# ENDOGENOUS GIBBERELLINS IN DEVELOPING APPLE SEEDS IN RELATION TO ALTERNATE BEARING

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*To my family*

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# ABBREVIATIONS

| | |
|---|---|
| ABA | abscisic acid |
| amu | atomic mass unit |
| ANOVA | analysis of variance |
| BA | benzyladenine |
| BSTFA | *bis*-trimethyl-silyltrifluoroacetamide |
| CEPA | (2-chloroethyl) phosphonic acid |
| CI | chemical ionization |
| C/N ratio | carbonhydrates/nitrogen ratio |
| cm | centimeters |
| CPPU | N-(2-chloro-4-pyridyl)-N-phenylurea |
| DNA | deoxyribonucleic acid |
| DAFB | days after full bloom |
| EI | electron impact |
| GA | gibberellin |
| GC-MS | gas chromatography – mass spectrometry |
| GUS | β-glucosidase |
| HPLC | high performance liquid chromatography |
| IAA | indoleacetic acid |
| IPGSA | International Plant Growth Substances Association |
| KRI | Kovats retention indices |
| LC-MS | liquid chromatography – mass spectrometry |
| m | meters |
| mm | millimeters |
| m/z | mass/charge ratio |
| MeTMS | methyl esters TMS ether |

| | |
|---|---|
| MIM | multiple ion monitoring |
| NAA | naphthaleneacetic acid |
| $NH_4^+$ | ammonia |
| $NO_3^-$ | nitrate |
| P | Phosphorus |
| PVP | polyvinylpyrrolilidone |
| RNA | ribonucleic acid |
| SIM | selected ion monitoring |
| TIBA | 2,3,5-triodobenzoic acid |
| TIC | total ion current |
| TMS | trimethylsilyl |
| TMSOH | trimethylsilylhydroxyl |
| UV | ultraviolet |
| WCOT | wall-coated open tubular |

# ABSTRACT

Tu, Yicheng, M.S., Purdue University, December, 2000. Endogenous Gibberellins in Developing Apple Seeds in Relation to Biennial Bearing. Major Professor: Peter M. Hirst.

It is believed that Gibberellins (GA) produced in apple seeds act as an inhibitory signal to flower induction in the neighboring buds. As an exception, $GA_4$ is not inhibitory and sometimes found to be promotive to apple flowering. The alternate bearing of apple cultivar is thus associated with the activity of endogenous GAs in the seeds of that specific cultivar. To elucidate the impact of GAs on flowering habit of biennial and non-biennial apple cultivars, freeze-dried embryos of 'Fuji' (biennial, on-year) and 'Gala' (non-biennial) were thoroughly scanned for GAs by GC-MS. Altogether, 17 different kinds of GAs were identified in 'Gala' and 'Fuji'. They are: $GA_1$, $GA_3$, $GA_4$, $GA_7$, $GA_{20}$, $GA_{31}$, $GA_{34}$, $GA_{35}$, $GA_{44}$, $GA_{45}$, $GA_{50}$, $GA_{54}$, $GA_{61}$, $GA_{63}$, $GA_{68}$, $GA_{80}$ and $GA_{88}$. In addition, we also traced 6 GA-like mass spectra that do not match any published GA mass spectrum reference. The possible structures of these GA-like compounds were also proposed. More types of GAs were found in 'Fuji' embryo samples than in those of 'Gala'. 'Fuji' sample contained significant amount of $GA_{88}$, which didn't appear in 'Gala' samples. This suggests higher GA activity in 'Fuji' considering most GAs identified are biologically active. However, quantitative analysis of $GA_1$, $GA_3$, $GA_4$, $GA_7$, and $GA_{20}$ for both cultivars in 1998 ('on-year' for 'Fuji') and 1999 ('off-year' for 'Fuji') indicated a central role of $GA_4$, a flower-promoting gibberellin, in control of apple flowering. We did not find higher $GA_1$, $GA_3$ and $GA_7$ activity in 'Fuji' seeds in 1998. In fact, 'Gala' even produced more $GA_7$ than 'Fuji' at certain time of the 1998 growing season. The $GA_4$ concentration in 'Gala' was constantly higher in 'Gala' than in 'Fuji' in 1998. We thus propose that regular bearing cultivars such as 'Gala' can use $GA_4$ to

protect themselves from the inhibitory effects of $GA_1$, $GA_3$ and $GA_7$. As expected, the activity of all 5 GAs quantified was found to be similar between 2 cultivars in 1999.

# LITERATURE REVIEW

## Introduction

As the initial step of reproductive growth of plants, the flowering process has drawn intensive attention from plant physiologists. For a long time people have realized that the flowering of plants is a complicated developmental process that involves a series of morphological and physiological stages under the control of a number of external signals and internal factors. Among all factors that control plant flowering, photoperiod and plant hormones have been studied in great detail. The availability of modern molecular techniques has made it more convenient and, moreover, fruitful to scrutinize the mechanisms of plant flowering. Genes have been identified that genetically control floral organ identity and development (Weigel *et al.*, 1992; Mandel *et al*., 1992; Mizukami & Ma, 1997). The expression of these genes was found to be regulated by photoperiod and hormones (Okamuro *et al.*, 1996).

In the horticultural world, flowering of crops is of great economic importance. For almost all fruit trees, yields depend on the number and quality of flower buds formed. An extreme example is the frosts in early spring in some areas makes peach growing a very risky business because it can lead to total crop loss in the same year by killing all the flower buds. Such a crop loss due to adverse environmental conditions can lead to a phenomenon known as alternate (biennial) bearing, which is characterized by large yields of small-size fruits in the "on-year" and low yields of oversized fruits in the "off-year" (Fig 1). Biennial bearing is one of the big problems in today's apple orchards. Alternate bearing can be triggered by a many factors (Monselise & Goldschmidt, 1982; Jonkers, 1979). However, the direct cause of alternate bearing was found to be lack of floral buds in the "off-year" rather than lack of fruit set (Jonkers, 1979). Detailed understanding of flower formation mechanisms is necessary to develop appropriate orchard techniques to reduce biennial bearing.

Plant hormones play a role in the flowering of fruit trees (Luckwill, 1970; Ma, 1987). The effects of 5 individual groups of plant hormones on flowering have been well described (Hoad, 1984). Among them, gibberellins (GA) were found to have more direct and significant influences on flower formation of many species than other plant hormones. As a general observation, exogenous GA inhibits apple flowering if applied during flower induction (Li *et al.,* 1995). However, the role endogenous GA plays in this issue is a source of contradiction and ambiguity. One of the difficulties of research on endogenous GA is the lack of a precise and efficient methodology to identify the type and amounts of the GAs in plant tissues. The development of high performance liquid chromatography (HPLC) as a separation technique and combined gas chromatography – mass spectrometry (GC/MS) as a molecular detection method has provided the technology necessary for efficient quantification of specific GAs (Crozier & Durley, 1983; Pearce *et al*., 1994).

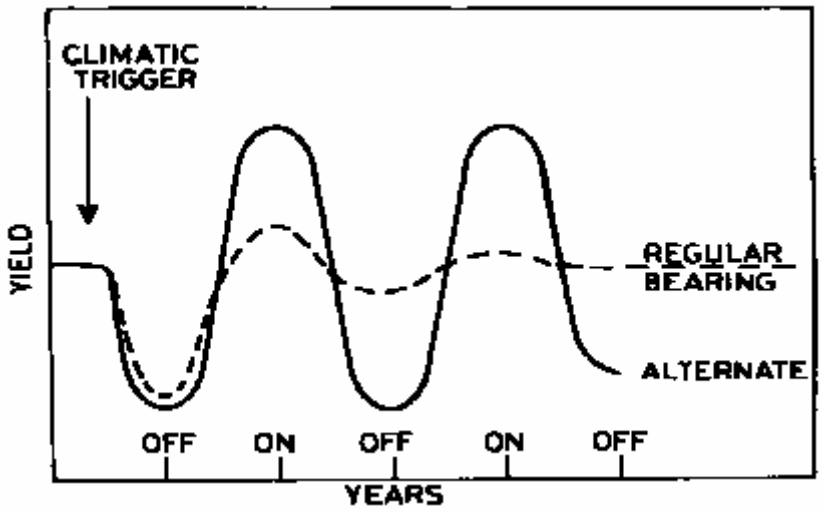


Fig 1**.** Schematic differences in cropping of a regular and biennial bearing cultivar reacting to the same environmental trigger. (Monselise & Goldschmidt, 1982).

The major objective of this research was to further understand the process of apple flowering as related to regulation by endogenous gibberellins. Through study of the types and amount of endogenous GA of different apple varieties (biennial bearing vs. non-biennial bearing), the GA activity pattern in these varieties, as a possible mechanism of control of alternate bearing, is investigated. Most previous works on endogenous GAs

in apples have used bioassays and are not qualitative. For those few studies that used GC-MS, the GA content was not related to flowering or biennial bearing. This is what makes this study unique.

**Basic morphology of apple flowering**

Apple (*Malus domestica* Borkh.), as a woody perennial species, is a typical temperate deciduous fruit tree. It forms terminal buds on the shoots in addition to lateral buds developed from the leaf axils. Even though apple shoots can grow up to 10 feet, the spurs, shortened shoots with length less than 5 cm, are the platform of fruit production and thus the focus of a lot of physiological research. Figure 2 shows the structure of an apple spur. The newly formed shoot in a spur is called a bourse. Like other shoots, a bourse is the location where buds are formed. The bourse buds, along with those on other shoots, are the potential flowering sites for the following year. Likewise, the flowers from which fruits develope were initiated during the prior growing season.

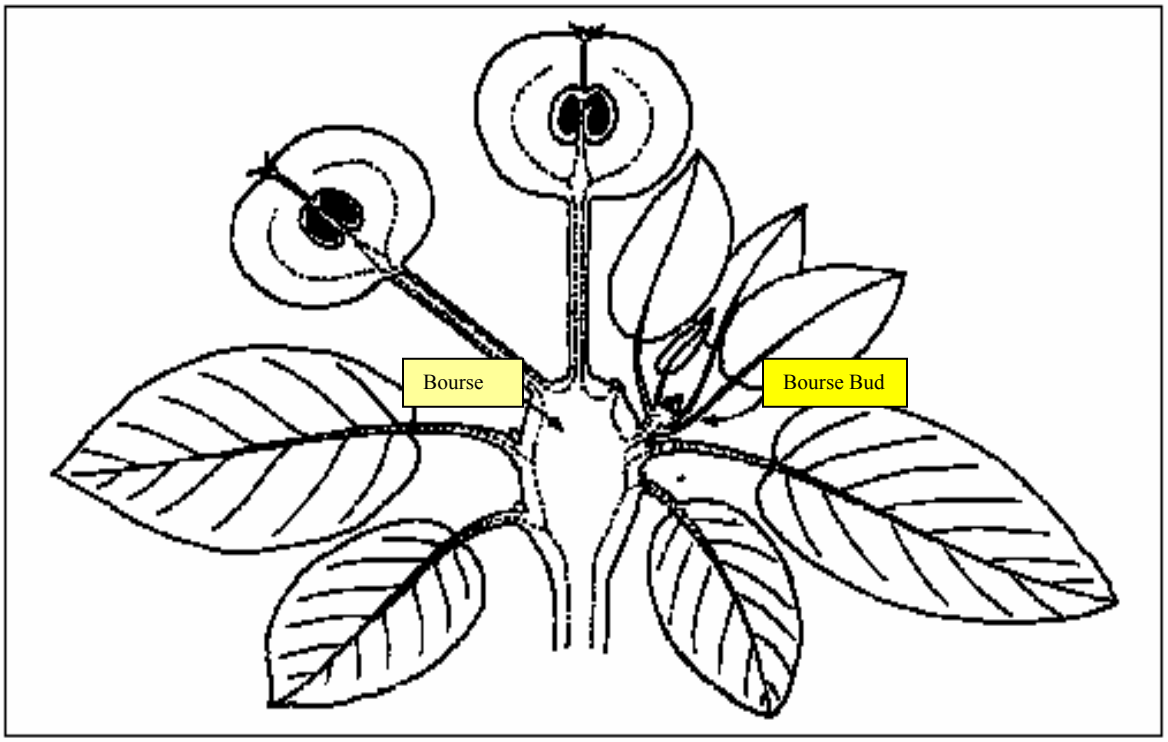


Fig 2. Structure of an apple spur. (Luckwill, 1970)

Flowering of apple is a process that consists of several stages including flower induction, flower initiation, flower differentiation and anthesis (bloom). **Flower induction** is the transition of meristem development from vegetative status to reproductive status. During this period, flowering signals are received by the apical meristem and the genes required for flower organ development are turned on. Flower induction is followed by **flower initiation** when a series of histological changes including active mitosis are underway but no visual difference can be observed. Because it is very difficult to distinguish the time of flower induction and flower initiation, these two terms are often used interchangeably in references. They are both used to describe the critical period when the buds are sensitive to stimuli which determine their fate. When a bud is induced to be reproductive, it will irreversibly undergo the process of floral organ development, regardless of the internal/external conditions that could affect flower induction (Miller, 1982; Janick, 1974). **Flower differentiation** is characterized by morphological changes of apple buds. It starts by the appearance of floral promordia in the bud (Abbott, 1977; Hirst & Ferree, 1995a), and ends with the development of the promordia of other parts of the floral organ. Flower differentiation is always marked by the appearance of the dome-shape apex in the bud that takes place about 12 weeks after full bloom (Abbott, 1977; Huang *et al.*, 1986). Then the rate of apex enlargement will increase. As a result, the central part of the apex becomes a 'King' flower surrounded by 4 lateral flowers (Hirst & Ferree, 1995a) and sepals, petals, stamens and carpels are differentiated subsequently. The development of flower lasts until the following spring, when anthesis occurs (Zeller, 1955, 1960, as cited by Verheij, 1996).

**Internal changes of bud during flower induction**

It's not until flower differentiation that visual evidence showing that flower induction has occured can be seen. Nevertheless, flower induction is not such a 'silent' process in terms of biochemical and histological changes occurring in the apex.

There are a number of reports showing that the nucleic acid (both DNA and RNA) content in the buds of fruitless spurs is higher than that of spurs bearing fruits (Buban & Simon, 1978; Buban & Hesemann, 1979, as cited by Buban & Faust, 1982). On the

contrary, the content of histone proteins is lower in fruitless spurs. The assumption here is that the terminal buds of fruitless spurs will be induced to form flowers (Feucht & Arancibia, 1970). In floral buds of 'Starkrimson', Bai *et al*. (1986) found 2 peaks of RNA content between which a DNA content peak is sandwiched. The second RNA peak ends about 2 weeks prior to the peak of floral organ differentiation. Studies on other fruit species such as orange (Huang *et al*, 1991) and almond (Kabeel *et al*., 1981) indicate a higher RNA/DNA ratio is favorable for flower induction, but there is no evidence for this in apples.

Proteins and amino acids are indispensable to the flowering of apple trees. There is a significant increase in protein synthesis associated with flower induction. This has been attributed to the requirement for a large amount of proteins during flower development (Marcelle, 1984). Similar results were obtained from experiments on citrus (Liu *et al*. 1984). In this research, a 5-10 fold increase in the concentration of 17 different kinds of amino acids was detected during flower induction. Subsequently, these amino acids were quickly converted into proteins when flower organ development commenced.

Histological activities are also visualized in the buds under flower induction. These activities are represented by active mitosis in the entire apex and the connection of central meristem and subdermatogen (Buban & Faust, 1982). It was also observed that the number of layers in the accessory tunica is changed from 4 to 2. These histological changes are immediately followed by the differentiation of flower primordia.

**Time of flower induction**

The level of alternate bearing can be greatly reduced by various methods among which the reduction of flower formation in the 'on-year' (induced in the previous 'off-year') is the most important one (Monselise & Goldschmidt, 1982). The primary method to reduce biennial bearing is thinning fruit in the on-year to try to increase bloom in the following 'off-year'. Since the fate of buds is determined during the flower induction period, most of the orchard techniques for the purpose of controlling apple flowering will be effective only if they are implemented during the period when flower induction is

possible. Thus, the studies on the flower induction period can provide important guidance to the scheduling of these techniques.

Flower induction and shoot growth. Barnard (1938, as cited by Ramirez, 1979) first reported the correlation of cessation of terminal growth and flower organ differentiation in apples. Huet (1973) had similar results from his research on pears. Other studies indicate that flower induction can take place either before or after shoot elongation ceases (Williams, 1973; Tromp, 1968; Zeller, 1960). Despite the contradictions of these reports, it is now generally accepted that flower differentiation occurs after the cessation of shoot growth (Luckwill, 1970; Huang et al. 1986). Huang and coworkers (1984, 1986) found a 3-7 week interval between shoot growth cessation and flower differentiation in 'Ralls Janet' apple and they proposed that this is exactly the time of flower induction. They also studied the duration of flower differentiation and shoot growth at a whole-tree level. Both processes lasted 5 weeks due to the lack of synchronization of buds on different branches. The length of the time interval depended on year, area and variety. It's unlikely that the cessation of shoot elongation is the direct cause of flower differentiation. The transition from vegetative growth to reproductive growth is a process highly determined by the physiological condition of individual buds.

Bud development before flower induction. Morphologically, a bud is an embryonic miniature stem bearing a number of leaves in various modified forms called 'appendages'. A typical apple bud has the following types of leaves from outside to inside (or from older to younger): bud scales, transition leaves, true leaves and bracts (Fig 3). The primordia of floral organs are developed from the meristem above the bracts. Our understanding of apple bud development is to a great extend attributed to the work of Abbott (1970) and Fulford (1965). Not only did they describe the process of appendage increase in apple buds, the concept of 'critical appendage number' was also introduced by them.

Some species in the grass family such as wheat, corn and sorghum, will differentiate inflorescences only when a certain number of appendages are formed in the buds (Zeng, 1990). This also holds true for eggplant, tomato (Zeng, 1990) and apple. Abbott (1970) gave the critical appendage number of some apple varieties in his paper,

indicating this number is inherently bound to the cultivar. This was confirmed by Hirst and Ferree (1995a, 1996) who showed that rootstock and year of investigation had little effect on the critical appendage number. Although a certain number of appendages are present prior to the visual signs that flower induction has occurred, this does not necessarily imply a causal relationship. The time interval between formation of 2 neighbouring appendages (plastochron) also has its significance. This number for apple floral buds is 7 days while being longer or shorter than 7 days favors vegetative growth (Fulford, 1965). Hirst and Ferree (1995a) found similar plastochron to Fulford for floral buds but longer plastochron for vegetative buds. Luckwill (1974) hypothesized that plant hormones control apple flowering by altering the length of the interval mentioned above. Unfortunately, no further experiments support his theory.


Fig 3. Cross section illustration through a bourse bud. From outer to inner appendages: budscales (thick solid structures), transition leaves (flecked structures), true leaves (unflecked structures), and bracts (thin lines) (Abbott, 1970)

After reaching the critical appendage number, floral buds will start flower initiation as well as differentiation of more appendages, while in leaf buds the appendage development slows down. This explains why floral buds have at least 2-3 more appendages than vegetative buds (Cao, 1988, as cited by Zeng, 1990).

Methods of investigating the period of flower induction. Flower induction in apple requires a certain number of healthy leaves. Removing leaves (defoliation) before

flower induction can effectively inhibit apple flower formation. Therefore, by removing leaves at different time after full bloom people have inferred the ending time of flower induction (Huet, 1972; Li *et al*., 1995). When defoliation has no inhibitory effect on flowering, the implication is that the buds have passed the flower induction period. Another way to study the flower induction period is removing fruits, which promotes flowering when practiced before flower induction (Bowman, 1941). The role fruits play in apple flowering will be discussed in a later part of this review. In a similar situation to leaf removal, buds are thought to have finished flower induction when defruiting no longer increases flower formation.

The combination of defoliation and defruiting gives reliable results but the method itself is devastating to the trees. Spraying gibberellic acid on the trees during flower induction significantly suppresses flowering and has little influence on flower organ differentiation (Hull & Lewis, 1959; Luckwill & Silva, 1979). By spraying $GA_3$ of certain concentrations at different times, results very close to those by defoliation-defruiting method were obtained (Huang *et al*., 1986; Li *et al*., 1988, 1989, 1995).

**Factors affecting flower induction**

Cultivar and rootstock. There is no doubt that different cultivars have different flowering habits. As far as flower induction is concerned, they differ in time of induction, duration of the period, density of floral buds and response to flowering signals (McLaughlin & Greene, 1991a). Due to its effects on flowering, genotype is probably the dominant reason for alternate bearing in fruit trees. For example, ‘Gala’ is generally a regular bearing cultivar (Hirst, 1999) while ‘Fuji’ tend to be very biennial bearing (Li *et al*., 1995). A list of the tendency of various apple cultivars to bear alternate crops was made available by Jonkers (1979).

Location of buds, different reactions to environmental stress, and various tendency for self-thinning were proposed to be the reasons for cultivar effects on biennial bearing (Monselise & Goldschmidt, 1982). Plant hormones also play important roles in regulation of apple flowering and alternate bearing. This will be reviewed at later sections of this thesis.

Rootstocks were rarely found to have direct effects on apple flowering. In several comprehensive rootstock evaluation projects, rootstock influences on flowering and bud development were carefully studied (NC-140, 1990, 1991; Hirst & Ferree, 1995a, 1996) and the effects of rootstocks on flower density of 'Gala' and 'Triple Red Delicious' apples were found to be insignificant. Occasionally, rootstocks can affect flowering in 'Delicious' apples by increasing the percentage of floral buds rather than producing more buds. However, the critical appendage number required for flower induction was not changed by rootstocks.

Environmental factors. There is evidence showing the influence of a number of environmental factors including light, temperature and water supply on flower induction of apple trees.

Light is required for flower formation. Tromp (1984) documented the flowering response of 'Cox's Orange Pippin' apple to light. He found that to form flower buds, high-intensity light within the first 7 weeks after full bloom was needed. Lower light density or illumination beyond 7 weeks decreased the proportion of flower buds. Exposure to red light during the night tends to increase flower formation (Tromp, 1993), but many believe the flowering response of fruit trees to be photoperiod insensitive therefore this observation needs further investigation.

Increasing soil temperature from 7°C to 28°C significantly increased return bloom (Tromp, 1992). However, whether this was caused by affecting flower induction or flower development is not known since the treatments were applied in August. Zhu *et al*. (1997) reported the enhancement of flower formation by increasing air temperature from 13°C to 20°C. This was inconsistent with the results of Tromp (1993, 1976). In recent research on apple flowering with respect to effects of temperature, Verheij (1996) found that the response to temperature was highly variety dependent. It was likely that temperature affected flower induction by changing the plastochron, which in turn has been associated with increased flowering.

Water stress during the growing season reduced growth and yield as well as flower induction of apple (Sritharan & Lenz, 1988). However, as a general observation, water stress at a lower level in summer can increase flower induction (Zeng, 1990).

Similarly, lower air humidity favors flower induction. Tromp (1984) documented the increase of flower induction when 'Cox's Orange Pippin' apple trees were treated with air humidity of 40%-50% instead of 80%-100%. Appropriate water stress may increase apple flowering indirectly by regulating the balance between vegetative and reproductive growth.

**Interaction of flower induction and growth of other organs**

Leaves. Leaves play a unique role in the formation of flowers. As discussed earlier in this review, defoliation leads to reduction of flower induction. The effects of leaves in flower induction may come from 3 aspects: 1) As the organ for assimilation, leaves provide the carbohydrates needed for flower induction; 2). Leaves are the key to keeping a favorable hormonal balance for flower induction. This is achieved by both foliar synthesis of hormones and the transportation of hormones from other organs via the transpiration stream. The fact that 2-3 times more leaves are required for flower induction on non-ringed branches than on ringed branches (Davis, 1957) indicates the efflux of flower promoting hormones on non-ringed branches. 3). Leaves are also the receptor of environmental signals related to flower induction.

Fruits. An excessive cropload will inhibit flower initiation. Heavy croploads are known to induce alternate bearing (Monselise & Goldschmidt, 1982). In apple trees, the development of fruits coincides with the time of flower induction. As strong sinks for assimilates, developing fruits were thought to suppress flower induction by competing for carbohydrates with buds. While this correlates with the finding that a certain leaf/fruit ratio is a prerequisite for flower induction (Huet, 1972), Chan and Cain (1967) showed this is not the case. The other reason why fruits inhibit flowering, and the primary interest of this research, is the flower-inhibiting signals from the fruits. The experiment of Feucht (1968, as cited by Zeng, 1990) dramatically shows the influence of fruits on flowering. Fruits bearing on the apical sites the branches significantly decreased flowering on the whole branch while fruits on the basal sites didn't. Ringing the middle of the branch counteracted the inhibitory effects of fruits on flower induction in the buds below.

Seeds. Seeds are strong inhibitors of flower induction in apples. The early experiments in this area were reviewed by Neilson (1998). Most of these pioneers attributed the inhibition of flowering by fruits to competition for nutrients. In other words, the buds were 'starved' to be vegetative. However, the classic paper of Chan & Cain (1967) demonstrated that the presence of seeds is the crucial element in fruit inhibition of apple flower induction. Using the apple cultivar 'Spencer Seedless', a facultatively parthenocarpic cultivar, they found seeded fruits inhibit flowering unless they are removed within 3 weeks after pollination while seedless fruits had little effect. This experiment, along with similar information on pear (Huet, 1973), indicates the role of some chemical signals other than nutrients – plant hormones – in the regulation of flowering. Since Dennis and Nitsch (1966) identified $GA_4$ and $GA_7$ in apple seeds and other reports showed the inhibitory effects of exogenous GAs (Dennis and Edgerton, 1962), much research effort was expended studying gibberellic activity in apple seeds.

For almost 30 years, people have believed that gibberellins generated in developing apple seeds constitute the major controlling signal for apple flower induction and the dominant cause of biennial bearing. The experiments of Chan & Cain (1967) were repeated recently (Neilson, 1998) with the conclusion that control of apple flowering was not as simplistic as earlier suggested. The length of the bourse shoot as well as the presence of seeds was suggested by Neilson (1998) as controlling factors. A positive relationship between bourse shoot length and return bloom was documented. The longer the bourse shoots, the less inhibition of flowering was brought about by seeds. Almost every spur flowered when the bourse shoot was longer than 9mm. This is actually consistent with the result of Chan and Cain that shows less inhibition by seeds when the bourse shoots are longer than 2cm. Several hypotheses can be proposed based on the discovery of Neilson: 1). Larger leaf area in longer bourse shoots provides more carbohydrates needed by flower induction; 2) Less inhibitory factors (gibberellins?) synthesized in seeds would arrive at the bourse bud due to the longer translocation distance when shoots are longer; 3). As suggested by Dennis and Neilson (1999), an unknown flower-promoting hormone (florigen) counteracts the inhibitory activity of seeds. This hormone may either be generated by bourse leaves or translocated by

transpiration stream from the lower part of the trees. Cytokinin would seem to be a likely candidate. More experiments need to be done in order to examine these theories.

**Nutrients and flower induction**

Before plant hormones were identified, the dominant theory about apple flowering centered on control by nutrients. This reflects the influence of the C/N ratio hypothesis proposed by Kraus and Kraybill (1918, reviewed by Cameron & Dennis, 1986). Even though it has generally been observed that high C/N ratio favors flower formation and excessive N application inhibits it, it seems that nutritional status is not the rate-limiting factor when nutrient supply reaches a threshold level. With the development of modern hormone theory in the 1970's, the C/N theory was gradually abandoned. However, the requirement of nutrients for flower formation was never doubted.

Carbohydrates. As stated by C/N ratio theory, flower bud formation requires a high level of carbohydrates. Heavy cropload in the on-year of biennial bearing apple trees leads to depletion of carbohydrate reserve of the tree, thus the flower formation of the following year could be inhibited by the lack of sufficient carbohydrates. Grochowska (1973) observed low level of carbohydrates in the spurs of off-year trees. Many of the flower-promoting orchard techniques such as ringing, summer pruning, root pruning and vigor control are accompanied by an increase in carbohydrate level. As an energy reserve, carbohydrates are necessary for floral organ development. Flower induction consumes a lot of carbohydrates and proteins (Dietz & Held, 1974). Sufficient carbohydrates alone, however, do not act as a signal to trigger the transition of buds from vegetative growth to reproductive growth.

Nitrogen. As the other component of the C/N ratio hypothesis, it is generally accpeted that excess nitrogen inhibits flower induction. Under this situation, nitrogen may indirectly affect flower induction by increasing the vigor of the whole tree. Conversely, N deficiency may reduce flower induction (Stiles, 1999). On the second year of soil nitrogen application, Tami et al. (1986) found a positive relationship between leaf N content and percentage of floral buds in 'Starkspur Golden Delicious' apple trees. The above studies demonstrate that, like carbohydrates, nitrogen is also required for flower

induction. It may be needed at a lower level than carbohydrates so that excessive nitrogen application is easily encountered in orchards.

The form of nitrogen applied is also an important factor to consider. Ammonia ($NH_4^+$) favors flower induction while nitrate ($NO_3^-$) does not. In apple trees that were continuously supplied with $NO_3^-$, flower induction can be increased by applying $NH_4^+$ for a short period of time (Grasmanis & Edwards, 1974). The effects of $NH_4^+$ can even spread to the lateral buds. Recent studies (Verheij, 1996) also found $NH_4^+$ application gives rise to shorter shoots and higher rate of flower induction. As mentioned earlier in this review, accumulation of free amino acids accompanies the onset of flower induction. Several reports indicate that $NH_4^+$ application leads to a higher level of amino acids in the floral buds, especially basic amino acids like Arginine (Zeng *et al*. 1987). The function of $NH_4^+$ is not only nutritional, but it can also trigger the activity of plant hormones. For example, Gao *et al*. (1992) detected an increase of cytokinin activity in apple xylem sap after $NH_4^+$ application. Cytokinins in turn are thought to promote apple flower initiation (Ramirez, 1979).

Phosphorus, Potassium and other elements. Phosphorus application was reported to increase apple flowering (Neilson *et al*., 1990; Taylor & Nichols, 1990). However, P application is not recommended in the orchard to promote flowering since the economic return could be very limited (Stiles, 1999). It is highly possible that P application only works when the P level in the trees drops below a critical value. Bould and Parfitt (1973) suggest such a value of 0.25%.

The roles of potassium and other mineral elements in apple flower induction are not very clear. Contradictory data have been published. It is believed that these elements affect apple flowering indirectly by affecting tree growth.

**Plant hormones and flower induction**

Since nutrients did not appear to be a controlling factor in apple flowering, more attention was paid to the theory of hormonal regulation. Chailykyan (1937, as cited by Neilson, 1998) proposed that flowering is determined in the bud by the presence of "florigen", which is probably a mixture of gibberellins and other flower-controlling

hormones. However, “florigen” has never been isolated and identified in spite of efforts lasting half a century. But we have had much useful information about hormonal control of flower induction from the studies aimed at elucidating the physiological functions of major plant hormones. It has also become clear that activity of no single hormone can give a perfect explanation to the observed plant response. Different hormones appear to regulate flower induction interactively.

Auxin. Both promotion (Grochowska & Karaszewska, 1978) and inhibition (Bangerth et al., 1986; Ramirez & Hoad, 1981) of flowering by auxin have been reported. The return bloom of apples was increased when the export of IAA from seeds was interrupted (Hoad, 1980). It is reasonable to deduce that the flower promoting effects of TIBA and $B_9$ are due to their inhibition of IAA biosynthesis and transportation. Bangerth (1997) reported that bending of vertical shoots decreased IAA export while GA application stimulated it. It seems that high concentrations of auxins act as a signal for vegetative growth rather than flower formation. But there are opposite reports on the role of auxin in flower induction of fruit trees. IAA application at low concentration increased flower induction of apple trees (Ebert & Bangerth, 1982). Similar results were obtained in other species (reviewed by Ma, 1987). The promotion of flowering by IAA may be explained by stimulation of ethylene synthesis or the diversion of nutrients towards buds (Sachs, 1977).

Gibberellins. Gibberellins are always regarded as inhibitors of apple flowering. Due to their unique role in apple flowering and being the major concern of this study, we will discuss them in a separate chapter.

Cytokinins. Cytokinins are generally associated with promotion of flowering in apples. Zeatin and benzyladenine (BA) application to apple trees after anthesis was found to promote flower induction (McLaughlin & Greene, 1984; Ramirez & Hoad, 1981; Luckwill, 1970). Grochowska and Karaszewska (1978) identified a significant peak of cytokinin concentration in the xylem sap during the critical period of flower induction. Other studies, however, suggested an inhibitory role of cytokinins. Girdling (Cutting & Lyne, 1993) and bending (Sanyal & Bangerth, 1998) of branches decreased rather than increased the cytokinin content in the xylem stream, but the return bloom was increased.

Furthermore, spraying of N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU), a synthetic cytokinin, inhibited flowering of apple trees (Bangerth & Schröder, 1994). However, it makes little sense to think of cytokinins as inhibitors of flower induction considering their strong antagonism to IAA transport (Bangerth, 1994) and well-known function in stimulating cell division and differentiation in meristems. The girdling and bending experiments only measured the cytokinin level in the shoots therefore the position for more meaningful quantification, the bourse bud, was ignored.

Abscisic acid. The effect of ABA on apple flowering remains obscure. Attempts to relate apple flower induction to endogenous ABA concentration were inconclusive (Hoad, 1984; Ramirez & Hoad, 1981). It is possible that ABA does not play direct role in apple flowering. However, it may affect flower induction by antagonizing GA and inducing cessation of shoot elongation.

Ethylene. It has been well documented that ethylene and ethylene-releasing compounds, when applied during flower induction, increases return bloom of apple trees (Williams & Fallahi, 1999). Bending branches to a horizontal position (Sanyal & Bangerth, 1998) and summer pruning (Klein & Faust, 1978) both significantly increased ethylene production in buds and return bloom. Compared with leaf buds, floral buds released twice as much ethylene (Klein & Faust, 1978). There would appear to be a positive relationship between ethylene level and the bud's ability to form flowers. Whether ethylene acts to promote flower induction directly or indirectly is not clear. The promotion of flower induction induced by ethylene was always accompanied by reduced shoot growth. Ethephon (an ethylene-releasing compound) is also used as a flower and fruit thinning agent since it favors the formation of abscission layers in the fruit pedicels. It is highly possible that ethylene affects apple flowering through mediating shoot growth and fruit drop.

Interaction of hormones. Hormones can induce *in vitro* flowering of over 30 plant species (Scorza, 1982). *In vitro* experiments showed that the fate of meristems is not determined by the activity of a single hormone. Bangerth (1997) and Williams (1973) illustrated the environmental and internal factors known to date that are required for apple flower induction. Both schemes emphasize the involvement of various plant

hormones. It seems IAA and GA are the negative signals for flower induction while other hormones can either enhance or mask these negative influences. There are no obvious positive signals for flower induction even though cytokinin is a candidate. It was hypothesized that flower induction is determined by the equilibrium of various hormones involved. Luckwill (1970) proposed a central role of cytokinin/GA ratio in apple flower induction, the higher ratio being more favorable towards flowering. This theory is supported by a number of studies. In an intensive study, Grochowska *et al*. (1984) found a clear correlation between cytokinin/GA ratio in spur leaves and the flowering ability of that spur. GA application led to a sharp decrease of cytokinin/GA ratio and inhibited flowering. On the contrary, the ratio and return bloom were dramatically increased by B-9 and CEPA, a cytokinin- and ethylene-releasing compound, respectively. Zhou *et al*. (1988) studied the GA and cytokinin content in both floral and vegetative buds of 'Ralls Janet'. They found that absolute concentrations of GA are roughly the same in both types of buds but the cytokinin/GA ratio in floral buds (4.02) was much higher than that in leaf buds (1.69).

**Gibberellins and apple flower induction**

History of gibberellins. Gibberellins(GA) were first discovered by Japanese scientists studying a rice disease characterized by excessive stem elongation named "bakenae" (foolish seedling). The symptom was found to be stimulated by compound(s) released by a fungus *Gibberella fujikuroi*. In 1935, the compound was isolated and named after the fungus as "Gibberellin". Further experiments showed that GA not only occurred in plants but also played a key role in various aspects of plant growth and development (the early history of gibberellins was reviewed by Croker, 1999).

Gibberellin is a huge group of tetracyclic diterpenoid carboxylic acids that share an *ent*-gibberellane skeleton (Fig 4). They are divided into 2 groups by the presence of the carbon at position 20. Most biologically active gibberellins are in the $C_{19}$ category while most $C_{20}$ gibberellins are biosynthetic intermediates of $C_{19}$-GAs. GA molecules are very labile to oxidation, especially hydroxylation at the 2, 3 and 13 positions. GAs with biological activity are normally hydrolyzed at 3β position but 2β-hydroxylation generally

leads to loss of activity. Trivial names ($GA_{1,2,3...n}$) based on the time order of discovery rather than chemical nomenclature of GAs are frequently used in the references. International Plant Growth Substances Association (IPGSA) has named 125 different types of GAs to date while a dozen others are on the waiting list.

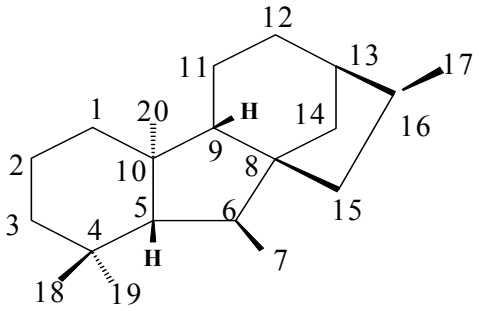


Fig 4. *ent*-gibberellane

Plant physiological responses to GA. GAs have profound whole-plant or tissue-specific effects on growth and development throughout the entire life history of plants. First of all, GAs were found to promote cell expansion and perhaps cell division that led to elongation of shoot tissues and development of a number of other morphological structures such as inflorescences and fruit. This displays the nature of GA as a positive regulator of plant growth. Some reports suggest that GAs promote cell expansion through alteration of biochemical properties of cell wall or/and the orientation of cell microtubules. The mechanism of GA's regulation of cell division is not clear.

Secondly, GA may regulate the mobilization of nutritional reserves and thus alter the course of some physiological processes such as the transition from vegetative to reproductive growth. Seed germination is highly dependent on the concentration of GA. In fact, failure of germination has been used as a standard selective criterion in identification of GA-deficient mutants. By stimulating the gene expression of hydrolases in the cell wall, GA can soften the endosperm of seeds (Groot *et al*., 1988) thus germination is made easier. Furthermore, GA induces the activity of other enzymes responsible for starch and protein degradation, which provides nutrients for seedling growth. Root development is also affected by GA. GA-deficient mutants *gib-1* (tomato) and *pkl* (*Arabidopsis*) both showed abnormal root growth and reduced degradation of nutrient precursors. Accumulation of triacylglycerol and fatty acids in *pkl* roots indicates

that GA promotes the transition of root from embryonic status to seedling status (Ogas *et al*., 1997).

GA and flower induction (in general). The role of GA in plant flower induction is a little confusing according to the results of conventional physiological experiments. Application of exogenous GA promotes flowering in some plant species but has no effects or even inhibits flower induction in other species. It seems that those promoted in flowering are normally long-day or cold-requiring plants. The phenotype of GA biosynthesis mutant *ga1-3* of *Arabidopsis* provided evidence for GA function in plant flower induction on a molecular level (Wilson *et al*., 1992). The *ga1-3* mutants never flowered under short days and took longer to flower in long days. In the presence of GA, the ability to flower is recovered. The lack of the flowering-deficient phenotype in other GA biosynthesis mutants indicates that flower induction is extremely sensitive to GAs. The *ga1-3* shows the defect in flowering only because it is blocked in early pathways of GA synthesis and contains very low level of endogenous GAs. More knowledge was obtained through studies of mutations in other loci related to flowering. LFY and AG are two floral homeotic genes responsible for floral identity and development in *Arabidopsis*. The floral meristems of heterozygous *lfy* and *ag* mutants are reverted into inflorescent meristems in short days but are maintained by GA treatment under the same conditions (Okamuro *et al*. 1993). It is likely that GAs affect flower induction via expression of the floral homeotic genes. In favor of this hypothesis, Blazquez *et al*. (1997) reported a markedly increase of LFY:GUS expression in *Arabidopsis* after $GA_3$ treatment.

Inhibition of apple flowering by GA. Regardless of its positive role in flowering of herbaceous plants and some conifers (Jackon & Sweet, 1972), GA negatively regulates the flower induction of many woody species. Exogenous $GA_3$ application reduces flower induction of a number of fruit trees including peach, pear, apricot, plum, cherry, citrus and apple (Ramirez, 1979).

Early reports on apple from Dennis and Edgerton (1962) and Guttridge (1962) showed that exogenous $GA_1$ applied within 2 months of full bloom, inhibits apple flower induction over a wide range of concentrations. Substances that interfere with GA biosynthesis and activity were found to promote flower induction. These substances

include diaminozide (Ramirez, 1979), paclobutrazol (Tromp, 1987) and chlormequat (Hedden, 1990). The above facts tend to support the postulation that flower induction is favored by lower levels of GA even though application of growth retardants does not always lead to lower GA content in the seeds (Stephens *et al*., 1991, 1992). Unlike other plant hormones, GA seems to affect apple flowering by acting as a direct message rather than enhancing vegetative growth. GA's suppression of apple flowering is effective only if the treatments are applied during flower induction when growth of most shoots has already ceased (Tromp, 1982). It is unconvincing as well to explain inhibitory effects of GA by nutrient diversion from the buds considering the similar nutritional status of seeded and seedless fruits (Chan and Cain, 1967).

In apple, immature seeds are the major source of GA *in vivo*. The GA content in seeds is 15-500 times that in leaves and shoots (Luckwill, 1974). Using bioassays, Luckwill *et al*. (1969) found that GA in apple seeds started to appear 2-3 weeks after full bloom and reached its maximum concentration during the $9^{th}$ week. This period coincides with the critical time of apple flower induction. Therefore, it is reasonable to postulate that GA synthesis or transport is the direct cause of biennial bearing and to expect different patterns of GA activity between seeds of non-biennial and biennial varieties. Studies attempting to relate the internal GA activity to the return bloom of apple varieties with different flowering habits failed in drawing any meaningful conclusion (Ebert & Bangerth, 1981). The major problem here was the poor precision of GA quantification by bioassay. Fortunately, the development of modern GA analysis techniques based on GC-MS and LC-MS provided a solution. The present study, aimed at better understanding GA regulation of apple flowering by investigating the GA content of immature seeds of biennial and non-biennial bearing apple cultivars and the relationship between GA content and flowering habits of these cultivars.

GA types and flower induction. The inhibitory effects of individual GAs are not identical. Tromp (1982) studied the effects of $GA_3$, $GA_4$ and $GA_7$ on flower induction of 'Cox's Orange Pippin' by spraying them at various times. The results demonstrated that the trees are most responsive to $GA_7$ application and least to $GA_4$. When $GA_4$ and $GA_7$ are used as a mixture the result was the same as spraying $GA_7$ alone. Furthermore, the

inhibitory effects of $GA_7$ (full bloom to 4 weeks after) lasted longer than that of $GA_3$ and $GA_4$ (only full bloom). More surprising information was presented by Looney *et al*. (1985) who found that application of $GA_4$ and C-3 *epi*-$GA_4$ promoted flowering of 'Golden Delicious'. There are reports showing that the types of GAs found in seeds are related to flowering habits. The dominant GA type in fruit exudates of seedless varieties 'Spencer Seedless' is $GA_4$ while $GA_3$ is the major ingredient in the biennial variety 'Elstar' (Steffan *et al*., 1997). A more extensive study (Steffan *et al*., 1999) confirmed the above results. The content of $GA_7$ was found to be trivial in these experiments. $GA_4$ seemed to be the critical factor in regulating apple flowering.

It is very difficult to interpret the different roles of $GA_4$ and $GA_7$ in apple flowering according to current knowledge. Differing only in presence of a 1'-2' double bond, the structures of GA4 and GA7 are so similar that the mixture of them was the only commercial product available for a long time. The unavailability of $GA_4$ and $GA_7$ standards also hampered attempts to isolate the more inhibitory GAs.

GA transport from seeds. GAs generated in the apple seeds, as a flowering signal, is unlikely to determine the fate of the buds if these compounds don't move to the buds. Therefore, the transport rather than absolute concentration of the GAs from seeds could be more promising in research of apple flowering (Bangerth, 1997). Even though many experiments (reviewed by Neilson, 1998) failed to detect transport of isotope-labeled GA from seeds, some did. Hoad (1978, 1980) identified 1.9 –2.5 times more GAs moving from seeds to buds in 'Laxton's Superb' (biennial) than in 'Cox's Orange Pippin' (non-biennial). Most of the studies about GA transport from apple seeds used exudates collected from cut pedicels. Whether or not this *in vitro* method simulates the real situation in apple fruits is doubtful. Labeling of GA standards seems to be the method of choice. In addition to biosynthesis, GA transport should be included in future exploration to tell a more complete story of apple flower induction.

# HYPOTHESES AND OBJECTIVES

## Hypotheses

Endogenous gibberellins produced in seeds play an important role in control of apple flowering and therefore are closely related to alternate bearing. There are different gibberellin activity patterns in the seeds of biennial and non-biennial bearing apple cultivars. They differ in the number of specific GAs produced, the concentration of these GAs, and the changes of GA concentration over time. For the biennial bearing cultivar, there are more flower-inhibiting GAs or less flower-promoting GAs (or both) in the 'on-year' than in the 'off-year'.

## Experimental Objectives

I. Investigate the flowering density of selected apple cultivars ('Fuji' and 'Gala') for 2 consecutive years and verify the level of their bienniality;

II. Look for the most abundant GAs in both cultivars by full-scan GC-MS on partially purified extracts;

III. Based on the knowledge of step II, quantify the GA types that are possibly related to biennial bearing using GC-SIM on samples spiked with deuterated internal standards;

IV. Relate the activity of flower-inhibiting and flower-promoting GAs to the flowering data and discuss their roles in the maintenance of flowering habits in biennial and regular bearing cultivars.

# MATERIALS AND METHODS

## Plant Materials

Apple cultivars 'Fuji' and 'Gala' were selected for this study. All apple trees were grown at the Horticulture Research Farm of Purdue University in West Lafayette, Indiana. 'Fuji' has a strong tendency for alternate bearing (Li *et al*., 1995). While yield and flowering data for 'Fuji' was unavailable, farm records clearly showed that 'Gala' bears uniform crops every year. Our investigation of flowering in the 1999 and 2000 growing seasons also confirmed the bearing behavior of 'Fuji' and 'Gala' (see Results and Discussion). Both 'Fuji' and 'Gala' were treated with fertilizer and normally pruned and irrigated similar to commercial orchards.

Fuji. Ten 'Fuji' trees on 'Mark' rootstock in Block 53A of the Purdue Horticulture Farm were selected for this study. These trees were planted in 1991 at a spacing of 2.5 × 4.5m. For each tree, we sampled all fruits from five randomly selected spurs. Samples from four of these ten trees were used for qualitative analysis of gibberellins. The other six trees were randomly grouped into 3 replications (each includes 2 trees) for quantification of gibberellins.

Gala. Ten 'Gala' trees in the same block were used. The planting year and spacing were the same as the 'Fuji' trees in the block. The trees were growing on various rootstock combinations including B9/P18, CG65/B118, PAT/P18, M9/MM111, and CG65/MM111. Flowering is mainly affected by cultivar rather than rootstock (Hirst & Ferree, 1995c) therefore 'Gala' was expected to be regular bearing regardless of rootstock. Again, fruits from four and six trees were used as the material for GA qualitative and quantitative analysis, respectively.

**Chemicals**

General chemicals. To ensure precision in GA analysis, most general chemicals were of HPLC grade. Deionized water and methanol were used to rinse the glassware throughout the whole research.

Group separation cartridges. Two kinds of group separation material were used to clean the plant extracts before they were applied to HPLC. The first was Preparative C18 (125Å, Waters Corporation). It was shipped as bulk-packing material that contains dimethyloctadecylsilyl bonded amorphous silica. Another cartridge, ICN adsorbentien (ICN Biomedicals) was also used after being hydrated for about a month.

Gibberellins. Dr. R. Coolbaugh in Department of Botany and Plant Pathology, Purdue University provided GA1, 3, 4 and 7 (produced by Abbott Labs, Inc.) for testing the retention time of individual GAs in HPLC. $GA_{20}$ was obtained from Dr. L. Mander's lab in the Research School of Chemistry, Australian National University, Canberra, Australia.

Deuterated gibberellins. Among all 5 deuterated GAs, [17-$^2H_2$]-$GA_1$, [17-$^2H_2$]-$GA_4$ and [17-$^2H_2$]-$GA_{20}$ were kindly provided by Dr. R. Coolbaugh. $^2H_2$-$GA_3$ and $^2H_2$-$GA_7$ were gifts from Dr. L. Mander. All deuterated Gibberellins were prepared by Dr. L. Mander's lab in Austrilia.

Tritiated gibberellins. These were only used for qualitative analysis of gibberellins in seed samples, $^3$H-$GA_1$, $^3$H-$GA_4$ and $^3$H-$GA_9$ were from Dr. R. Pharis' lab in Department of Biological Sciences, University of Calgary, Alberta, Canada.

Rice seeds. Dwarf rice *Tanginbozou* seeds were from the stock of Dr. Pharis. They were originally produced in Dr. Koshioka's lab in National Research Institute of Vegetables, Ornamental Plants & Tea, Ano, Mie 5142392, Japan.

**General Methods**

Collection of apple samples.

From May 18, 1998 to June 29, 1998, fruits of each cultivar were collected weekly. From July 6, 1998 to August 3, 1998, sample collections were bi-weekly. Each

time, all apple fruits from 5 randomly selected spurs in each selected tree were removed and frozen instantly by liquid Nitrogen. Fruits were dissected in the lab and all seeds were contained in seed envelopes and stored in a –80ºC freezer. A similar routine was used in the 1999 growing season except that sample collection was narrowed down to 5 dates ranging from June 13 to July 11.

Freeze-drying of samples.

Seeds were freeze-dried before GA analysis. When the temperature of the condenser drops to -50ºC, the seed envelopes were placed loosely in the flask of the freeze dryer (LABCONCO 4) and the vacuum was turned on. Seeds were left in the running freeze dryer for approximately 10 days.

Investigation of flowering situation.

In the spring of 1999 and 2000, five shoots on the periphery of each tree were randomly selected for investigation. The length of 2-year-old part of the shoots was measured and flowering clusters on those sections were counted.

**Qualitative analysis of gibberellins**

A portion of seed samples collected on June 8, 1998 for both ‘Gala’ and ‘Fuji’ were analyzed for the identification of individual gibberellins. Specifically, samples from 4 trees of each cultivar were used. The experiment was done in Dr. Richard Pharis’ lab in the Department of Biological Sciences, University of Calgary, Alberta, Canada.

Gibberellin extraction.

Methanol is the most recommended reagent for GA extraction while acetone is sometimes used (Hedden, 1987; Pearce et al., 1994). The same references also suggested partitioning of most GAs into ethyl acetate at pH 2.5-3.0. Our experiment utilized a relatively simple method to accomplish the extraction of GAs.

Dried samples were put into a mortar and ground into fine powder in the presence of 10ml 80% methanol. The powder/methanol mixture was then filtered through a piece of Whatman #1 filter paper under vacuum. The plant material was placed back in the

mortar and extracted again with 10ml 80% methanol. The latter procedure was repeated twice to make a total number of 4 extractions by 80% methanol. The filtrate was then immediately subjected to group purification.

Group separation.

PVPP, charcoal-celite, silica and Sephadex reversed-phase and ion-exchange cartridges are the most popular forms of group separation of GAs (Hedden, 1987; Pearce *et al*., 1994). Among them, purification using reversed-phase or ion-exchange cartridges has become a part of GA analysis routine in several major labs. In our protocol, CELITE and 2 cartridges, Waters Preparative $C_{18}$ and ICN adsorbentien were used.

A 30ml syringe was prepared as a chromatography column by washing it with 100% and 80% methanol alternatively. A small piece of Whatmant#1 was placed on the bottom of the syringe and filled with approximately 1 gram of Waters Preparative $C_{18}$. Plant extracts were then applied to the column under vacuum. The container holding the extracts was rinsed with 10ml 80% methanol to ensure complete transfer. The solution obtained through the column was then transferred to a small flask. Methanol was evaporated using a rotary evaporator with 35ºC water bath. The dried plant extracts were either immediately subjected to later steps of purification or sealed and stored in a freezer to avoid further chemical reactions.

In a small beaker approximately 1 gram of CELITE 545 was loaded. By washing the flask with deionized water and pure methanol, plant extracts were transferred to CELITE. When the CELITE is soaked with solution, hot air was blown against the beaker using a hair dryer. More water and methanol were used to rinse the flask and the drying process was repeated till transfer is complete.

The CELITE/sample mixture was dissolved in 20-30 ml of ethyl acetate–hexane (95:5) solvent saturated in 0.5M formic acid. No clots were left. Five grams of ICN adsorbentien was loaded to a 100ml chromatography column rinsed by methanol and ethyl acetate-hexane in advance. The sample/CELITE mixture was then applied to the column followed by 90ml ethyl acetate-hexane solvent. The elute flowing from the column was collected in a beaker and dried on a rotary evaporator. Again, the

chromatography column was washed with 100ml methanol and the elute collected in another beaker and evaporated to dryness under vacuum. The ethyl acetate-hexane partition contained most GAs in the sample and thus was the focus of following steps of analysis.

Reversed-phase HPLC separation.

The flask containing dried samples (ethyl acetate-hexane partition) was transferred with 3 pipettes (about 2ml each) of pure methanol to a small vial. When the transfer was completed, the sample in the vial was evaporated under $N_2$. Then the sample is dissolved in 150µl methanol followed by 1.35ml (0.675ml×2) 1% acetate. The solution was passed through a 10ml syringe connected to a Dynagard .45µm filter (Spectrum). This should protect the HPLC column from getting clogged.

In qualitative analysis of GAs, a Waters HPLC system was used for group separation. The lack of selective physicochemical means (gibberellins only absorb weakly in the UV range) makes HPLC a poor approach for GA qualitative analysis (Hedden, 1987). The identification of individual peaks was accomplished by locating radioactive GA standards added after extraction (See next paragraph for details). Furthermore, bioassay was used to determine which fraction(s) were biologically active. By passing samples through a 20mm × 150mm $C_{18}$ column (contains bonded octadecylsilica) under an increasing gradient of methanol in acidic water as polar mobile phase, the more polar GAs elute faster than the less polar ones. In our experiments, 2 solvents, 10% methanol with 1% acetate and 100% methanol, were prepared separately for the 2 pumps which formed a solvent gradient. With a flow rate of 2ml/min, the exact solvent gradient is as following:

| Timing | Methanol concentration |
|---|---|
| 0 – 10 min | 10% |
| 10 – 40 min | 10% - 73% |
| 40 – 50 min | 73% |
| 50 – 70 min | 73% - 100% |
| 70 – 75 min | 100% |

An automatic fraction collector was hooked to the HPLC system and was programmed to collect the elute of first 60 minutes into 60 test tubes, one fraction per minute. All fractions were labeled for future analysis.

From each fraction, a 20µl aliquot was added to a vial containing 5ml EcoLume™ scintillation liquid. All 60 vials were labeled and covered with caps. The radioactivity of each fraction was measured by liquid scintillation. The radioactivity values were plotted against the time when the corresponding fraction was collected. Each peak indicates the appearance of a $^{3}H_3$-GA standard. The positions of these standards gave an approximation about when certain GA groups came out.

All 60 fractions were then applied to rice *Tanginbozou* bioassay (Crozier & Durley, 1983). A second round of HPLC was needed to further separate and clean those fractions showing significant activity. The column for this separation was a Nucleosil $N(CH_3)_2$ (5µ) manufactured by AllTech Associates, Inc. With flow rate of 1ml/min, 99.9% methanol + 0.1% acetate was used as mobile phase throughout the separation. Like the first round, fractions for the first 50 minutes were collected and applied to the rice bioassay. Radioactivity of each fraction was also measured and the unused solution dried and stored in freezer. The GAs in physiologically active fractions were saved for GC-MS analysis.

Bioassay of GAs

There are a number of bioassays that are applicable to GAs. However, the responses of these bioassay systems are different to various GAs. Dwarf rice *Tanginbozou* leaf sheath bioassay is generally regarded as the best choice for studying GA-like activity in plant extracts due to its high sensitivity and the number of plant GAs it responds to (Crozier & Durley, 1983).

Rice seeds were planted in 1% alga 2 days before treatment. Altogether 8 seeds were planted in each bioassay vial and thinned to 6 for uniformity on the third day. Each HPLC fraction tested by bioassay was dissolved in 0.1ml ethanol. For each HPLC fraction, 2 dilutions (1µl and 0.5µl for fractions from the C18 HPLC, 1µl and 2µl for Nucleosil HPLC fractions) were applied to 2 different vials of seeds in case of toxic

effects caused by high level of GAs in the plant samples. The solutions were applied directly to the axils of the first and the second leaf sheath with a 10μl Hamilton syringe. Same amount of ethanol (5μl) was used to treat other 6 vials as control. $GA_3$ standards with the following concentrations were also applied to seeds in 3 vials each for the purpose of calibration: $0.1M$, $0.01M$, $3.3\times10^{-2}M$, $3.3\times10^{-3}M$, $10^{-3}$M, $3.3\times10^{-4}M$, $10^{-4}M$, $3.3\times10^{-5}M$, $10^{-5}M$, $3.3\times10^{-5}M$ and $10^{-6}M$. Leaf sheath length in mm of every seedling were measured 3 days after treatment and the data plotted against the retention time of each fraction.

Identification of GAs by GC-MS.

Dried HPLC fractions were transferred to small GC vials with approximately 2ml methanol and dried under $N_2$ before derivatization.

Diazomethane is widely used as the methylation reagent. It is a yellow gas dissolved in ether and is explosive and toxic. To make the reaction complete, excessive diazomethane (200 - 300μl per vial) was added. A persistent yellow color indicates sufficient diazomethane. After 15-30 minutes, the vial was dried under $N_2$ and then about 45μl pyridine and 60μl BSTFA (both from SIGMA) were added to the vial. The reaction requires at least 40 minutes to complete. The reaction solution was either directly injected to the GC-MS or dried and redissolved by 10μl hexane before injection.

The GC-MS system is a bundle of 5890A Series II gas chromatography and 5970A mass selective detector manufactured by Hewlett-Packard. All GC-MS data were processed and analyzed by ChemStation™ software that comes with the GC-MS hardware. Every time 1 or 2μl of samples were injected into a fused silica DB-1 capillary column (30m × 0.22mm × 0.25μm) installed in the GC. The temperature gradient for GC-MS is listed below.

| | |
|---|---|
| 60ºC | 0 – 1 min |
| 60 - 200ºC | 2 – 8 min with an increase rate of 20ºC/min |
| 200-260ºC | 9 – 23 min with an increase rate of 4ºC/min |
| 260-280ºC | 24 – 25 min, 20ºC/min |

At 10 minutes after injection, positive ion electron impact mass spectra were acquired, scanning from 60 to 550 atomic mass units.

**Quantitative analysis of $GA_1$, $GA_3$, $GA_4$, $GA_7$ and $GA_{20}$**

Most steps for the quantitative analysis were the same as those in qualitative study. However, the objective of quantitative analysis was focused on only 5 GA types and some procedures were simplified in order to ensure high productivity.

Deuderated standards, [17-$^2H_2$-$GA_1$, [17-$^2H_2$]- $GA_3$, [17-$^2H_2$]-$GA_4$, [17-$^2H_2$]-$GA_7$, and [17-$^2H_2$]-$GA_{20}$ were added to the sample as internal standards during tissue grinding and samples were extracted as described above. The amounts of standards were adjusted for each sample according to the results from preliminary experiments. After purification by Waters $C_{18}$ preparative, the samples were taken into dryness at 35°C by a rotary evaporator and dissolved in 5ml of acidified water (1% acetate). Then the aqueous phase was partitioned 3 to 4 times against an equal volume of acidic ethyl acetate (1% acetate). All ethyl acetate phases were combined and reduced to dryness in vacuo. The dried ethyl acetate phase was transferred to a small vial with 3 × 0.5ml methanol. Nitrogen gas was blown over the vial till the methanol was evaporated and the content in the vial was dissolved in 0.5 ml of 10% methanol (with 1% acetate). The solution was then filtered (Dynagard .45μm filter) and injected into an Ultrasphere $C_{18}$ reversed-phase HPLC column (10mm × 250mm), which was packed the same material as the one used in qualitative study but yields higher resolution in analysis. The HPLC system used was a Beckman System Gold driven by SystemGold 8.0 software, running the same solvent gradient as described above. Retention times of various GAs were found by injecting GA standards through the system and identifying components of each fraction by GC-MS.

$C_{18}$ HPLC fractions that correspond to retention times of $GA_1$, $GA_3$, $GA_4$, $GA_7$ and $GA_{20}$ were dried and methylated by adding excessive diazomethane/ether solution. Instead of drying the ether solution in the same vial after methylation, the solution was transferred to a clean vial and then taken to dryness. Again, BSTFA and pyridine were used to convert the sample into TMS derivatives. GA quantification was accomplished using a Hewlett-Packard 5890 gas chromatograph coupled to an HP5970 mass selective

detector. The GC column was a fused silica DB5 (J&W Scientifics) capillary column (25 m x 0.22 mm x 0.25 um film thickness). After equilibration at 60°C for 1 min, temperature was increased at a rate of 20°C $min^{-1}$ to 220°C and then at 10°C $min^{-1}$ to 260°C followed by a 20°C $min^{-1}$ increase to 280°C. Finally, the temperature was held at 280°C for 6 minutes. Characteristic ions ($GA_1$m/z 506; [17-$^2H_2$]-$GA_1$ m/z 508; $GA_3$, m/z 504; [17-$^2H_2$]-$GA_3$, m/z 506; $GA_4$, m/z 418; [17-$^2H_2$]-$GA_4$, m/z 420; $GA_7$, m/z 416; [17-$^2H_2$]-$GA_7$, m/z 418; $GA_{20}$, m/z 418; [17-$^2H_2$]-$GA_{20}$, m/z 420) were monitored with dwell times of 50 ms (Croker, 1998).

Calculation of GA content

The following equation was used for GA quantification in this research:

*ng GA in plant sample = S/100[BCE/(D-FC)-A]*

A = % of unlabelled molecules in the internal standard.

B = % of labeled molecules in the internal standard.

C = measured intensity of $M^+$ of unlabelled GA.

D = measured intensity of $M^+$ of completely labeled GA.

E = a factor calculated from the relative intensities of ions in the $M^+$ cluster of unlabelled GA and the amount of partly-labeled GA relative to fully-labeled GA in the internal standard, which accounts for the partly-labeled species not measured.

F = the intensity of the ion of m/z equivalent to $M^+$ of the fully-labeled GA in the $M^+$ cluster of the unlabelled GA, relative to the intensity of $M^+$.

S = ng of internal standard added.

Developed by Dr. David Pearce in Dr. Pharis' group, the above equation was a modification of the method of Fujioka *et al*. (1988). It is applicable to all [17-$^2H_2$] labeled gibberellins prepared by Mander's lab. For the calculation of each GA in a sample, we only need to know the values of C, D and S because all other items in the equation were pre-caculated and the same for each deuterated GA standard.

## RESULTS AND DISCUSSION

### I. Flowering density of 'Fuji' and 'Gala'

The flowering density of both cultivars in 1999 and 2000 are shown in Table 1. Apparently, 'Fuji' was in its 'off-year' in 1999. As indicated by the table, the flowering density of 'Fuji' in 1999 was significantly lower than the following year and than that of 'Gala' in the same year. 'Gala' flowering was regular through the 2 years, the difference between 2 years' data is simply caused by variance. In the 'on-year' of 'Fuji' (2000), it bore as many flowers as 'Gala' did.

Table 1. Flower density of 'Fuji' and 'Gala' in 1999 and 2000 (flower clusters/cm of branch)

| | Fuji | Gala | *P-value* |
|---|---|---|---|
| 1999 | 0.04 | 0.295 | 0.0002 |
| 2000 | 0.174 | 0.189 | 0.44 |
| *P-value* | $3.95\times10^{-9}$ | 0.11 | |

From the above information as well as the performance of both cultivars in past years provided by orchard records (data not shown), it appears certain that at the Purdue Horticulture Farm, 'Fuji' was an extremely biennial-bearing cultivar while 'Gala' was a regular bearing cultivar.

### II. Qualitative results of GA analysis

Bioassay of $C_{18}$ HPLC fractions. For both 'Fuji' and 'Gala' samples, the dwarf rice '*Tanginbozou*' leaf sheath bioassay detected significant GA-like activity in various fractions (Fig 5). Both 'Fuji' and 'Gala' displayed 3 major peaks of reaction in term of

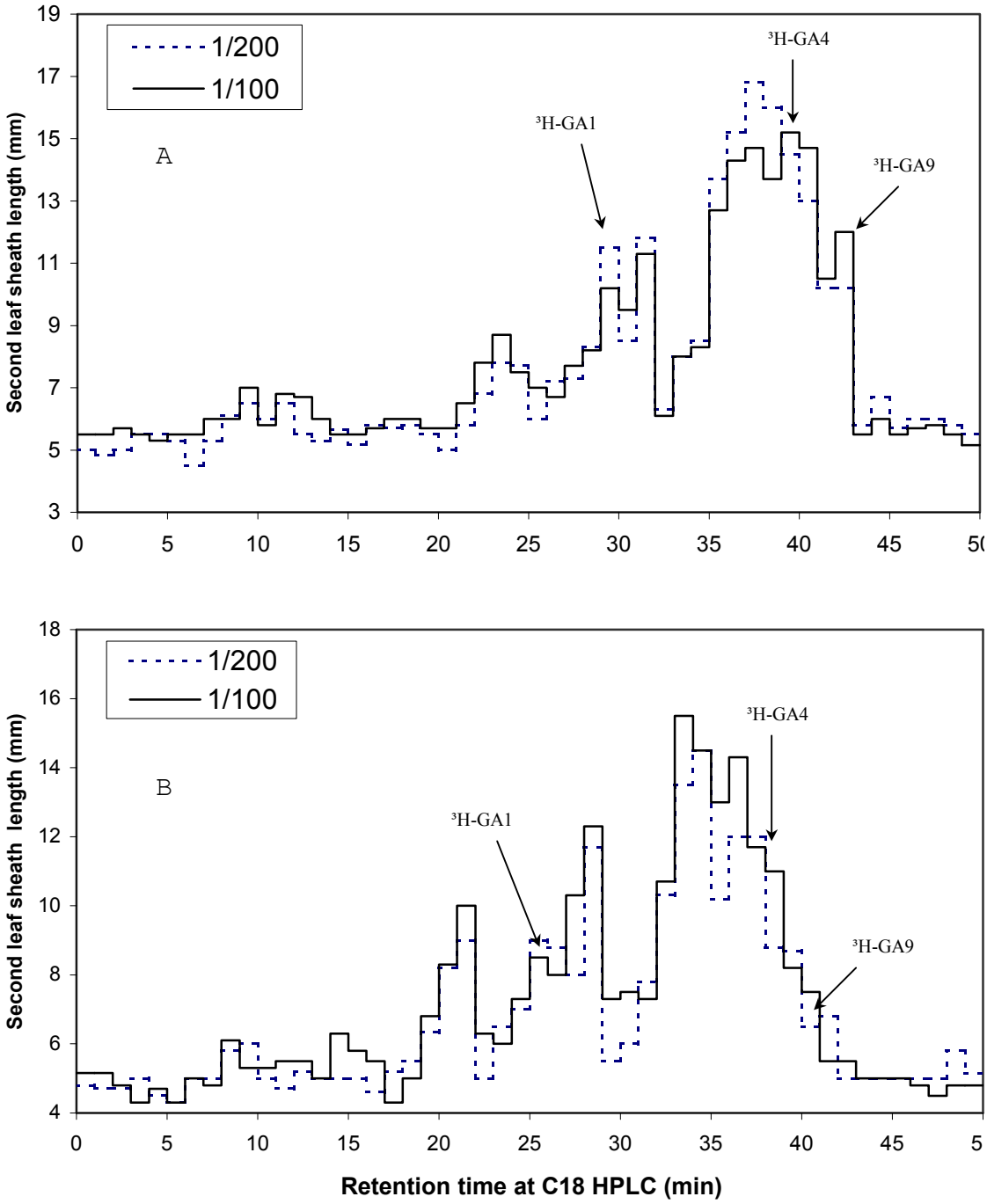


Fig 5. Dwarf rice *'Tanginbouzu'* bioassay activity of $C_{18}$ HPLC fractions of 'Fuji'(A) and 'Gala'(B) samples. Bioassay activity is expressed as length of second leaf sheath of rice seedling. Filled line and dotted line show the results of applications with 2 different concentrations. The retention time of tritiated GAs is also marked in the graph.

rice leaf sheath length. In 'Fuji', the peaks are fractions 23-25, 27-32 and 33-43 while fractions 20-21, 25-29 and 32-41 (numbers used for each fraction are retention time in minutes) have showed relatively high activities in 'Gala'. All fraction numbers here and in the following text were based on their retention time from HPLC. Radioactivity measurements of each fraction indicated that the possible retention times of $^3$H-$GA_1$, $^3$H-$GA_4$ and $^3$H-$GA_9$ were 30, 41 and 43 minutes respectively in 'Fuji'. In 'Gala', these retention times were 26, 39 and 41 minutes. The peaks of bioassay reaction and tritiated GAs were delayed about 2-3 minutes in 'Fuji' samples as compared to those in 'Gala' samples. This may have been a system error caused by starting the fraction collector at a wrong time. However, this discrepancy didn't affect the decision of choosing the right fractions in $C_{18}$ HPLC for future separation and analysis. For both 'Fuji' and 'Gala', the $^3$H-$GA_1$ peak coincided with the second bioassay reaction peak (fraction 27-32 in 'Fuji', fraction 23-31 in 'Gala') and $^3$H-$GA_4$ and $^3$H-$GA_9$ with the third bioassay peak (fraction 33-43 in 'Fuji', fraction 32-41 in 'Gala'). Therefore, all fractions from the second and the third bioassay reaction peaks of both varieties were subjected to Nucleosil HPLC for further analysis. For the convenience of description in the following text, fractions in the second peak are labeled as '$GA_1$ group' and those in the third peak as '$GA_{4/9}$ group'. The first bioassay peaks were not included because it is unlikely that fractions before 25 minutes will contain any GAs according to previous experience. This prediction was confirmed by GC-MS analysis of an aliquot of fractions from those 2 peaks – no GA–like spectra were identified from these aliquots.

Bioassay of Nucleosil HPLC fractions. The major objective of running the $GA_1$ and $GA_{4/9}$ groups through Nucleosil HPLC was to separate clusters of GAs in the sample and thus achieve greater purity in fractions subjected to GC-MS. The results of separation can be observed in the graphs where bioassay activity of various fractions is plotted against retention time (Figs 6 & 7). For both $C_{18}$ GA groups of both varieties, more than one biologically active group was identified. This indicates that Nucleosil column does

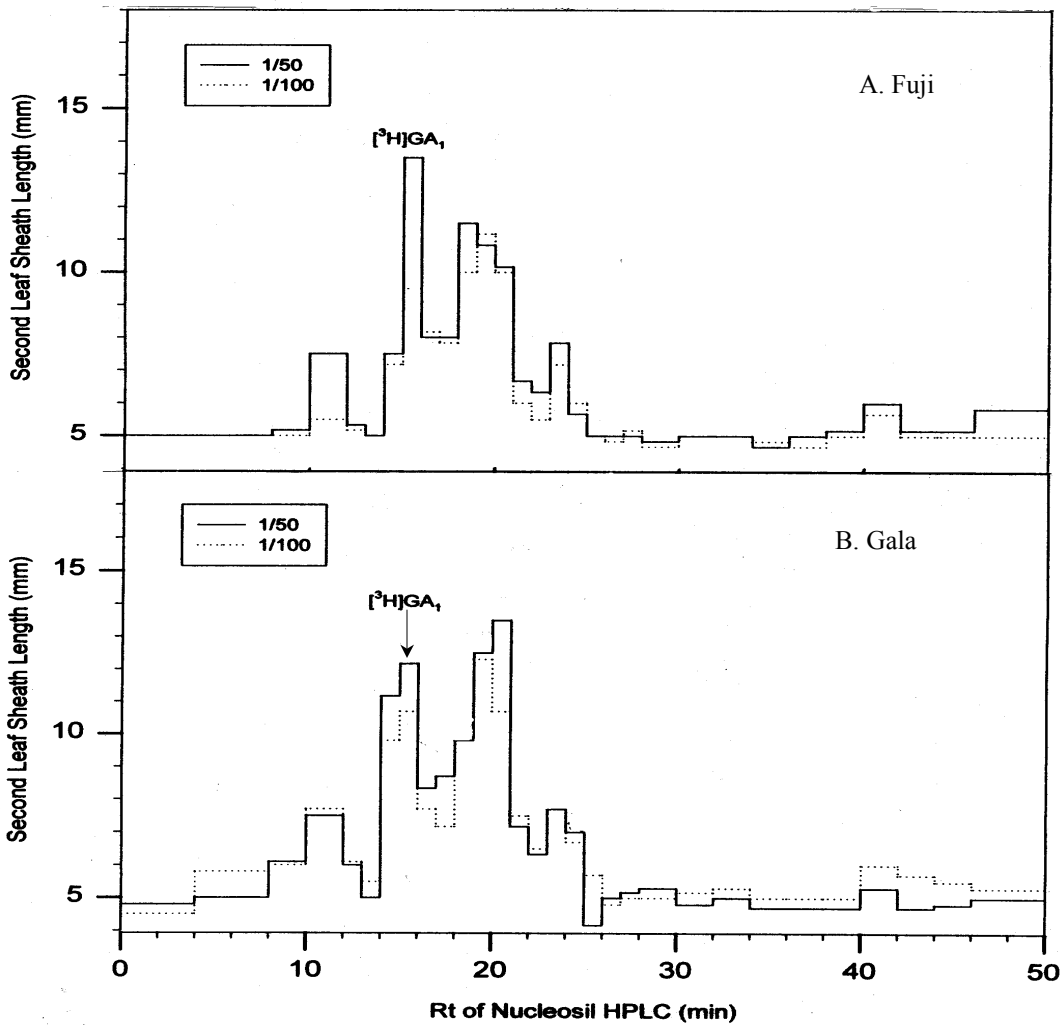


Fig 6. Dwarf rice *'Tanginbouzu'* bioassay activity of Nucleosil HPLC fractions of $GA_1$ group obtained from $C_{18}$ HPLC for 'Fuji' and 'Gala'. Bioassay activity is expressed as length of second leaf sheath of rice seedling. Filled line and dotted line show the results of applications with 2 different concentrations. The retention time of $^3H$-$GA_1$ is also marked in the graph.

further separate GA-like substances from broad peaks obtained from $C_{18}$ HPLC into different groups.

The $GA_1$ group of $C_{18}$ HPLC showed similar active groups in 'Fuji' and 'Gala' (Fig 6). In 'Fuji', the highest activity was found on fraction 16 with a leaf sheath length reading of 13.5mm. The counterpart of this fraction in 'Gala' is also within a group of high activity except that the group includes one more fraction (fraction 15). Similarly,

fraction 19-21 made up another group of significant GA-like activity in both 'Fuji' and 'Gala'. Among them, fraction 21 is the zenith of all fractions of 'Gala' $GA_1$ group. With almost the same bioassay readings, fractions 11-12 and 24-25 appeared to be 2 other peaks in both 'Fuji' and 'Gala' $GA_1$ group plots. Radioactivity data (not shown here, but indicated in Fig 6 and Fig 7) suggested the retention time of $GA_1$ be 16 minutes in Nucleosil HPLC.

For the $GA_{4/9}$ group, altogether 5 activity groups were observed in both varieties (Fig 7). Fractions 14 -19, which was divided into 2 groups in both plots, showed the highest activity among all peaks. A majority of active GAs may elute at retention times close to those of $GA_4$, $GA_9$ (for $GA_{4/9}$ group from $C_{18}$ HPLC) and $GA_1$ (for $GA_1$ group from $C_{18}$ HPLC). That is the exact reason why we only used tritiated $GA_1$, $GA_4$ and $GA_9$ for positioning of possible active GA groups (Zhang, personal communications). Therefore, the fact that $^3$H-$GA_4$ (16min) and $^3$H-$GA_9$ (18min) were positioned within the range of fraction 14 to fraction 19 indicate that most GAs in the $GA_{4/9}$ group, if there are any, may be found by running GC-MS of fractions 14 – 19. In 'Fuji', the largest peak appeared to be fractions 41 – 44 that contained the highest bioassay reading (16mm for fraction 44). This was not expected because very few GAs will be washed out after 40 minutes from the $N(CH_3)_2$ column according to past experience. This can be explained by an excessive amount of a single GA in those fractions or other substances in the plant extract that can trigger sheath elongation. Fractions 41 – 46 in 'Gala' also showed some activity even though it is not so pronounced as its counterpart in 'Fuji'. The other 2 intermediate peaks, fraction 21-24 (21-23 in 'Gala') and fraction 28 (28 – 30 in 'Gala') have lower bioassay readings than those described above. No identifiable GAs were detected when we run GC-MS of aliquots of these 2 peaks.

Identification of Gibberellins by GC-MS. Altogether, 17 GAs were identified from 'Fuji' and 'Gala' samples (Table 2). The identification of GAs is usually accomplished by comparing the mass spectra and retention time of GC peaks in the sample and those of authentic GAs from the references. Due to the difference of absolute retention time of a certain compound in different gas chromatography systems, Kovats

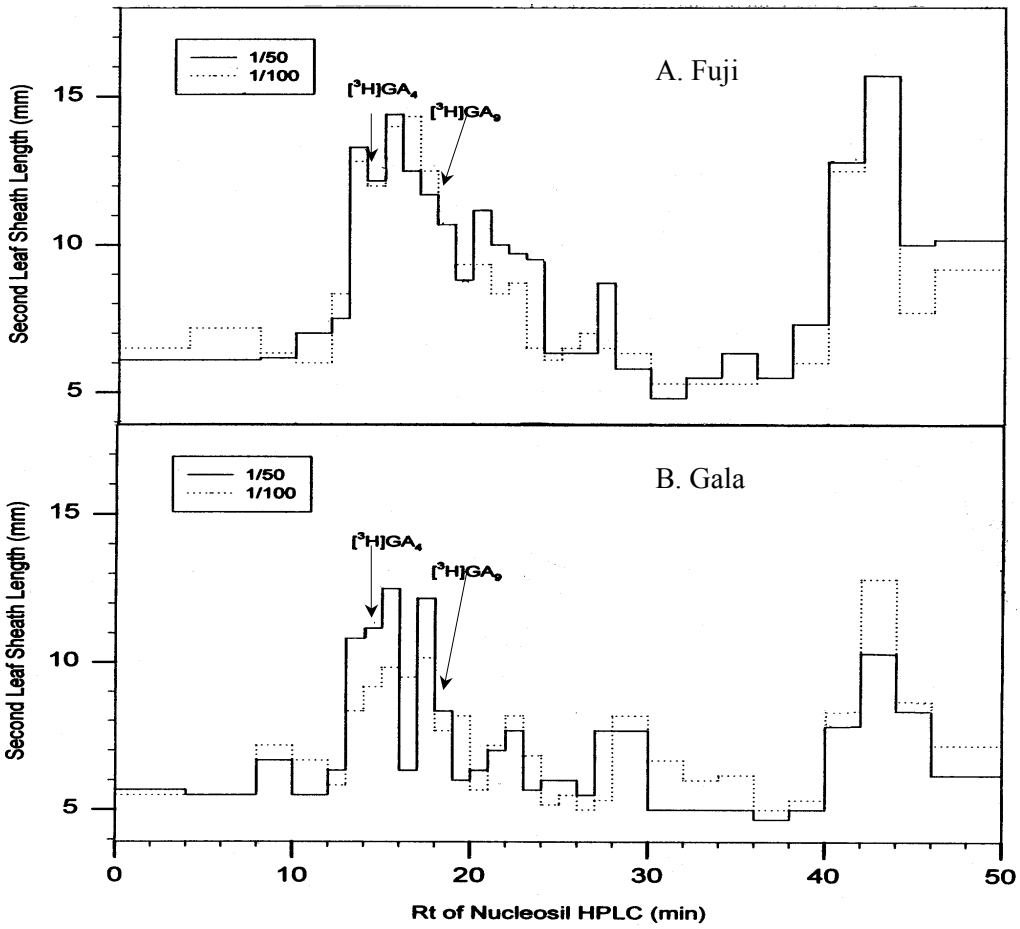


Fig 7. Dwarf rice *'Tanginbouzu'* bioassay activity of Nucleosil HPLC fractions of $GA_{4/9}$ group obtained from $C_{18}$ HPLC for 'Fuji' and 'Gala'. Bioassay activity is expressed as length of second leaf sheath of rice seedling. Filled line and dotted line show the results of applications with 2 different concentrations. The retention time of $^3$H-$GA_4$ and $^3$H-$GA_9$ are also marked in the graph.

Retention Indices (KRI), an index obtained by comparing retention time of GAs and saturated hydrocarbons co-injected, is generally used. However, KRI was not used in this research because the absolute retention time in GC and 2 rounds of HPLC as well as the mass spectra provided enough information to identify specific GAs in the apple samples.

Compared with the recent qualitative research of GAs in apple seeds (Oyama *et al*., 1996; Hedden *et al.*, 1993), fewer specific GAs were identified in our samples. Thirty-two GAs and twenty-four as well as a number of novel GAs were traced in the study of Oyama *et al.* (1996) and Hedden *et al*. (1993), respectively. One reason for this discrepancy is that we used only embryos as experimental material for qualitative research while the testa were removed before GA extraction. According to past experience, apple seed testa contain very limited amount of GAs and may be a major source of contaminations in GA analysis (Pharis, personal communications). However, testa were not removed in our quantitative study of apple GAs. In these experiments, the level of contamination brought by testa was found to be acceptable due to the utilization of GC-SIM, which showed much higher sensitivity than full-scan GC-MS used for qualitative analysis. Another reason why more GAs were found by Oyama *et al.* (1996) is that they completely scanned each fraction from HPLC. Therefore, some non-active GAs that act as biosynthetic intermediates were also included.

Among the 17 GAs identified, 11 were found in both cultivars, 5 GAs ($GA_{20}$, $GA_{45}$, $GA_{61}$, $GA_{88}$ and $GA_{34}$) appeared only in ‘Fuji’, while $GA_{68}$ was unique in ‘Gala’ (Table 2). The abundance of $GA_{80}$, $GA_{63}$ and $GA_{35}$ were found to be relatively larger than those of other GAs in both ‘Fuji’ and ‘Gala’. This is inconsistent with the results of Dennis and Nitsch (1966) who found that $GA_4$ and $GA_7$ were the major GAs in developing apple seeds. The abundance of most GAs found in both cultivars was higher in ‘Fuji’ than in ‘Gala’. This fact, as well as the discovery of a larger number of GAs in ‘Fuji’, suggests higher gibberellin activity in ‘Fuji’. However, the abundance of GAs in full-scan GC-MS can only give us an approximation of the amounts in the samples. The most significant difference between 2 samples comes from the sole existence of $GA_{88}$ in ‘Fuji’ (Fig 8, which also showed the absence of $GA_{34}$ from ‘Gala’). Being detected by GC-MS as a peak with height over 3,000,000 and known as a metabolic precursor of $GA_7$ (Oyama *et al*., 1996), $GA_{88}$ seems to be another piece of evidence supporting our hypothesis that there are more types of flower-inhibiting GAs produced in the seeds of on-year biennial bearing apples.

Table 2. Retention time and peak size of gibberellins identified by GC-MS in ‘Fuji’ and “Gala’.

* Unit for retention time is minute. '+' : confirmed biological activity, "-": no avtivity, “?”: activity unknown; In sample cultivar, G: Gala, F: Fuji.

| C18 HPLC | Nucleosil HPLC | Sample | GC | GA | 'Fuji' sample | | 'Gala' sample | | Biological |
|---|---|---|---|---|---|---|---|---|---|
| retention time | retention time | Cultivar | retention time | | Peak height | Peak area | Peak height | Peak area | Activity |
| 23 - 32 | 15 - 16 | G, F | 16.01 | $GA_{35}$ | 2,033,138 | 116,509,793 | 2,154,931 | 120,100,046 | + |
| (GA1 group) | | G, F | 16.33 | $GA_{1}$ | 321,904 | 15,439,369 | 1,316,806 | 63,619,565 | + |
| | 19 - 21 | G, F | 16.34 | $GA_{80}$ | 5,229,161 | 303,402,947 | 5,754,923 | 364,091,157 | + |
| | | G, F | 16.68 | $GA_{3}$ | 297,810 | 24,874,483 | 181,482 | 10,401,258 | + |
| | | G, F | 18.94 | $GA_{50}$ | 1,301,953 | 91,712,447 | 1,023,845 | 80,339,968 | - |
| | | G | 19.21 | $GA_{68}$ | -- | -- | 343,419 | 29,478,242 | + |
| 32 - 43 | 14 - 16 | G, F | 14.01 | $GA_{4}$ | 3,484,358 | 142,018,492 | 1,061,964 | 30,412,452 | + |
| (GA4/9 group) | | G, F | 18.05 | $GA_{44}$ | 659,148 | 33,359,066 | 355,685 | 28,124,872 | + |
| | | G, F | 12.85 | $GA_{31}$ | 224,478 | 9,167,821 | 493,645 | 21,049,455 | ? |
| | | F | 12.69 | $GA_{61}$ | 1,226,805 | 44,691,246 | -- | -- | ? |
| | 16 - 19 | G, F | 14.29 | $GA_{7}$ | 2,288,313 | 105,363,391 | 740,307 | 34,006,692 | + |
| | | G, F | 15.93 | $GA_{54}$ | 1,992,797 | 107,056,971 | 157,436 | 7,109,796 | + |
| | | F | 12.94 | $GA_{88}$ | 3,236,279 | 156,550,897 | -- | -- | + |
| | | F | 13.74 | $GA_{20}$ | 261,000 | 10,700,000 | -- | -- | - |
| | | F | 16.25 | $GA_{34}$ | 574,203 | 29,869,554 | -- | -- | - |
| | 41-44 | F | 13.77 | $GA_{45}$ | 504,735 | 23,459,472 | -- | -- | ? |
| | | G, F | 16.83 | $GA_{63}$ | 3,541,805 | 204,345,633 | 2,098,990 | 154,503,160 | + |

In addition to the 17 GAs identified, we also obtained 6 GA-like mass spectra that do not match any published GA mass spectra. Among 6 such novel MS patterns found in the GC-MS spectra of 3 HPLC fractions, 2 were from both cultivars (Fig. 9A-B) and other 4 were exclusively from in "Fuji" (Fig. 9C-F).

Analysis of the 6 novel MS patterns allows the chemical structures of these 6 GA-like substances to be proposed. The way structural information of GAs was derived from mass spectra was thoroughly discussed by Peter Hedden (Hedden, 1987). Generally, EI (electron impact) mass spectra are regarded as the first evidence for discovery of a novel GA. Even though the structure of a GA cannot be determined purely by its mass spectrum, high-resolution EI mass spectra are very informative in structure assignment. In Fig 9, both spectra A and B are MeTMS derivatives of dihydroxy $C_{19}$-gibberellins, as indicated by their molecular ions of 506 *m/z* as well as ions at 416 *m/z* that were formed after loss of a TMSOH group (90amu). Intensive ions at 147 *m/z* suggests that both A and B have hydroxyl groups at 2' and 3' positions therefore they are unlikely to be biologically active GAs. Spectra C and D represent either $C_{20}$-GAs or $C_{19}$-GAs with an extra ketone group. Again, loss of activity can be deduced from the presence of high-intensity ions at 147 *m/z*. Molecular ions at 418 *m/z*, as shown in spectrum E, is a typical feature of monohydroxy $C_{19}$-GA MeTMS derivatives. A strong fragment ion at 129 m/z, as accompanied by molecular ion of 416 or 418 *m/z*, indicates a 3' hydroxy group (Oyama *et al*., 1996). Relatively high intensity of 129 *m/z* ions in E and F is therefore the evidence for biological activity of the GAs they represent. As a result of dehydration, F shows molecular ions at 416 *m/z* instead of 418 *m/z*.

From the qualitative analysis of the 1998 results, clearly the GA activity involves a larger number of GAs in 'Fuji' than in 'Gala'. Generally, it is reasonable to deduce that the presence of more GAs in a plant organ means more active biosynthesis of GAs in that organ because most GAs are metabolic precursors of active moieties. In plants, GA biosynthesis is a very complicated process with many pathways intertwined, especially for the oxidative reactions after $GA_{12}$-aldehyde (Graebe, 1987). Therefore, more GAs indicates that more biosynthetic pathways are involved. Actually, most of the GAs found in our samples are physiologically active, as evidenced by the existence of hydroxyl group at the 3β or 13' position (Table 2). The higher GA-like activity in extracts of 'Fuji'

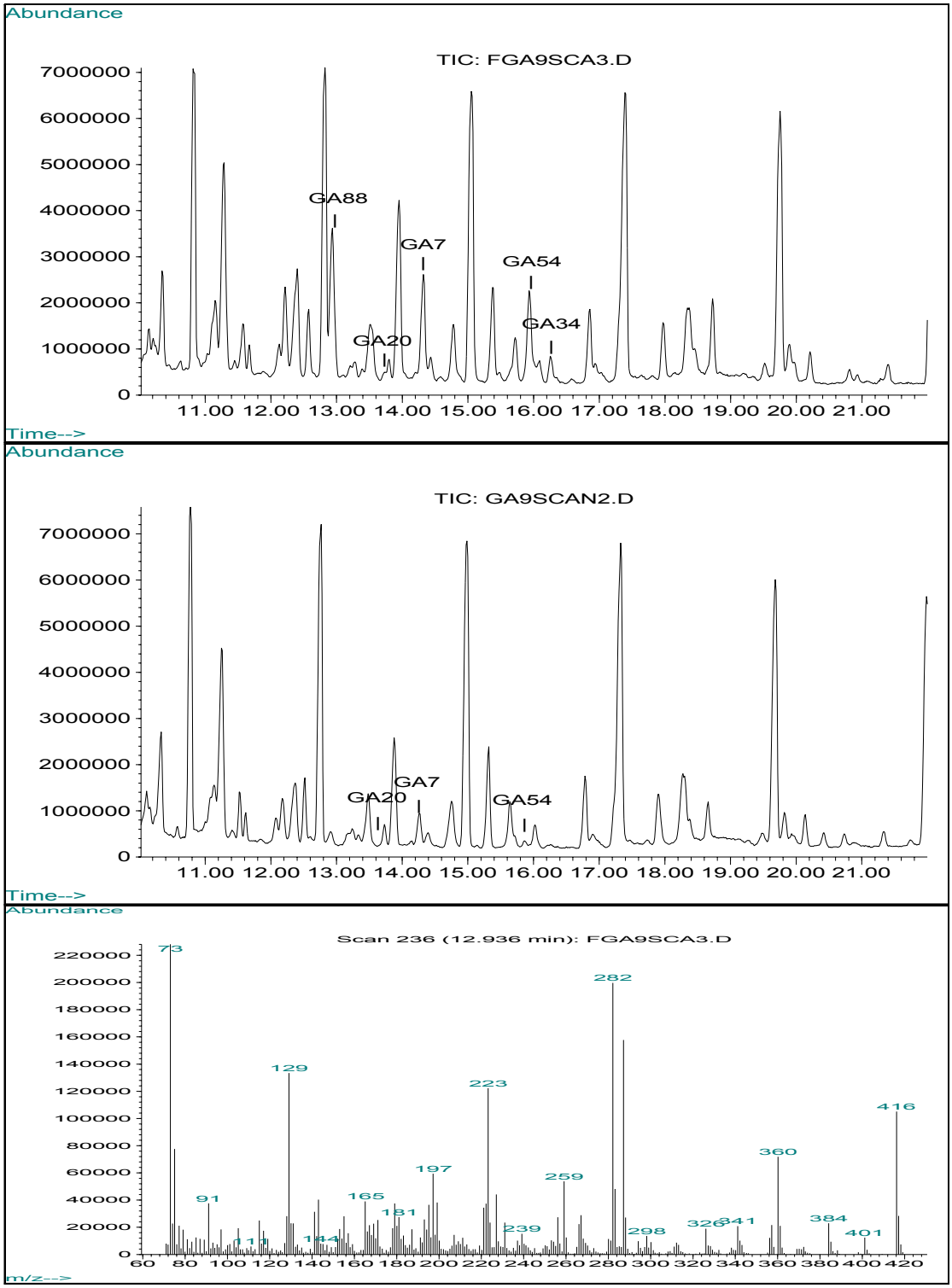


Fig 8. Total ion chromatogram of fraction 16-19 in $GA_{4/9}$ group of 'Fuji' (upper), 'Gala' (middle), and mass spectrum of $GA_{88}$ (lower).

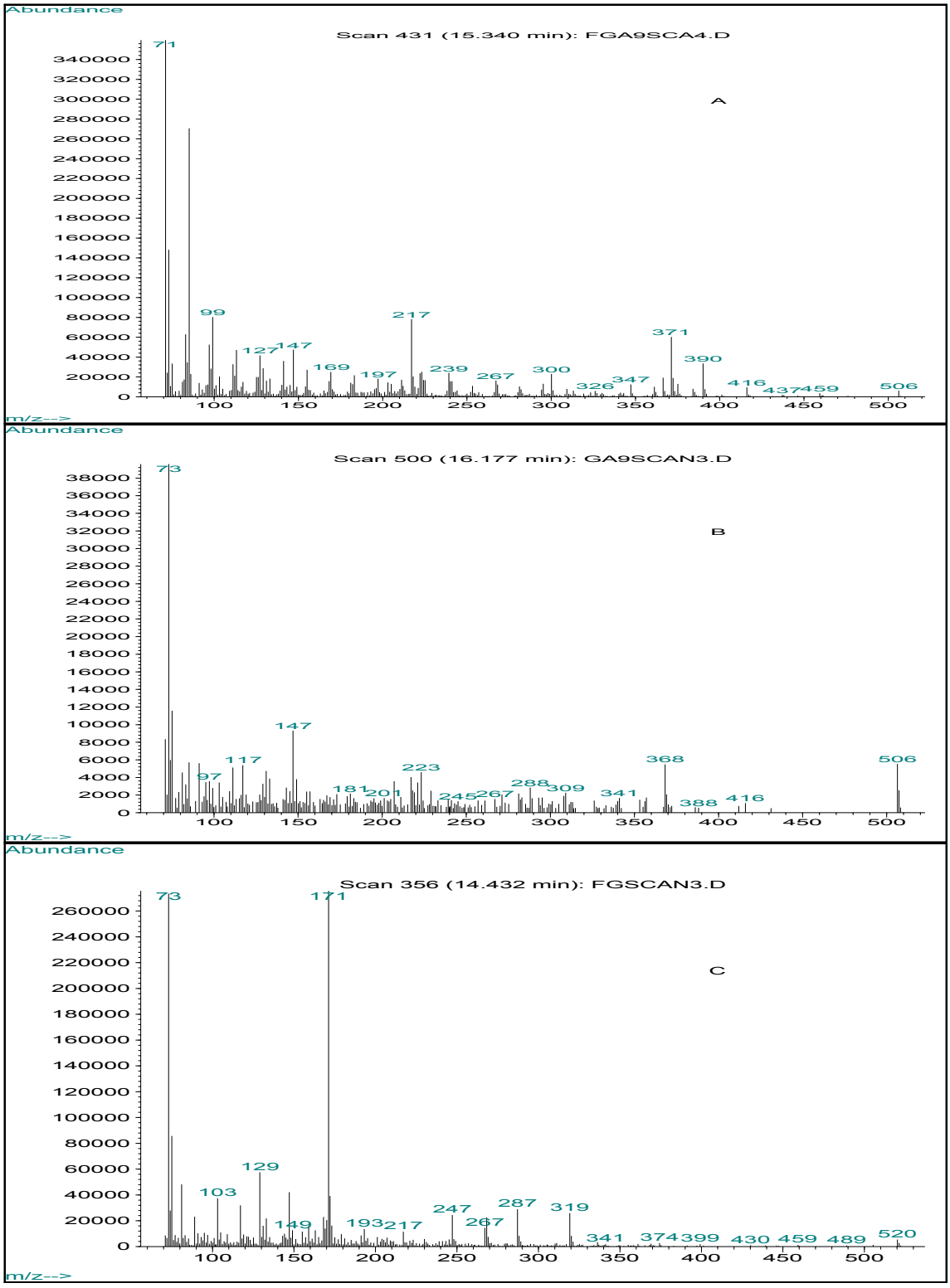


Fig 9. MS spectra of novel GA-like substances found in qualitative analysis

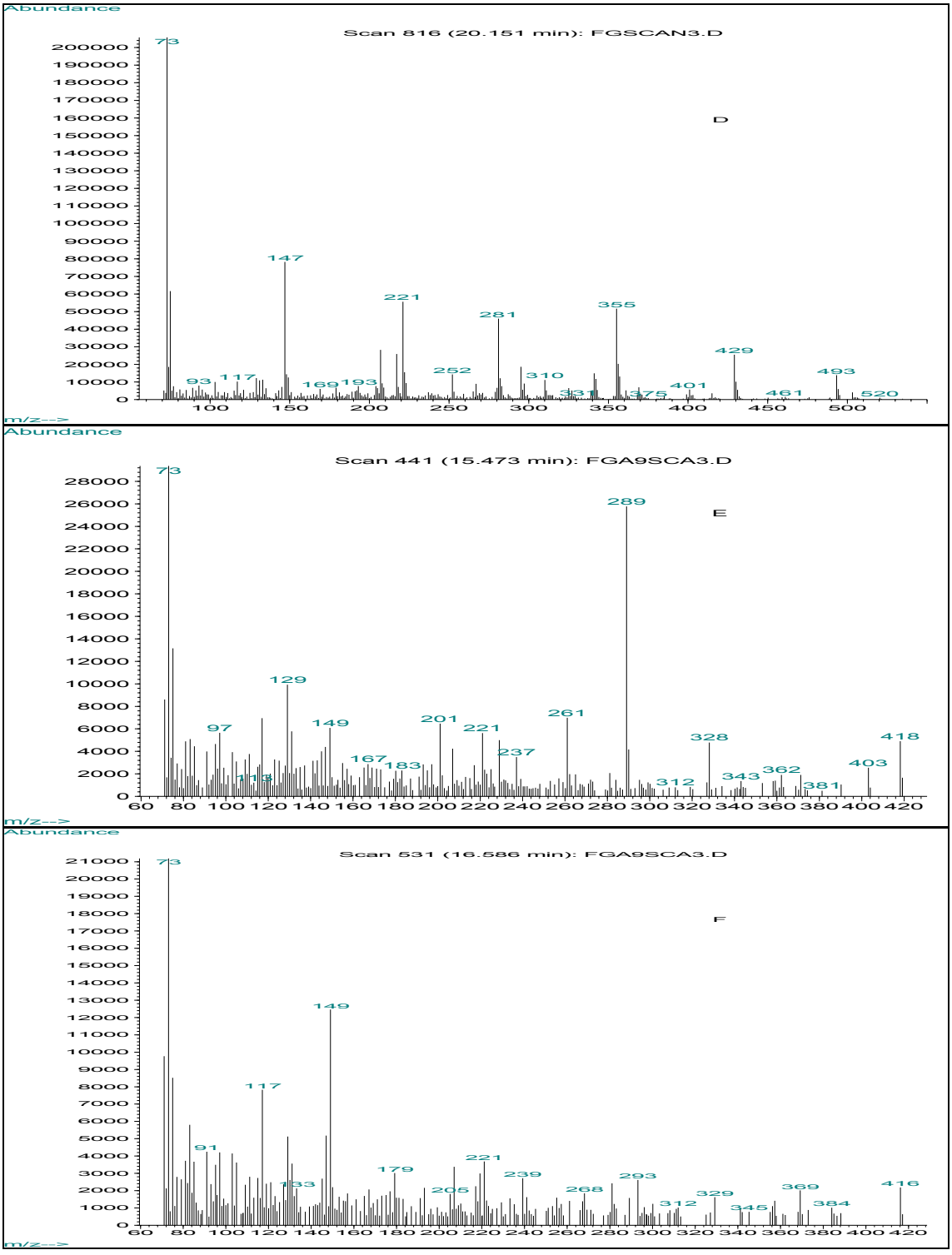


Fig 9 (continued). MS spectra of novel GA-like substances found in qualitative analysis

tends to support our hypothesis that the seeds of biennial bearing apple cultivars may produce GAs that inhibit flowering. This conclusion, however, requires confirmation by a more quantitative approach.

The height and area of GA peaks shown in gas chromatogram are usually not used as indexes for precise quantification because it is difficult to reproduce the same operating conditions from one injection to another. Another reason is that, even under the same chromatographic conditions, the ionization level of various GAs may be different. This is why internal standards are needed. GA standards that were labeled by $^{2}H$, $^{13}C$, and $^{14}C$ in various positions have very similar chemical and chromatographic features as their unlabeled counterparts (Pearce *et al*., 1994). When known amounts of these internal standards are injected during GA extraction, the relative amount of the internal standard and the GA of interest can be calculated by the abundance of characteristic ions in mass spectra derived from both molecules. GAs deuterated in the 2 hydrogen atoms at the 17' position are widely used and have a molecular mass that is 2 units greater than the natural hormone.

The next phase of this study was to precisely quantify those GAs that may have effects on flowering of biennial or non-biennial apple cultivars. Therefore, those GAs being uniquely or primarily found in one cultivar were considered first. Furthermore, the GAs chosen were 3β-hydroxylated and not 2β-hydroxylated as the former are biologically active. Based on the above information and the availability of deuterated internal standards, we decided to study further the concentrations of $GA_1$, $GA_3$, $GA_4$, and $GA_7$ in 'Fuji' and 'Gala' seeds over the growing season. The immediate precursor to $GA_1$ and only found in 'Fuji' by qualitative analysis, $GA_{20}$ was also included. The unavailability of deuterated $GA_{88}$ as internal standard precluded the further study of this particular GA.

### III. Quantification of $GA_1$, $GA_3$, $GA_4$, $GA_7$ and $GA_{20}$

$\underline{GA_1, GA_3, \text{ and } GA_{20}}$ The concentrations of $GA_1$ for both cultivars in both years are plotted in Fig. 10. To be compatible with previous research on GA contents in apple

seeds (Luckwill & Weaver, 1969; Ebert & Bangerth, 1981; Stephens *et al*., 1991, 1992), the unit for GA concentration was ng GA/g seed in dry weight, and the sampling date was translated into days after full bloom. The same units were utilized for interpretation of other GA data. For most of the growing season in 1998, $GA_1$ content was under 500ng/g except one peak at 37 DAFB for 'Fuji' and one at 44 DAFB for 'Gala' (Fig 10). Although the $GA_1$ peak occurs a week earlier in 'Fuji', no overall difference was found by 2-factor (sampling date vs. cultivar) ANOVA test between 'Fuji' and 'Gala'. Physiologically, the one-week difference between cultivars are likely to have similar effects on flowering since flower induction is generally believed to occur within 3 to 10 weeks after bloom (Buban & Faust, 1982; Li *et al*., 1995). The GA content readings of both cultivars in 1999 were relatively low and similar to each other except there was a huge peak for 'Fuji'. However, this peak was due to a single reading that was extremely high. The large variance caused by this reading makes this peak at 59 DAFB statistically insignificant. Since we only started sampling from 40 DAFB in 1999, the early $GA_1$ peaks found in 1998 were not observed. Despite the ambiguities described above, we can still see that the $GA_1$ activities in both 'on-year' (1998) and 'off-year' (1999) of 'Fuji'.

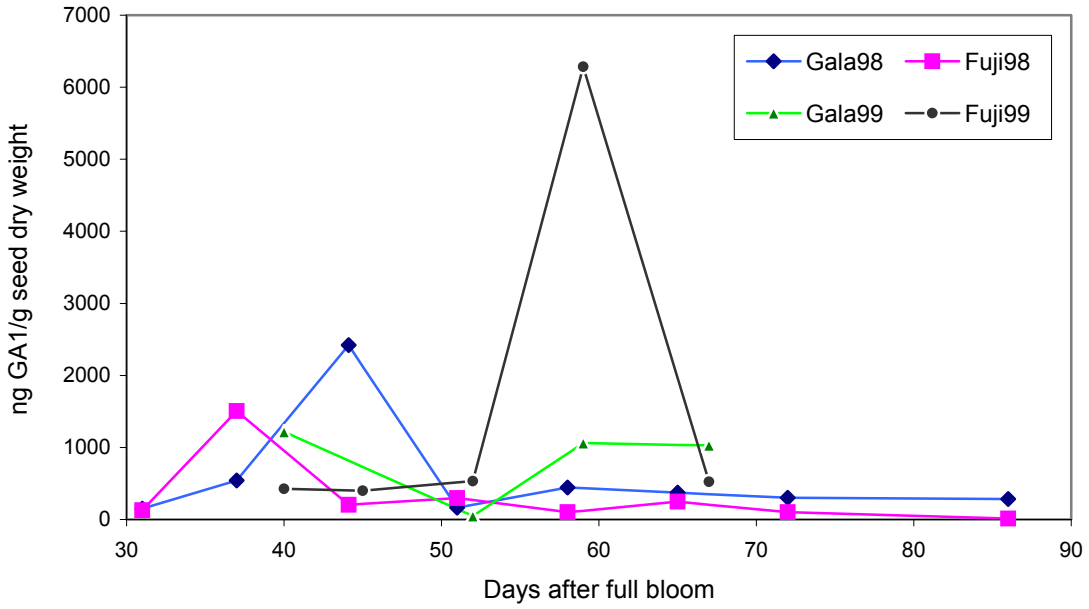


Fig 10. $GA_1$ concentration in "Fuji" and "Gala" seeds in 1998 and 1999

From the $GA_3$ data (Fig 11), we can also observe early concentration peaks between 40 and 50 DAFB. In 1998, there was a second peak for both 'Fuji' (72 DAFB) and 'Gala' (58-65 DAFB), and the peak height of 'Fuji' at both peaks was lower than that of 'Gala'. In 1999, the curves for both cultivars were very similar in shape except that the early concentration peak for ‘Gala’ seems to be of longer duration than that of ‘Fuji’. These facts do not favor the hypothesis of $GA_3$ acting as a negative signal to apple flowering because 'Gala' showed strong return bloom while 'Fuji' had much fewer flowers in 1999. One can argue that the second $GA_3$ peak occurred beyond the critical period of flower induction and even the difference between 2 cultivars in the first peak is not statistically significant, but more $GA_3$ in ‘Fuji’ seeds would be expected in the ‘on-year’ if we propose inhibitory GAs such as $GA_3$ plays the controlling role in apple flowering. For the same reason, the similar $GA_1$ activity patterns between both cultivars in 1998 excluded the central role of $GA_1$ in this issue, too. The results of $GA_1$ and $GA_3$ in 1999 turned out to be no surprise since the 2 cultivars were similar in terms of GA concentration as well as return bloom.

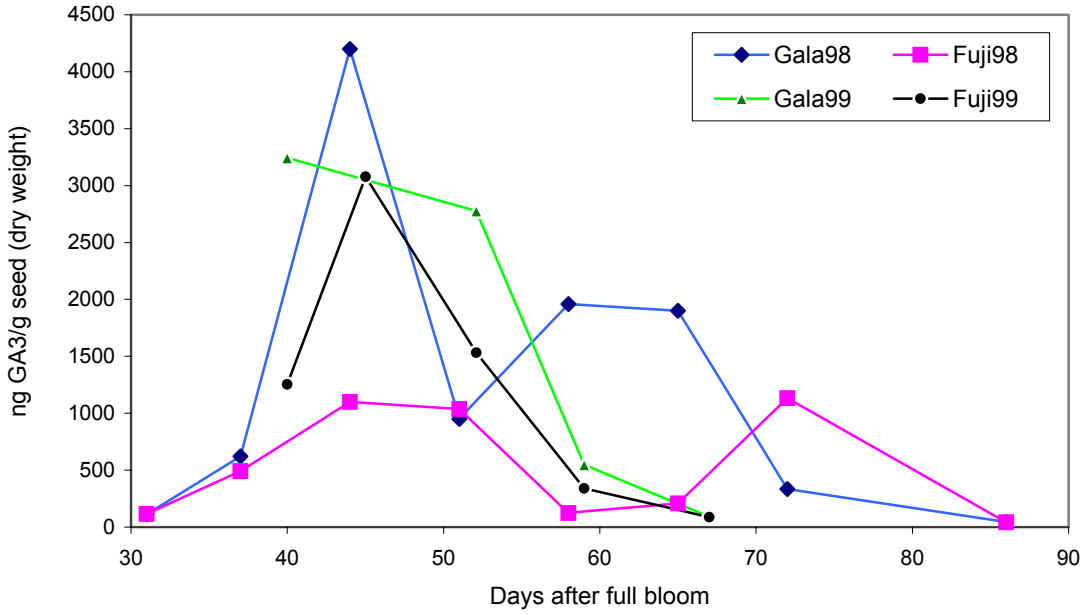


Fig 11. $GA_3$ concentration in "Fuji" and "Gala" seeds in 1998 and 1999

$GA_{20}$ concentrations were always low compared with those of other GAs (Fig 12). A $GA_{20}$ peak was found between 37 and 50 DAFB for both cultivars in 1998, similar to the pattern observed for $GA_1$ and $GA_3$. There was no significant difference between ‘Fuji’

and 'Gala' in terms of $GA_{20}$ concentration even though the height of the concentration peak appeared to be higher in 'Fuji'. The 1999 data for $GA_{20}$ is more ambiguous: the peaks occurred later than those in 1998 while a high reading at 40 DAFB for 'Fuji' suggests the possibility of a missed early peak. Although 'Fuji' produced significantly more $GA_{20}$ than 'Gala' at 67 DAFB, there was no overall difference between cultivars. $GA_{20}$ is a biosynthetic precursor to $GA_1$ rather than an active hormone therefore there is little point in looking for a relationship with return bloom. However, there was no correlation between $GA_1$ and $GA_{20}$ data although there was visual similarity between the shape of $GA_1$ and $GA_{20}$ curves for both cultivars in 1998.

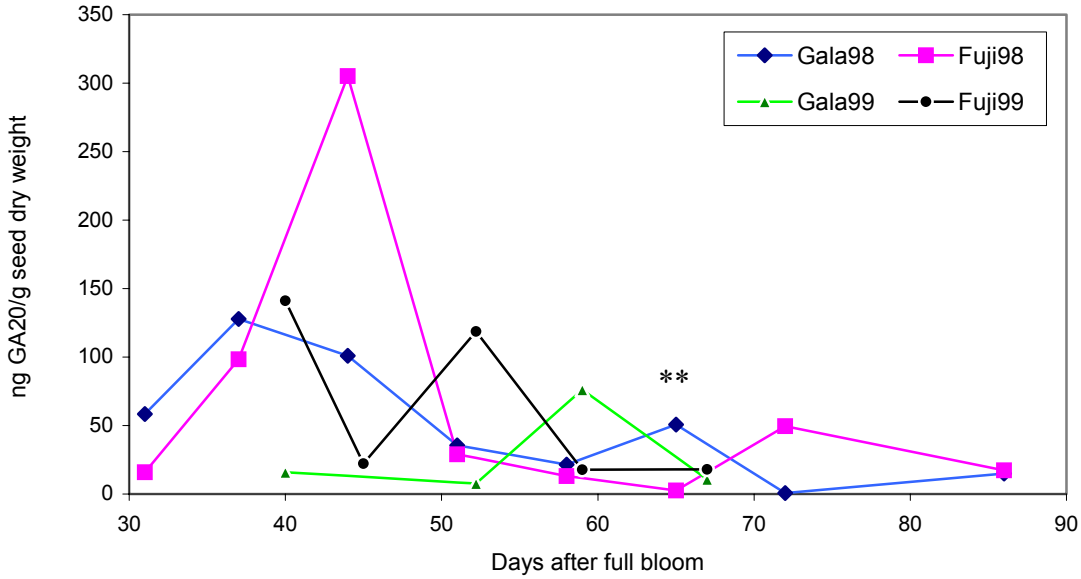


Fig 12. $GA_{20}$ concentration in "Fuji" and "Gala" seeds in 1998 and 1999
** indicates significant difference at 0.01 level

<u>$GA_4$ and $GA_7$</u> As mentioned in the Literature Review of this thesis, the concentration (absolute and relative) of $GA_4$ and $GA_7$ and their relationship with return bloom was the main focus of this study. While the results of $GA_1/GA_3$ quantification did not point to any clear relationship with the biennial bearing behavior of 'Fuji', the $GA_4/GA_7$ data point to a more interesting role in the control of apple flowering.

Like $GA_1$ and $GA_3$, $GA_7$ is a strong inhibitor to apple flower induction (Tromp, 1982; Unrath & Whitworth, 1991) when applied exogenously. More $GA_7$ produced by 'Fuji' seeds in its 'on-year' would therefore be expected. On the contrary, we found higher concentrations of $GA_7$ in 'Gala' in 1998 (Fig 13). Although the ANOVA test

didn't find a general difference between cultivars, at 44 DAFB in 1998 'Gala' had a significantly higher concentration of $GA_7$ than 'Fuji'. Previous studies (Tromp, 1982; Li *et al*., 1995) have shown that a burst of inhibitory GA concentrations occurring in a short period of time can lead to markedly reduced return bloom. Therefore, the higher $GA_7$ content at 44 DAFB in 'Gala' could affect its flower induction if all the $GA_7$ produced in 'Gala' seeds acted as a negative signal to flowering in the bud. But the fact is 'Gala' still formed the same amount of flower buds in 1998 as it did the next year when the $GA_7$ concentrations were lower. This suggests that the relatively high concentrations of $GA_7$ in 'Gala' were not inhibitory to flower induction in 1998. $GA_7$ may either be antagonized by other hormones (maybe flower-promoting GAs) or not effectively transported to the site of flower induction at all.

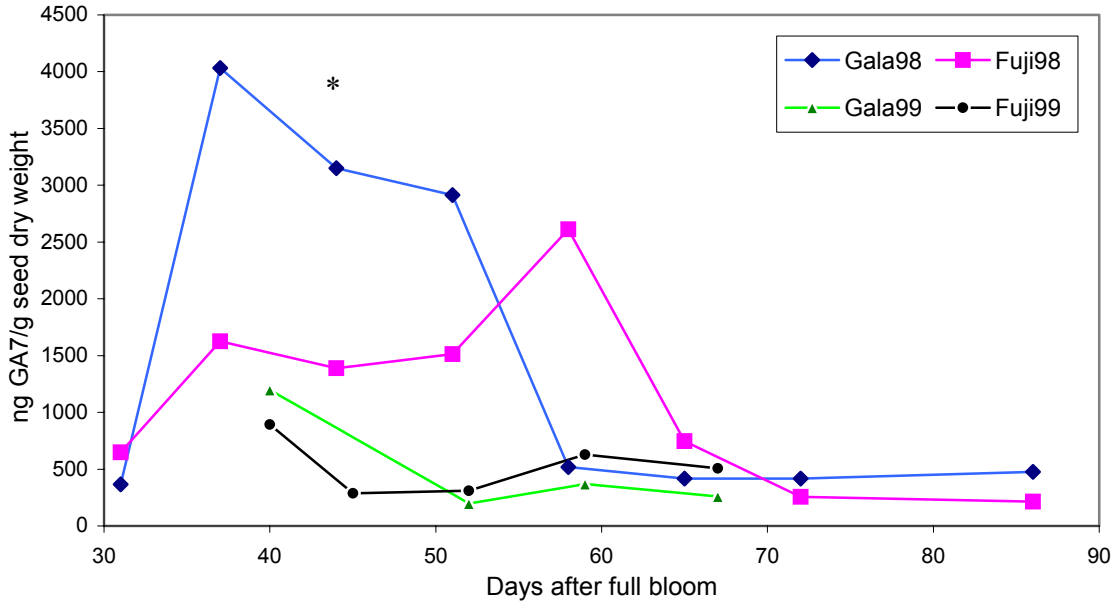


Fig 13. $GA_7$ concentration in "Fuji" and "Gala" seeds in 1998 and 1999
* indicates significant difference at 0.05 level

Very interesting results were obtained when we analyzed the $GA_4$ data for 1998 (Fig 14). First, we can easily observe a concentration peak for both cultivars before 50 DAFB. This peak in 'Gala' is followed by a smaller peak at 65 DAFB. Secondly, the first peak in 'Gala' was much higher than the corresponding peak in 'Fuji'. Statistical analysis (2-factor ANOVA) demonstrated that both sampling date and cultivar ('Gala' and 'Fuji') had significant effects on $GA_4$ concentration. Since the interaction between sampling date

and cultivar was insignificant, we can conclude that 'Gala' constantly produced more $GA_4$ than 'Fuji' in the growing season of 1998. As $GA_4$ is thought to be less inhibitory and in some instances promotive to apple flowering, it is appropriate to relate the high concentrations of $GA_4$ in 'Gala' to its regular bearing behavior from 1999 to 2000. Because 'Gala' also produced larger or the same amounts of $GA_7$, $GA_1$, and $GA_3$ as 'Fuji' in 1998, its consistent return bloom in 1999 may be explained as the result of its $GA_4$

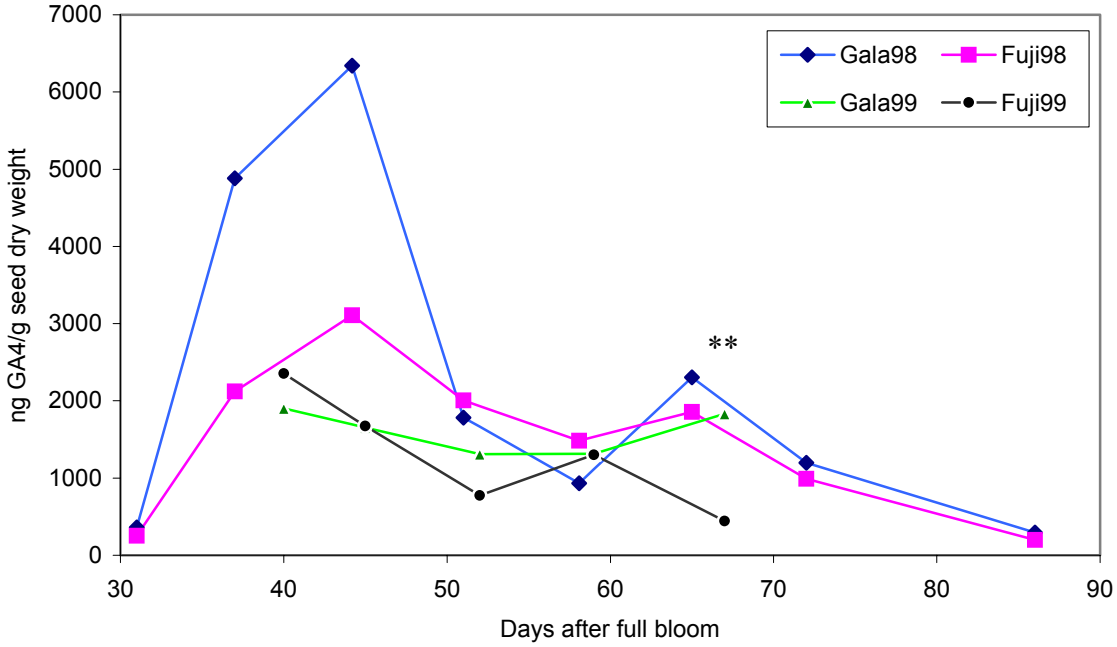


Fig 14. GA4 concentration in "Fuji" and "Gala" seeds in 1998 and 1999
** indicates significant difference at 0.01 level

activity. In a recent paper, Stephan *et al*. (1999) reported quantitative results of GAs exported from apple fruits. They found significant $GA_4$ efflux in apple cultivars showing no or little biennial bearing but not in the biennial bearing cultivar 'Elstar'. Although more study is required, the current evidence strongly suggests a controlling role of $GA_4$ in apple flowering. It is possible that inhibitory GAs play subordinate roles in flower induction and the activity of $GA_4$ determines how effectively the inhibitory GAs can affect the process of flower induction. $GA_4$ may accomplish such regulation by either suppressing the transportation of other GAs to the bud or competing with them for opportunities to bind with receptors on the cell membrane. However, more experiments need to be done for evidence supporting these hypotheses. The concentration changes of

$GA_4$ in 1999 were very similar to those of $GA_7$. Actually, no obvious concentration change over time was found in either cultivar. The difference between the 2 cultivars was significant only at 67DAFB when flower induction of most buds has accomplished.

Ratio of inhibitory GAs to $GA_4$. The GA concentration data could also be interpreted in other ways. One of our original hypotheses was that the ratio of $GA_7$ to $GA_4$ is a prominent factor in the regulation of apple flower induction. Low $GA_7/GA_4$ ratios may be favorable to induction of floral buds. However, our results do not support such a theory. For both 1998 and 1999 growing seasons, the change of $GA_7/GA_4$ ratio in 'Fuji' coincides with that in 'Gala' except 31DAFB in 1998 and 67DAFB in 1999 when 'Fuji' has a much higher $GA_7/GA_4$ ratio (Fig 15). For 4 of the sampling dates in 1998 the $GA_7/GA_4$ ratio is higher in 'Fuji' while the same ratio in 'Gala' is higher for the other 4

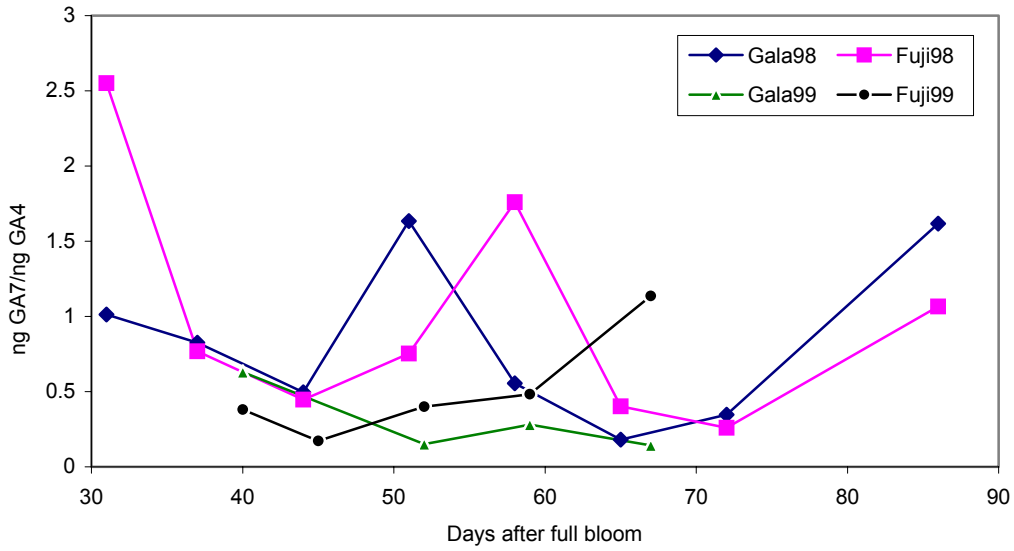


Fig 15. Concentration ratio of $GA_7$ to $GA_4$ in "Fuji" and "Gala" seeds
Three replications were pooled to avoid misleading curve shape caused by individual readings that were extremely high.

dates. Therefore the reduced return bloom of 'Fuji' in 1999 can hardly be explained by or correlated to the $GA_7/GA_4$ ratio in 1998 even though the difference at 31DAFB in 1998 between 2 cultivars may look significant. The $GA_7/GA_4$ ratio of 2 cultivars in 1999 further rejected our original hypothesis of the negative effects of $GA_7/GA_4$ ratio on apple flowering. The $GA_7/GA_4$ ratios tend to be higher in 'Fuji' for 3 of the 5 sampling dates

and a little lower in ‘Fuji’ for the othert 2 dates. According to our hypothesis, we would expect the relative concentration of $GA_7$ to $GA_4$ in 1999 to be the same in both cultivars since they formed the same density of floral buds. The high ratio in ‘Fuji’ at 67DAFB in 1999 is obviously contradictory to this expectation. As shown in Fig 13 and Fig 14, ‘Gala’ produced more $GA_4$ as well as $GA_7$ than ‘Fuji’ in 1998. This explains why the $GA_7/GA_4$ ratio in ‘Gala’ was not significantly lower than that in ‘Fuji’. In this instance, the $GA_7/GA_4$ ratio was a poor indication of the role endogenous GA relations play in the control of apple flower induction. The statistical analysis of Data about ratios is not straightforward. In the calculation of mean, we always have to consider the numerator and denominator separately to see whether the sum of either one has a physical meaning (Rego, 2000). If so, it would be less meaningful to average the ratios. The results shown in Fig 15 were obtained by dividing the sum of $GA_7$ concentration by the sum of $GA_4$ concentration in all 3 replications. When we first analyzed the data by averaging ratios for 3 replications, the difference between cultivars at all sampling dates was found to be accompanied by large variance therefore it is difficult to draw any conclusion. The variance of 1998 data is smaller, but again we could not find any significant difference between ‘Gala’ and ‘Fuji’, especially at the early part of the growing season (30 to 50 DAFB) when flower induction was underway.

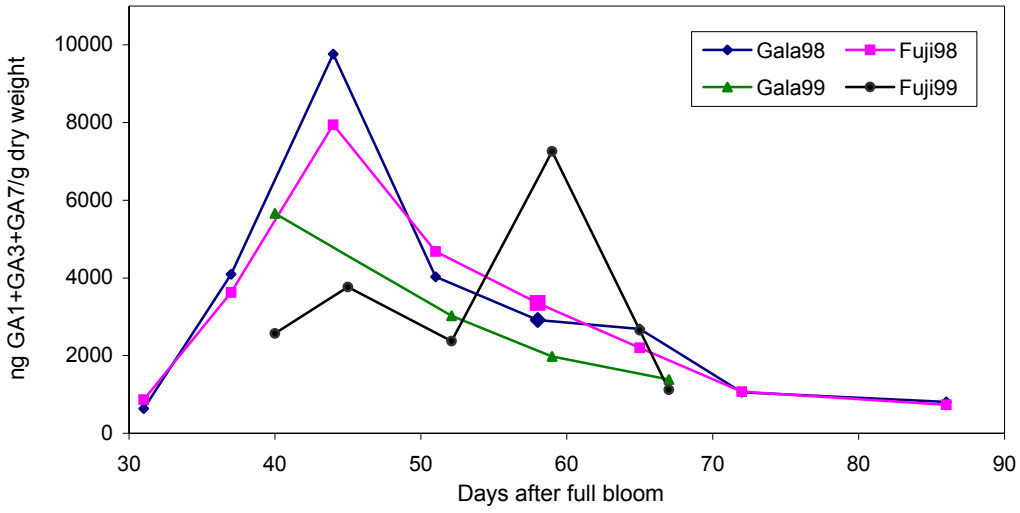


Fig 16. Total concentration of inhibitory GAs (GA1,3,7) in 'Fuji' and 'Gala' seeds in 1998 and 1999

Besides the $GA_7/GA_4$ ratio, the ratio of all inhibitory GAs quantified ($GA_1+GA_3+GA_7$) to $GA_4$ were also calculated. First of all, the sum of $GA_1$, $GA_3$, and $GA_7$ concentrations was plotted in Fig 16. We can easily see that the change of the total concentration of these 3 inhibitory GAs is strikingly similar between 'Fuji' and 'Gala' in1998. The concentration difference between 2 cultivars at any single sampling date is very small even though 'Gala' produced a little more inhibitory GAs than 'Fuji' at 44DAFB. This shows that flower induction of apples is not directly determined by the total amount of $GA_1$, $GA_3$, and $GA_7$ considering the extremely low level of flower induction in 'Fuji' in 1998 compared with 'Gala' in the same year. We do not know whether the amount of all inhibitory GAs in seeds has any correlation with the level of flower induction since only 5 GAs were quantified. However, the total amount of inhibitory GAs should be well represented by the sum of $GA_1$, $GA_3$, and $GA_7$ concentrations because $GA_1$, $GA_3$, $GA_4$, and $GA_7$ were found to be the major endogenous

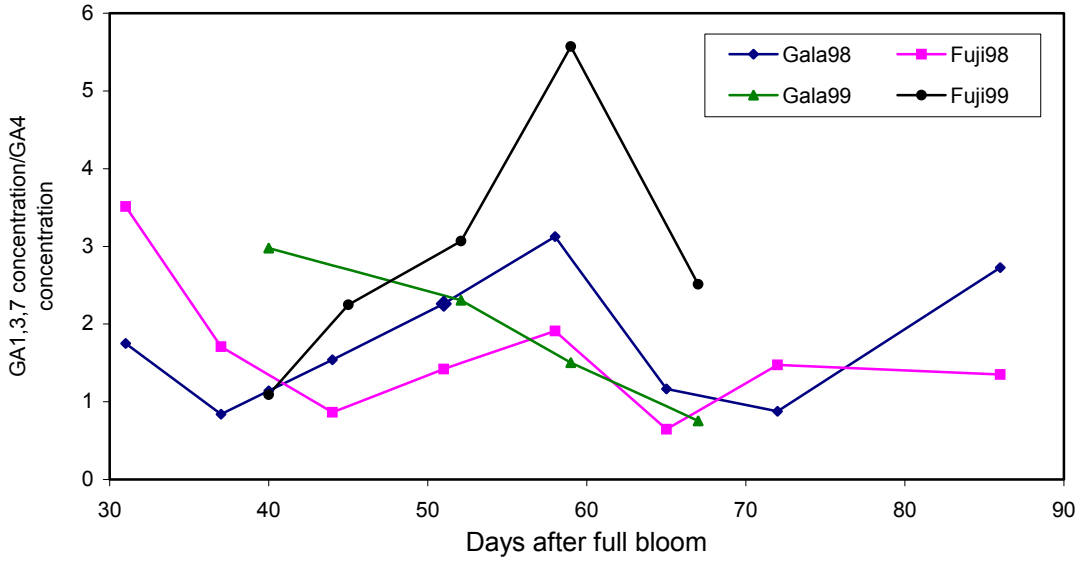


Fig 17. Concentration ratio of GA1,3,7 to GA4 in 'Fuji' and 'Gala' seeds in 1998 and 1999

GAs in seeds (Dennis and Nitsch, 1966) and fruit exudates (Stephan *et al*., 1999). Therefore, it is unlikely that the low return bloom of 'Fuji' in 1999 was caused by higher concentrations of flower-inhibiting GAs altogether. The data of 1999 also demonstrated that total amount of inhibitory GAs makes little sense in interpretation of GA regulation of apple flowering. Instead of similar trends of GA concentration change between 2 cultivars, we found that the total concentration of $GA_1$, $GA_3$, and $GA_7$ is much higher in

'Fuji' than in 'Gala' at 59DAFB and 67DAFB. But the return bloom of both cultivars in the next year was the same.

Previous discussion around Fig 15 did not favor the existence of any relationship between $GA_7/GA_4$ ratio and flower induction. Naturally, another question we asked was whether the ratio of total concentration of $GA_1$, $GA_3$, and $GA_7$ to that of $GA_4$ is useful in explanation of GA regulation of apple flowering. Such ratios for both cultivars at different sampling time are illustrated in Fig 17. In 1998, the ratio of $[GA_1] + [GA_3] + [GA_7]$ to $[GA_4]$ (hereinafter called 'the ratio') for both 'Fuji' and 'Gala' experienced fluctuations within the range from 0.8 to 3.5. For most of the flower induction period in 1998, the ratio was slightly higher in 'Gala' than in 'Fuji' and it was lower in 'Gala' for the first 2 sampling dates. Like $GA_7/GA_4$, we do not observe an overall difference between the curves for 2 cultivars in Fig 17. The only point that is worth further consideration is that 'Fuji' had a higher ratio of inhibitory GA concentration to $GA_4$ concentration at the early part of 1998 growing season in both figures. However, whether the effects of the ratio at 31DAFB lasted long enough to alter the process of flower induction for the whole summer is questionable. For the ratio in 1999, the shape of the curves for 2 cultivars are totally different: the readings for 'Gala' kept decreasing while those for 'Fuji' first increase and then drop down. The highest point reached 5.7 for 'Fuji' at 59DAFB. Although this peak was produced by an unusual high reading of $GA_1$, the overall trend observed in the 1999 data strongly suggests that flower induction is under the control of factor(s) other than the ratio of inhibitory GA concentration to $GA_4$ concentration. The high activity of inhibitory GAs in 'Fuji' in 1999 was not reflected by its return bloom in 2000.

Content of $GA_4$ and $GA_7$. From $GA_4$ and $GA_7$ concentrations in the seeds, and seed dry weight per fruit, the total $GA_4$ and $GA_7$ content per fruit could be calculated (Fig 18). The trends were almost identical to those found for seed GA concentrations. For the content data of $GA_4$, a significant difference was again found between 'Fuji' and "Gala' in 1998 by 2-factor (cultivar and sampling date) ANOVA test and the interaction between cultivar and sampling date was negligible. In 1999, the fluctuations of $GA_4$ content in 'Fuji' synchronize with those in 'Gala' with almost the same amplitude till 67DAFB

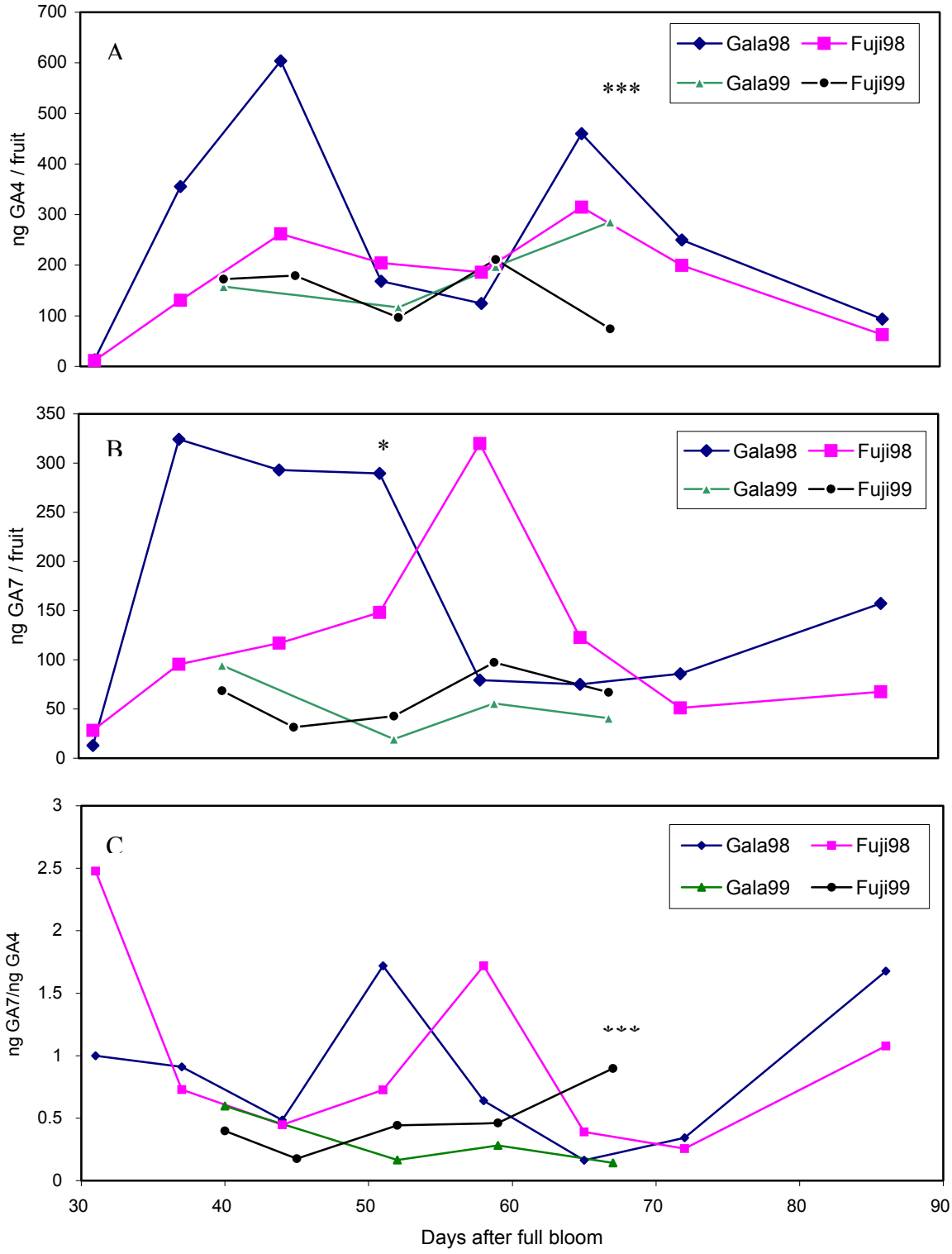


Fig 18. Content of $GA_4$ (A) and $GA_7$ (B), as well as ratio of $GA_7$ content to $GA_4$ content (C) in 'Fuji' and 'Gala' seeds in 1998 and 1999

*, **, *** Significant at $P \leq 0.05$, 0.01, 0.001, repectively.

when ‘Gala’ evidently had higher content of $GA_4$ ($P = 0.0005$). No statistical difference was demonstrated by ANOVA between the $GA_7$ content in ‘Fuji’ and ‘Gala’ in 1998. However, the $GA_7$ content in ‘Gala’ was constantly higher than in ‘Fuji’ from 30 to 51DAFB even though *t*-test showed difference at 0.01 level only for readings at 51DAFB. The content ratios of $GA_7$ to $GA_4$ were also calculated (Fig 18C). It was very similar to the seed concentration data in Fig 15 thus no more information can be derived from it. The reason why the results were not altered when GA concentrations were translated into GA contents is easy to understand. From our records, the dry weight of seeds per fruit did not change dramatically (data not shown) or at least it didn’t change as quickly as the concentration of GAs over the sampling time. Most importantly, the gain of dry weight of seeds per fruit occurred at the same speed in ‘Fuji’ and ‘Gala’. Therefore, the shape of GA concentration – sampling time curve remained approximately unchanged and the comparison between 2 cultivars led to the same conclusions when GA concentration data was converted into GA content per fruit.

One point that needs to be emphasized is that both GA concentration and GA content used here are parameters focusing on the ‘density’ of GAs in certain area of the tree. These are superior to other parameters such as total amount of GAs (partial or whole tree) in the studies of hormonal regulation of apple flowering because flower density has been used to describe flower induction. If we use number of flowers and total GA amount in a branch or a tree, the effects of the tree/branch size can not be ignored unless we are sure that the size of trees/branches we chose are uniform among treatments and replications. The utilization of flower density and GA concentration/content can easily eliminate the variance brought by such difference in tree/branch size and other factors that may affect tree/branch size.

Comparison of our data to others’ work. Using bioassays, Ebert and Bangerth (1981) and Luckwill *et al*. (1969) found the highest GA activity occurred at 8-10 weeks after full bloom for various apple cultivars. On the contrary, our data show the concentrations of the GAs discussed above in 1998 were the highest between 40 to 50DAFB that corresponds to 6-7 weeks after full bloom. The 1999 data did not show a clear trend. But the limited sampling time in 1999 implies the existence of missed GA

peaks early in the growing season. Such difference between our data and previous work can be the result of 2 factors: first, the cultivars involved in these experiments are all different from ours. Ebert and Bangerth used 'Golden Delicious' and 'King of the Pippins' while 'Cox's Orange Pippin', 'Emneth Early' and 'Laxton's Superb' were the materials in the study by Luckwill *et al.* Secondly, the GA quantification data obtained by bioassay and GC-MS are incomparable. What bioassay measured was an approximation of total GA-like materials (in term of equivalent $GA_3$ / g seeds). The accuracy of measurements is highly dependent on the sensitivity of specific assays to particular GAs. These shortcomings of bioassays can be overcome by GC-MS that shows much higher specificity and sensitivity. Therefore, we tend to keep confidence on the GC-MS data and regard bioassay data as an auxiliary source of information.

The GA concentrations obtained in our experiments are about 2 orders of magnitude higher than the data of Stephan *et al*. (1997) and Steffens *et al*. (1992, 1991). The GA concentrations in both studies were generally under 300ng/g dry weight but we encountered many readings over 3000ng/g dry weight seed. Upon scrutinizing all these data, we found that the major discrepancy between our results and those of Steffens *et al*. comes from the time the seeds are sampled. Steffens collected seed samples only at 75DAFB and at this date the GA concentrations we measured are on the same level as their data. Both studies mentioned above only measued GA concentrations at one single date of the growing seacon. To our knowledge, no experiment about GA analysis at different times in a year has been reported. Other factors such as cultivars, GA analysis protocol and internal standards may also affect the quantification of GAs. The samples used in Stephan *et al*. 's experiments were collected 7 weeks after full bloom when our sample trees were shown to produce large amounts of GA. However, they used a different set of internal standards (synthesized in Bangerth's lab) and analysis methods (LC-MS instead of GC-SIM). It is possible that these different experimental setups can give rise to some dissimilarity of data but it is unlikely to bring such dramatic changes in results. How internal standards and MS analysis methods impact the output of GA quantification is beyond my scrutiny. But there is one thing that is very difficult to explain in the data of Stephan *et al.* : more $GA_3$ and $GA_{20}$ were found in the exudates of

'Elstar' than in its seeds. This could either be the result of GAs produced in tissues other than seeds or an unreliable GA analysis protocol.

Summary of quantitative analysis. Based on the knowledge that GAs can be either inhibitory or promotive to apple flower induction, we assume apple flowering can be regulated by either 1) the presence and activity of inhibitory GAs only, or 2) the presence and activity of promotive GAs only, or 3) the relative content of inhibitory to promotive GAs. Our data tend to support the second case.

The concentration of $GA_1$ and $GA_3$ were found to have little correlation with the return bloom of either 'Fuji' or 'Gala'. In 1998, we did not observe higher $GA_1$ or $GA_3$ concentration in 'Fuji'. On the contrary, the $GA_3$ concentration was even higher in 'Gala' at most of the times tested. The 1999 data for $GA_1$ and $GA_3$ concentration was ambiguous due to the high level of variance. The higher $GA_7$ concentration in 'Gala' in 1998 is another piece of strong evidence rejecting the first possibility stated above. It is not surprising that the sum of $GA_1$, $GA_3$, and $GA_7$ concentrations was not related to the flowering data considering the presence of none of the 3 inhibitory GAs can be related to the return bloom.

'Gala' continuously produced higher concentrations of $GA_4$ than 'Fuji' in 1998 (Fig 14). Comparison of the $GA_4$ data was the only occasion that an overall difference between 2 cultivars over the whole growing season was identified statistically. This leads to our conclusion that the second case stated above could be correct. If this is true, the effects of endogenous and exogenous $GA_4$ on apple flower induction are not quite the same. It seems from our data that endogenous $GA_4$ itself has deterministic impact on the flowering process while it cannot mask the negative effects of $GA_7$ when applied exogenously to the trees (McLaughlin and Greene, 1984).

Neither the concentration ratio of $GA_7$ to $GA_4$ nor that of total inhibitory GAs to $GA_4$ was found to be indicative to the endogenous GA status that controls the flower induction of 'Fuji' and 'Gala'. If we recognized that the concentration of inhibitory GAs has no control at all over flower induction, the fact that the relative concentration of inhibitory GA(s) to $GA_4$ is not informative would be understandable. Again, the

exclusion of the possible effects of concentration ratio of inhibitory GAs or $GA_7$ to $GA_4$ may have on apple flowering points to the dominant role of $GA_4$ in this issue.

The GA concentration data were also interpreted in the form of GA content per fruit. It turned out that GA content was as informative as GA concentration in the study of gibberellic effects on apple flowering. Since the change of seed dry weight over the growing season was relatively small as compared with the fluctuations of GA concentration, the overall trends of GA concentration was maintained during the conversion from concentration to content.

# CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

A larger number of specific GAs (16 vs. 12) and novel GA-like substances (6 vs. 2) were identified by GC-MS in 'Fuji' than in 'Gala'. This clearly demonstrated that in its 'on-year' (1998), 'Fuji' undergoes more active GA biosynthesis and /or metabolism than 'Gala'. To elucidate the effects of total number of GAs on flowering habit, qualitative analysis of GAs in the 'off-year' should be included in further research.

In 1998, quantitatively more GA was found during the early part of the growing season for both 'Gala' and 'Fuji'. This is in contrast to the bioassay data reported by both Luckwill & Weaver (1969), and Ebert and Bangerth (1981), which showed a GA biological activity peak at about 9 weeks after full bloom. Bioassay data show the amount of GA-like response to substances in a extract and are variable for different GAs and different bioassays. This lack of specificity renders bioassays inferior to GC-MS and they are not recommended for GA quantification. The concentrations of GAs identified in this study are comparable to those reported by Stephens *et al*. (1991, 1992) using GC-MS. However, they only sampled apple fruits at very late stages of the growing season (10 and 14 weeks after bloom).

Whether or not there are early GA peaks in 1999 is not known. It is highly likely that GA peaks appeared before our first sample date in 1999 and therefore was not detected. In future research, sampling of fruits should be concentrated on the time period between 20 and 50 DAFB when GA activity was found to be maximal and flower induction was at a critical stage. To avoid the situation of having no fruits to collect during 'off-year', bigger or more trees are suggested. Another problem we encountered was that our GA quantification data showed a high level of variance due to limited number of replications (3). This was because we had to pool samples so that each replication contained enough seeds for GA analysis. This problem could also be solved by collecting more fruits from bigger trees.

As expected, the activity of all 5 GAs quantified was similar between cultivars in 1999 when flower initiation of both 'Fuji' and 'Gala' was found to be on the same level. For $GA_1$ and $GA_3$, similar trends were shown in 1998: no significant difference was

found between ‘Gala’ and ‘Fuji’. Therefore, the biennial bearing behavior of ‘Fuji’ cannot be explained by the activity of $GA_1$ or $GA_3$.

‘Gala’ produced more $GA_4$ and $GA_7$ than ‘Fuji’ in 1998. It was surprising to find a higher concentration of GA7 in ‘Gala’ since $GA_7$ inhibits apple flowering. This leads to our conclusion that $GA_4$ can be used by regular bearing cultivars to protect their buds from the negative effects of other inhibitory GAs during flower induction. Among all the GAs, $GA_4$ may play a more important controlling role in regulation of apple flowering.

The quantification of endogenous GAs in this study only showed one aspect of the entire puzzle. More cultivars and GAs (such as $GA_{88}$) should be taken into account to make sure our conclusions can be extended to other cultivars and GAs. The study of GAs exported from apple fruits seems to be a promising avenue in the research of apple flowering in association with biennial bearing (Stephan *et al*., 1999). Future research should focus on the selected transportation of GAs by various cultivars and the GA transportation over time. Furthermore, efforts should be made to improve this method by looking for a way to better simulate the natural export of GAs through pedicels to the buds.

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