulting in promotion of stem growth under non-vernalized condition. Therefore, cold treatment may lift the block by promoting the production of another type of GA 20-oxidase production that is responsible for promotion of stem growth in vernalized plants. More experiments such as *in situ* hybridizations are needed to provide new evidence and to confirm whether or not each GA 20-oxidase is only expressed in some particular cell types and under specific environmental conditions.

The main function of the GA 20-oxidase is to oxidize and eliminate the C-20 of the GA_{20} in the GA biosynthesis pathway. Different research groups have used

various approaches to illustrate the importance of the GA 20-oxidase in plant development. For instance, the expression of the GA 20-oxidase mRNA in the northern blot analysis was found higher in spinach plants under LD than SD (Wu et al., 1996). This result indicated that the expression of GA 20-oxidase mRNA was regulated by day length.

Moreover, over-expression of citrus GA 20-oxidase mRNA in *Nicotiana tabacum* resulted in significant changes in the phenotypes of the transgenic plants (Vidal et al., 2001). Hypocotyl lengths of transgenic tobacco plants were 4 times longer than the wild type plants. The over-producers were twice as tall as the wild type plants and had much larger inflorescences. These phenotypes were correlated with the elevated GA_4 levels found in the over-producers. This indicates that over-expression of the GA 20-oxidase gene in transgenic plants resulted in an increase in endogenous GA_4 level, which in turn resulted in a tall phenotype.

Two research groups have successfully introduced different GA 20-oxidase genes into *Arabidopsis* to further investigate the effects of these genes on the phenotypes of transgenic plants (Huang et al., 1998; Coles et al., 1999). Both groups reported similar phenotypes with longer hypocotyls, increased stem elongation and earlier flowering. The endogenous GA_4 level increased by two to three-fold in the transgenic line.

On the other hand, plants that under-express GA 20-oxidase displayed a shortened hypocotyl and reduction in stem elongation. This evidence suggests that stem growth is regulated by the activity of the GA 20-oxidase gene in transgenic

plants. As previously described, the application of a C-20 GA such as GA_{12} has no effect on stem growth under non-induced condition, whereas plants subjected to the same GA treatment under thermoinductive condition showed strong stem growth (Fig 3.2 and 3.3). This result was consistent with findings published by other groups that GA 20-oxidase plays a critical role in the regulation of the GA biosynthesis pathway via vernalization.

Although GA 20-oxidase is believed to be associated with the multiple steps of the oxidation reactions in the GA biosynthesis pathway, it is unclear whether the regulated step is located at the conversion of the C-20 methyl to an alcohol, or the conversion of the alcohol to aldehyde, or both. More studies using exogenous GAs, coupled with the quantitative analysis in the content of GAs are needed to better understand the processes.

Application of various GAs under non-vernalized condition would also provide insights in understanding which pathway(s) may play an important role in the GA biosynthesis process in *Arabidopsis*. Strong stem growth was promoted only by GA_4, which has a hydroxyl group at C3, under non-vernalized condition (Fig 3.2). GAs including GA_{14} and GA_{36} (both of which are C3 monohydroxylated GAs) also promoted stem growth (Fig 3.4). Plants treated with GA_{14} or GA_{36} demonstrated stronger stem growth than those treated with GA_{12} and GA_{19} (Fig 3.4). This result suggests that the 3ß-hydroxylation reaction plays an extremely important role in determining the bioactivity of GAs in *Arabidopsis*.

Evidence from other studies also gives support to the idea that 3ß-hydroxylation plays an important role in determining the biological activity of GA in *Arabidopsis*. Two mutant lines, *ga4* and *ga5,* which are associated with the 3ß-hydroxylation and the GA 20-oxidase reaction, respectively, were treated with GA_9 to determine its effect on stem growth (Talon et al., 1990b). Their results showed that stem growth was only observed in the *ga5* mutant line. The 3ß-hydroxylation reaction in the *ga4* mutant line might have been blocked rendering GA_{20} or GA_9 incapable of being converted to the bioactive form of GA_1 or GA_4, respectively.

GA_4 is the Most Biologically Active GA in Promoting Stem Growth of *Arabidopsis*

Applications of various C-19 GAs and GA precursors demonstrated that stem growth was strongly promoted when plants were treated with GA_4 or GA_9 (Fig 3.5). However, applications of GA_3 resulted in greater stem growth than GA_1. They are slightly different in their structures. Both GA_1 and GA_3 contain hydroxyl groups on C-3 and C-13 but GA3 contains a C-2,3 double bond. The explanation for why plants treated with GA_3 had greater stem growth than those treated with GA_1 is as follows. GA_1 may have been deactivated following C-2 hydroxylation to GA_8 whereas GA_3 cannot be converted to an inactive form by the same enzyme because of the C-2,3 double bond. Therefore, the lower rate of metabolism presumably results in a longer period of time in which tissues contain biologically active concentrations of GA_3 than GA_1. It is also possible that the C-2,3 double bond of GA_3 may cause some changes in

its three dimensional structure and allow GA_3 to have a stronger interaction with GA receptors than GA_1 leading to greater stem growth.

The results from dose response experiments further demonstrate that GA_4 is more bioactive than GA_1 in *Arabidopsis* (Fig. 3.8). Based on the following reasons, it is reasonable to suggest that GA_4 is the bioactive form of GA responsible for stem growth in *Arabidopsis*. First, GA_{20} and GA_1, products of 13-hydroxylation, have little or no effect on stem growth in CR plants under non-vernalized condition whereas GA_4, a product of 3-hydroxylation, has the ability to promote stem growth in the non-vernalized plants.

Second, the response of stem growth from both LD treated CS933 and vernalized CR plants demonstrated that GA_4 was the most active form in *Arabidopsis* under non-inductive conditions. Third, GA_9, a precursor of GA_4 and highly biologically active GA in *Thlaspi*, also gives support to the idea that GA_4 has intrinsic biological activity in *Arabidopsis* (Metzger, 1990; Kobayashi et al., 1993).

Stem height was significantly less in non-vernalized than vernalized plants treated with the same GA. For example, stem height of the vernalized plants with GA_4 treatment was six times greater than that of non-vernalized plants (Fig 3.2 and 3.3). Increasing the amount of GA_4 to 10 μg and applying it to plants under non-vernalized condition resulted in stem growth that was only one half of that with the 1μg application on the vernalized plants (Fig 3.3 and 3.8). It is apparent that CS 933 plants are more sensitive to exogenous GA than the CR plants when comparing average stem heights of these two lines under non-inductive condition (Fig 2.8 and Fig 3.2). There

may be two possible reasons to explain this. First, the regulation of GA biosynthesis may not be the key factor in control of thermoinductive stem growth. Second, CR plants may need vernalization to: 1) lift the block in GA biosynthesis and 2) to increase GA sensitivity.

The first idea is supported by a study in which only sustained application of GA promoted flowering in the CR line under non-inductive conditions (Sheldon et al., 1999). However, expression of the *FLC* gene, which is one of the critical genes for vernalization, was not affected (Sheldon et al., 1999). Mutant *ga1-3,* which has a defect in kaurene synthase, does not flower under SD (Michaels and Amasino, 1999b). Kaurene synthase is known to catalyze the first step in GA biosynthesis. Further investigations indicate that the block to flowering in SD cannot be overcome by vernalization, but can be reversed in LD. This suggests that GA may not play a key role in the thermoinduced flowering.

Evidence from experiments on the cold-requiring species *Raphanus sativus* showed that GA levels increased in response to vernalization (Suge, 1970). Moreover, results obtained from studies using *Thlaspi*, a relative of *Arabidopsis*, showed that GA metabolism in shoot tips was changed by the cold treatment (Hazebroek et al., 1993). Contrary to the data described in earlier discussions, these results suggest that GA biosynthesis is under thermoinductive control. One may conduct an experiment in which the expression of the *FLC* ortholog in *Thlaspi* is compared between induced and non-induced plants. If transcript levels of *FLC* do not change during or after the cold treatment, then GA may play a role in controlling part of the vernalization response

that is not associated with the *FLC* expression since it has been shown that *FLC* plays the central role in the vernalization.

The second idea is that cold treatment not only can lift the block in GA biosynthesis but also can increase the sensitivity of CR plants to GA. Several genes that that have been known to be associated with GA response such as *SPINDLY* (*SPY*), repressor for ga*1-3 (RGA*), *GIBBERELLIN INSENSITIVE* (*GAI*) and *SHORT INTERNODES* (*SHI*) have been cloned by different groups (Jacobsen and Olszewski, 1993; Jacobsen et al., 1996; Peng et al., 1997; Silverstone et al., 1998; Fridborg et al., 1999). All these genes are negative regulators of GA signal transduction pathway (Sun, 2000).

To date, there has been no report that addresses the question of whether or not vernalization response lifts the block of the GA biosynthesis and suppresses the expression of both *RGA* and *GAI* genes, key components in the GA signal transduction pathway.

Evidence from research on the function of *RGA* strongly indicates that *RGA* is a repressor of GA signal transduction (Silverstone et al., 2001). A recessive *rga* mutation in the GA-deficient mutant line *ga1-3* resulted in partial reversal of the dwarf phenotype (Silverstone et al., 1997a; 1997b). Sequence analysis suggests that *RGA* is a member of the VHIID family, which functions as a transcriptional regulator (Silverstone et al., 1998; Di Laurenzio et al., 1996). Quantitative analysis showed the content of GA in *rga* mutant plants is similar to that of the wild type. However, in *rga* the transcripts of *GA4*, the gene that encodes the C-3 hydroxylase, declined 10% when

compared with wild type plants. This suggests that the decrease in the expression of *GA4* gene was the result of impaired feedback regulation caused by the mutation in *RGA*. The expression of the fusion protein containing a green fluorescent protein and the promoter of *RGA* was also investigated in the same study. The level of both fusion protein and the endogenous *RGA* protein dropped rapidly after the GA treatment. This suggests *RGA,* the GA response repressor protein, was degraded by a component in the GA signal transduction pathway.

Finally, *GAI* is a GA insensitive mutant. High levels of GA_1 were detected in *GAI* plants suggesting that the GA response pathway was partially inhibited (Peng et al., 1997). DNA sequencing analysis revealed that *RGA* and *GAI* share 82% sequence identity (Peng et al., 1997; Silverstone et al., 1998). Both genes are members of the GRAS family known for their regulatory functions in transcription (Peng et al., 1997, 1999; Silverstone et al., 1998; Pysh et al., 1999). A unique conserved region was found in both *RGA* and *GAI* near the N-terminal called DELLA. It has been confirmed that deletion in the DELLA region results in a constitutive repressor of the GA response and confers a dwarf phenotype (Peng et al., 1997). The loss of *GAI* function also increases the resistance to GA biosynthesis inhibitors such as paclobutrazol. Based on these observations, Peng et al. (1997) suggests that *GAI* is a repressor in the GA signal transduction pathway. Transgenic rice plants overexpressing *GAI* have been created (Fu et al., 2001). They exhibit a dominant dwarf phenotype, which is believed to be associated with high levels of *GAI* (Fu et al., 2001). This evidence further

supports the idea that *GAI* is one of the important components in the GA signal transduction pathway.

To elucidate the interaction of GA signal transduction and vernalization, the following experiments can be conducted. First, perform quantitative analysis to measure the endogenous levels of GAs in CR plants. The result of this experiment can help confirm whether or not GA biosynthesis is blocked in the GA 20-oxidase reaction. If high levels of the bioactive form of GAs such as GA_1 or GA_4 accumulate in non-vernalized plants, this may indicate that CR plants are less sensitive to GAs as a result of an impairment in GA signal transduction.

Second, the expression of the *RGA* gene can be investigated since *RGA* is down-regulated by GA. If *RGA* expression levels do not decrease after GA treatment under non-inductive conditions, this may indicate that exogenous GA has reduced ability to degrade *RGA,* and thus has less effect on stem growth. Since the knowledge about GA signal transduction is still very limited, more research is needed to elucidate whether GA signal transduction also plays a crucial role in the vernalization response.

GAs and Flowering

Vernalization is one of the most important environmental signals to control the transition from the vegetative to the reproductive state. Several methods have been established to measure the flowering response of plants such as the time required for the first flower to bloom, the number of nodes formed before the first flower and the number of leaves before the first bloom (Lang, 1965; Bernier et al., 1981).

Counting the number of leaves is a more applicable method in measuring the flowering response of *Arabidopsis* in this study. Plants exhibiting a stronger response to GA treatment tend to generate fewer rosette leaves whereas plants that exhibited little or no response to GA treatment have more rosette leaves (Blázquez et al., 1998; Martinez-Zapater and Somerville, 1990; Reeves and Coupland, 2001; Wilson et al., 1992). Another method in determining the effects of GA on stem growth is to measure stem height after receiving the treatment.

In this research, several GAs were applied to vernalized and non-vernalized CR plants. Stem growth was promoted with the applications of various GAs under non-vernalized condition (Figs 3.2, 3.4 and3.7). However, the various GA treatments failed to promote flower development at the end of the experiment. All plants had aerial rosettes at the top of the elongated stem and resembled palm trees.

One possible explanation of this phenomenon is that GA biosynthesis may not have a direct relationship to flower initiation. Stem elongation and flower development, which often occur around the same time in rosette plants, may be two separate developmental events. The late flowering phenotype of CR plants can be overcome by vernalization, which apparently is related to the decrease in the expressions of the *FLC* gene. However, the expression of the *FLC* gene did not change after GA application (Sheldon et al., 1999; 2000). These findings support the suggestion that GA biosynthesis and vernalization are independent processes (Sheldon et al., 1999). The same report also stated flowering was induced with continuous GA_3 treatments. This may also help to explain why no flower induction was observed in my

research because the GA application period was not long enough to stimulate flowering. It is possible that increasing the duration of the GA treatment under non-vernalized condition might have triggered flower development in my studies.

Some research data suggested that vernalization promotes flowering through both *FLC*-dependent and a *FLC*-independent pathways (Michaels and Amasino, 2001). Flowering of the *FLC* mutant line under vernalized condition showed evidence that vernalization promotes flowering via a *FLC*-independent pathway (Michaels and Amasino, 2001). Moreover, a vernalization-responsive gene, *EARLI1,* has been identified by a gene expression screening method (Wilkosz and Schläppi, 2000; Gendall et al., 2001). Unlike the decline of the expression of the *FLC* gene in vernalized plants, the expression of *EARL11* increased during the cold treatment and remained at a high level until after vernalization. Thus, it is worthwhile to determine whether the GA biosynthesis pathway is related to the *FLC*-independent vernalization pathway or if the GA biosynthesis pathway is related to another vernalization gene.

Data obtained from other research also shows that GA may interact directly and modulate the expression of meristem identity genes such as *LEAFY* (*LFY*) (Blázquez et al., 1998; Blázeuez and Weigel, 2000; Nilsson et al., 1998). *LFY* is required for the transition of inflorescence to floral meristems and the specification of floral meristem identity in *Arabidopsis* (Weigel et al., 1992). Blázquez's group (1998) found that the activity in the promoter region of *LFY* decreased in the *ga1-3* mutant, which has a mutation in the first step of GA biosynthesis pathway and is unable to

flower in SD. Only transgenic plants overexpressing *LFY* in *ga1-3* background can overcome late flowering in SD (Blázquez et al., 1998).

The promoter activity of *LFY* was investigated in both SD and LD using transgenic plants containing a *GUS* reporter gene with various lengths of the promoter region of *LFY* (Blázquez and Weigel, 2000). An eight base pair motif was found in the proximal promoter region of *LFY*. This sequence is known as a consensus binding site for MYB transcription factors of animals and of the plant R2R3 family (Wang, 1997). Plants with mutation in this region showed less *GUS* activity under LD. However, *GUS* activity remained low throughout the course of the study in both SD and GA_3 treatment under SD, whereas plants with the normal 8-bp motif showed an increase in *GUS* activity during the course of the study in both conditions. This evidence provides a clue that the endogenous GA enables the interaction with the meristem identity gene that controls flowering.

It is not clear whether the interaction between GA and the meristem identity gene also plays a particular role in regulating the vernalization response. Application of GA in CR plants neither promoted stem growth nor stimulated flowering in non-induced condition. This indicates the activity of *LFY* may not change during the experiment. Therefore, it is necessary to investigate the activity of the meristem identity gene in CR plants under non-vernalized and vernalized conditions in order to understand whether vernalization interacts with the meristem identity gene via GA biosynthesis.

This study concludes that GA 20-oxidase is the rate-limiting step in GA biosynthesis under non-inductive condition and GA_4 is the GA responsible for biological activity, at least for stem growth in *Arabidopsis*. Also, vernalization treatment not only lifts the block of GA biosynthesis but also increases plant sensitivity to GA.

CHAPTER 4

GENERAL DISCUSSION

Photoperiodism and vernalization are the two common phenomena of plants requiring exposure to certain day-lengths or low temperatures for floral initiation (Thomas and Vince-Prue, 1984).

In addition to the external factors such as light and temperature, some plant hormones such as GAs have been shown to modify flowering behavior in a wide range of plants. It is well known that GA plays a crucial role in the daylength and temperature mediated regulation of stem elongation in rosette long day and cold requiring plants.

It is important to identify the regulatory step(s) in the GA biosynthesis pathway that control plant growth and development. Evidence of GA involvement in regulating stem elongation in my study was obtained through various physiological experiments performed on two late-flowering lines of *Arabidopsis* plants. The regulatory step(s) in GA biosynthesis pathway was further identified through quantitative analysis of endogenous KA levels extracted from plants grown under inductive and non-inductive conditions and experimental results collected from plants treated with various GAs under two different growth conditions. The findings from

these studies suggest GA_4 is the most bioactive form and that GA20-oxidase plays a key role in the regulation of GA biosynthesis pathway in *Arabidopsis.* The evidence for these conclusions is summarized in the separate sections below.

The Role of GA 20-Oxidase in GA Biosynthesis Pathway

It has been suggested GA 20-oxidase plays a key role in the GA biosynthesis pathway in *Arabidopsis*, tobacco and potato plants (Huang et al., 1998; Gil and Martinez- Garcia, 2000; Carrera et al., 1999; Wu et al., 1996; Jackson et al., 2000; Phillips et al., 1995). There are several unique characteristics of GA20-oxidase. First, a single GA 20-oxidase enzyme can catalyze several steps of oxidation reaction in the GA biosynthesis. Second, there are at least three GA 20-oxidase genes that have been cloned in *Arabidopsis*, legumes and potato plants (Phillips et al., 1995; Martinez-Garcia et al., 1997; Carrera et al., 1999). The expression of these GA 20-oxidase genes is tissue specific, which gives the indication that each GA 20-oxidase gene may play different roles or catalyze different GA conversions in the GA biosynthesis pathway that regulates plant growth and development. Finally, the application of GAs to *Arabidopsis* and potato plants, led to the suppression of the GA 20-oxidase gene, suggesting the enzymes may be involved in the regulation of GA biosynthesis.

To date crucial evidences regarding the significance of GA 20-oxidase in the regulation of GA biosynthesis came from molecular studies in transgenic *Arabidopsis* plants (Coles et al., 1999). Plants overexpressing the GA 20-oxidase gene showed an

elongated hypocotyl and early flowering phenotype. The endogenous content of GA_4 in the vegetative shoot tips of the over-producers was 2-3 fold higher than the wild type.

My research data suggests that GA 20-oxidase is limiting under non-inductive conditions. Responses of non-induced plants (SD and non-vernalized plant) to the treatment of either GA_{12}, GA_{14}, GA_{24} or GA_{36} gave support to this idea. More stem growth was elicited when plants were treated with GA_{24} and GA_{36} (the intermediate product of GA 20-oxidase) than with GA_{12} and GA_{14} (Fig. 2.14 and Fig. 3.4). Talon et al. (1990b) suggested that applied [^{14}C] GA_{12} was converted into either GA_{53} or GA_{15} by the 13-hydroxylation or oxidation pathway, respectively. No GA_{14} (3β-hydroxy GA_{12}) was detected by Talon et al. 1990b. In my research, some stem elongation was observed with exogenous GA_{14} whereas no stem growth was observed with GA_{12}. This indicates that GA_{14} can be used as a substrate for GA 20-oxidase to be converted to some bioactive form of GA.

Do GAs Other Than GA_1 Have Intrinsic Biological Activity in *Arabidopsis*?

Significant differences in stem growth of *Arabidopsis* plants following application of various GAs under non-inductive condition suggested stem elongation of rosette plants might be regulated by the biological activity of various GAs. GA_4 appears to have intrinsic biological activity based on results obtained from my research work as follows. Only plants treated with GA_4 and GA_9 exhibited strong stem growth under SD or non-thermoinductive conditions (Fig. 2.8 and Fig 3.2). CS933

lines were not as responsive to GA_1 under non-inductive condition as plants treated with GA_9. Non-vernalized plants of the CR line were also insensitive to exogenous GA_1. Greatest stem growth was observed in plants of both lines treated with GA_4 (Fig 2.19 and 3.8).

The fact that GA_4 is more biologically active than GA_1 may be an indication it has intrinsic biological activity, while GA_1 is thought to be the GA responsible for GA responses in most species (Fujioka et al., 1988; Ross et al., 1992; Swain and Reid, 1992). Work from other laboratories has provided evidence that this is not always the case. For example, GA_4 is believed to be the major biologically active form of GA in plants including tobacco and *Mathiola incana* (Hisamatsu et al., 2000; Vidal et al., 2001). Young *Arabidopsis* seedlings with GA_4 treatment had stronger hypocotyl growth than with GA_1 treatment (Cowling et al., 1998). Furthermore, applications of GA_4-related, 3ß-hydroxy GAs, including GA_{14} and GA_{36} elicited more stem growth than those of 13C-hydroxylated GAs like GA_{19} (Fig. 2.14 and Fig. 3.4). This evidence suggests that the 3ß-hydroxylation pathway leading to GA_4 and/or GA_1 may play a key role in regulating stem growth in *Arabidopsis*.

Young seedlings treated with GA_1 and GA_4 in both maize and rice had similar stem growth (Kobayashi et al., 1993). However, GA_1 has been suggested as the most bioactive form of GAs in both maize and rice. I think it is possible that plants may contain more than one GA with intrinsic biological activity. Each individual bioactive form of GA may be involved in different physiological reactions such as stem growth, leaf expansion and internode elongation. The distribution of GAs in different parts of a

plant was investigated in *Salix pentandra* (Olsen et al., 1995). Higher concentrations of GA_1 were detected in young leaves than in any other part of the plant. Moreover, the effects of photoperiods on GA levels in shoot tips of *Salix pentandra* plants were also conducted by Olsen et al. (1995). GA_1 levels were reduced by more than 50% when plants were removed to SD condition. The content of several GAs in expanding stem internodes and young ears of wheat was analyzed by GC-MS (Webb et al., 1998). Only GA_4, GA_9 and GA_{34} were detected in expanding stem internodes whereas GA_1, GA_{20} and GA_{29} were present in both internodes and ears. This indicates that the non-13-hydroxylation GAs have different distribution patterns in wheat.

The Relationship between GA Biosynthesis and Environmental Factors

In my research, *Arabidopsis* plants with two phenotypes (requiring LD and vernalization to promote stem growth) were selected to investigate the relationship between GA metabolism and stem growth under various environmental conditions. Physiological responses such as stem growth in the long day requiring line (CS933) were, in general, different from those shown on plants of the cold requiring (CR) line. The differences in their responses were more pronounced when they received the same GA treatment under non-inductive conditions. All CS933 plants reached their final stage in plant development (flowering stage) at the end of the experiments. However, the CR plants did not flower at the end of the study, even with the GA_4 treatment.

It is interesting to note that regulation of the metabolism of GA seems to be a necessary step in growth and development in *Arabidopsis* despite the fact that CS933

and CR lines may be using different mechanisms to reach and accomplish the flowering stage (Nakayama et al., 1995; Xu et al., 1995; Huang et al., 1998). However, bolting (stem elongation) and flowering (flower initiation) are separate developmental phenomena. Bolting is part of inflorescence development, but it occurs after flower initiation. Therefore, GA-regulated stem growth may not result directly from LD or vernalization exposure, but rather as an indirect consequence of flower induction or flower initiation.

In summary, my research data suggests that stem elongation is the result of the activation of GA 20-oxidase especially the conversion of $GA_{12} \rightarrow GA_{24} \rightarrow GA_4$. The increase in GA4, which appears to have intrinsic biological activity, results in stem growth in Arabidopsis. In addition, data in this study show that GA_{24}, GA_{36}, GA_9 and GA_4 are much more active than their 13-OH analogs suggesting the non-C3, 13 hydroxylation pathway is an important step in GA biosynthesis for plant growth and development. More research is needed to identify the signal transduction pathway(s) between flower initiation under inductive conditions and the regulation of the GA biosynthesis pathway.

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