Influence of Branch Bending under Joint Tree Training System on Endogenous Levels of Phytohormones and Flowering in Japanese Plum

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Influence of Branch Bending under Joint Tree Training System on Endogenous Levels of Phytohormones and Flowering in Japanese Plum

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Table of Contents

Abbreviations list

ABA	=	abscisic acid
BEH	=	Ethylene Bridged Hybrid
BR	=	brassinosteroids
cDNA	=	complementary deoxyribonucleic acid
CK	=	cytokinin
Ct	=	threshold cycle
C_{18}	=	carbon 18
DMRT	=	Duncan ’s multiple range test
E	=	efficiency of amplification
eV	=	electron volt
FAA	=	formaldehyde, acetic acid, and ethanol, in v/v
FW	=	fresh weight
FT	=	*FLOWERING LOCUS T*
g	=	gravity
GA	=	gibberellic acid
GA2ox	=	gibberellin 2-oxidase
GA3ox	=	gibberellin 3-oxidase
GA20ox	=	gibberellin 20-oxidase
HCOOH	=	formic acid
IAA	=	indoleacetic acid
$L\ h^{-1}$	=	liter per hour
M	=	Molar
MeOH	=	methanol

min = minute

MRM = multiple reaction monitoring

OsGA20ox = *Oryza sativa gibberellins 20-oxidase*

PCR = polymerase chain reaction

PslAct = *Prunus salicina actin*

PslGA2ox = *Prunus salicina gibberellin 2-oxidase*

PslGA3ox = *Prunus salicina gibberellin 3-oxidase*

PTFE = polytetrafluoroethylene

qRT-PCR = quantitative real-time polymerase chain reaction

RNA = ribonucleic acid

s = second

SE = standard error

SIR = selected-ion reaction

SRM = selection reaction monitoring

t-butyl = *tert*-butyl

UPLC-ESI-MS/MS = ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry

v/v/v = volume per volume per volume

List of tables

List of figures

Chapter 1 : General introduction

Chapter 2 : The effect of joint tree training system on endogenous phytohormone levels and flowering

Chapter 3 : The changes of endogenous phytohormone, bud development and gene expression levels in flower induction period

Chapter 4 : The effect of different position among three-tree joined on endogenous phytohormone, gene expression levels and flowering

Chapter 5 : General discussion and conclusion

Chapter 1

General introduction

Flowering indicates the transition from vegetative to reproductive growth, which is an important phase change in angiosperm. In the transition, a combination of endogenous and environment cues induces floral development (Simpson and Dean 2002). Flower induction is defined as the morphogenetic transition of stem cells in apical- and lateral-central meristems into differentiated floral buds (Bangerth, 2009). In cultivated fruit trees, flower induction is a vital determinant of success in commercial orchards because of its influence on the quantity and quality of fruits (Buban and Faust, 1982; Link, 2000). Horticultural procedures including branch bending are applied to promote flower induction. Branch or shoot bending has long been established and widely used as a horticultural intervention to enhance flowering and increase fruit in high-density pear and apple orchards (Lin et al., 1990; Goldchmidt-Reischel, 1997). However, it varies with genotype and time of bending (Lauri and Lespinasse, 2001), as well as with the angle of bending and duration of bending time (Colaric et al., 2007). In Japan, joint tree training system was established to achieve a high production in high density orchards. However, the changes of phytohormones and flower bud development under joint tree training system have not been clarified.

Flowering in fruit trees

Flowering in fruit trees comprises physiological and morphological stages controlled by internal factors (i.e. genotype and endogenous hormones) and external or environmental factors (Hanke et al. 2007). Phytohormone signaling regulate suites of morphogenic processes, influence on the transition

to reproductive phase. The effects of phytohormone appear positively or negatively in plant depending on the type of phytohormones and growth condition (Davis 2009). Environmental factors exert inductive effects by the changes in phytohormone levels; therefore, flowering is usually associated with hormonal changes (Campos and Kerbauy 2004). Wilkie et al. (2008) showed that flower initiation in tropical fruit trees such as mango responses to an environmental stimulus, whereas in temperate fruit tree flowers are initiated autonomously. The overall mechanism remains unclear, but there is strong evidence suggests that phytohormones play a central role in flower induction and differentiation (Jiang et al. 2010).

Phytohormone signals and regulation of flowering

Phytohormones are classified into gibberellic acids (GAs), auxin, cytokinins (CKs), ethylene, abscisic acid (ABA), brassinosteroids (BR), jasmonates and salicylic acid (Bajguz and Tretyn 2003). The effect of phytohormones on plant development and the regulation of flowering varied between plants, in which inducing flowering in one while having the opposite effect in another species (Galvao and Schmid 2014).

For GAs, more than a hundred GAs were indentified in plant. Only small of them are thought to function as bioactive form including GA_1, GA_3, GA_4 and GA_7. In the GA metabolism pathway, GA_{12} is converted to a bioactive GA4 through oxidations on C-20 and C-3 by GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox), respectively. GA_{12} is also a substrate for GA13-oxidase (GA13ox) for the production of GA_{53}, which is a precursor for GA_1 and GA_3 in the

13-hydroxylated pathway (Yamaguchi, 2008) (Figure 1.1). Endogenous GAs influence a wide variety of development process. In reproductive development, GA can affect the transition from the juvenile to the mature stage, as well as floral initiation, sex determination, and fruit set (Taiz and Zeiger, 2002). The field of flowering time in model plant (*Arabidopsis*) has been organized to four pathways, with the vernalization and photoperiod pathways mediating the response to environmental cues and autonomous and the GA pathways acting largely independently of these external signals (Mouradov et al. 2002; Dornelas et al. 2007). Taiz and Zeiger (2002) also indicated that, flowering is influenced by environmental factors, such as photoperiod and nutritional status, and these environmental effects may be mediated by GA. GAs affects to promote flowering in long-day and biennial plants (Gocal et al. 2001), whereas in other plants including fruit trees, they inhibit it (Goldberg-Moeller et al. 2013).

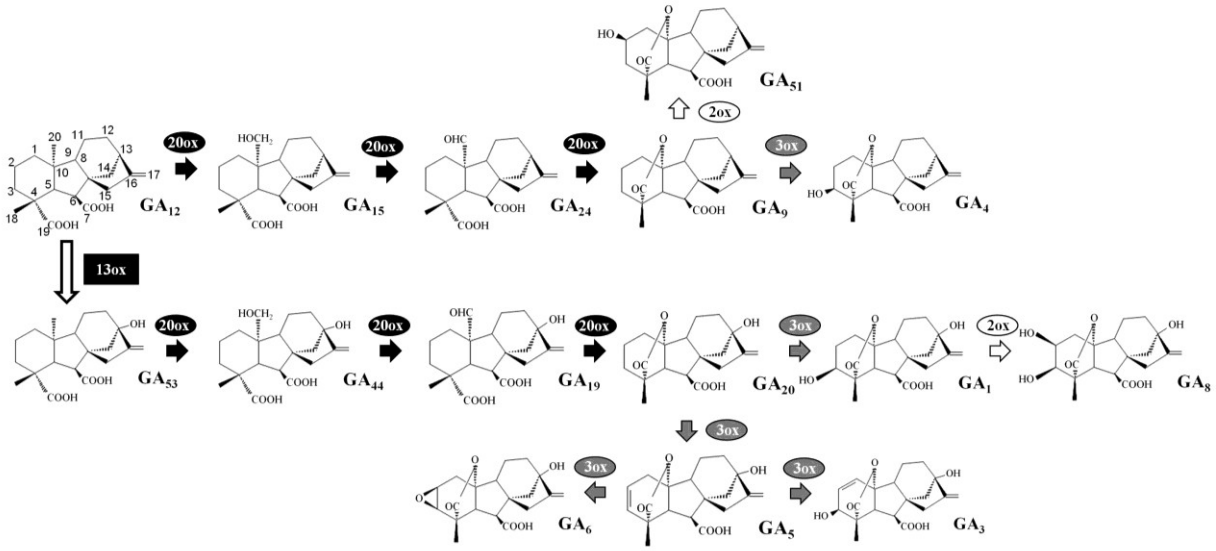


Figure 1.1 GA biosynthetic and catabolic pathway in plant, modified from Yamaguchi (2008)

Auxin plays an important role in basically all aspects of plant development. IAA has been reported to induce cell elongation in stem and leaves, and increase photosynthesis activities in plants. In lentil, IAA showed beneficial effect on flower retention and subsequently on yield (Khalil et al. 2006). In the regulation of flowering, auxin signaling component regulated flower by interaction with GA. It was shown that auxin delay flowering by reduced expression of GA biosynthesis gene, which would result in a reduction of bioactive GA in *Arabidopsis* (Mai et al. 2011; Galvao and Schmid 2014).

CKs comprise a class of plant phytohormones obtained from adenine that were first indentified as factors controlling cell proliferation (Galvao and Schmid 2014). In addition to the control of cell division, CKs are involved in other events during plant life cycle, for instance photomorphogenesis and regulation of organ growth (Kieber 2002). Similar to GA, CKs signaling seems to regulate flowering both in leaves and at the shoot apical meristem through the homologue genes of *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) (Bonhomme et al. 2000). Cytokinins have been shown to participate in the regulation of numerous aspects of plant development including initiation of buds, flowering, abscission and yield by enhancing the cell expansion (Morris et al. 1990).

Ethylene plays an important role throughout the entire plant life cycle, from germination and photomorphogenesis to senescence and fruit ripening (Galvao and Schmid 2014). Ethylene has either a positive or negative effect on flowering, depending on the species analyzed. For example, in mango and

Arabidopsis, ethylene has been shown to repress flowering, while it induces flowering in pineapple (Taiz and Zeiger 2002; Wuriyanghan et al. 2009). Moreover, ethylene signaling might act to decreased active GA_1 and GA_4 while increased their precursor in GA biosynthesis (Achard et al. 2007).

ABA is a phytohormone that plays a prominent role in adaptation to environmental stress in plant. ABA has been reported to play a role in flower induction and differentiation (Liu et al. 2007), while the regulation of ABA biosynthesis is responsive to many and varied environment or development cues (Osborne and McManus 2005). ABA acts as a repressor of flowering in *Arabidopsis* (Domagalska et al. 2010), while it has been reported that ABA had a positive effect on flowering in long-day plant (Riboni et al. 2013). Besides, ABA acts as a transcriptional repressor of some GA-regulated genes (Hoecker et al. 1995).

Changes in endogenous phytohormone levels influence flower induction (Martinez-Fuentes et al. 2004). A low concentration of auxin and GA were observed during flower induction in *Chtysanthemnm morifolium* (Jiang at al. 2010). Goldberg-Moeller et al. (2013) found that spraying GAs during flower induction reduces the percentage of flower buds formed in citrus. In chrysanthemum, a reduction in indoleacetic acid (IAA) content occurs during cessation of shoot tip growth after flower induction, leading to flower differentiation (Jiang at al. 2012). In general, GAs inhibit flower induction, which is consistent with the reported large decrease in GA_3 level during the flower induction stage (Koshita et al. 1999; Jiang at al. 2010). Documented cases show that GAs may directly affect molecular events in the apical bud to determine

whether the shoot apices are programmed for vegetative or reproductive growth (Hisamatsu and King 2008).

Among the main five classical phytohormones, it was found that the signaling of Auxin, ethylene, and ABA are tightly interconnected with GAs. Therefore, it is possible that other phytohormones control the flowering indirectly by modulating GA biosynthesis and signaling (Galvao and Schmid 2014).

Branch bending and joint tree training system

Exogenous or environmental factors, such as temperature and photoperiod, and stress conditions also influence flower induction (Valiente and Albrigo 2004). Branch or shoot bending is a type of mechanical stress commonly used by commercial growers to increase flower bud number. In Japanese pear, IAA and GA were decreased while CK and ABA were increased by bending (Ito et al. 2001). Recently, the 'joint tree training system' was introduced in Japan for Japanese pear to replace conventional training systems that suffered from reduced tree vigor and dwarfing with increasing tree age (Seki et al. 2008; Shibata et al. 2008). The joint tree training system is achieved by grafting the apical leader of scaffold branches of one tree onto the bent portion of the main stem of a neighboring tree in a row of three or more trees. The joint tree training system can achieve a high yield more precociously than that attained under a conventional training system in Japanese pear (Shibata et al. 2008). Recently, this system has been applied to the other fruit trees such as apple, Japanese plum, peach etc. In 'Kiyo' Japanese plum, this system was utilized to replace the traditional training system that trees are old causing to decreased fruit

production. It was found that under joint tree training system not only enhanced fruit production in early year but yield per area was higher than traditional training system, moreover, this system can reduce the cost of labor because the time for manage such as pruning, pollination, fruit thinning and fruit covering less than the individual bending trees (Hirai et al. 2013). Now, this system is in interesting for farmer in Japan.

Figure 1.2 The joint tree training system that has been done in apple (A), Japanese pear (B), and Japanese plum (C).

Japanese plum

Oriental plum or Japanese plum (*Prunus salicina*) were first discovered in China but largely cultivated in Japan (Westwood 1978). In 2012, growing area is about 3,150 ha in Japan, main production area is Yamanashi, Nagano, Wakayama, and Yamagata prefectures which about 934, 411, 303, and 245 ha, respectively (MAFF 2014). ‘Kiyo’ and ‘Taiyo’ are 2 main cultivars that have good taste and big fruits (Hirai et al. 2013). ‘Kiyo’ Japanese plum (*Prunus salicina* Lindl.) is a triploid (2n=3x=24) cultivar that bears large fruit (≅ 200 g) (Obayashi et al. 2009). Plum flowers initiate in lateral buds of both current season shoots and new growth on older spurs, initiation occurs mostly in late summer. One to three flowers without leaves are produced in each flower bud (Westwood 1978) (Figure 1.3). In recent years, ‘Kiyo’ Japanese plum has become increasingly popular on account of the large fruit and good fruit quality (total soluble solids of ≥15° Brix). However, the production of this cultivar is hampered by its inherently low fruit-set rate being a triploid non fertile cultivar. Jun et al. (2010) reported a low fruit set in ‘Kiyo’ of only 2.5% and 7.0% after pollination with the diploid plum ‘Hollywood’ and ‘Simka’, respectively. Further, the incidence of fruit drop is very high at 95.5% within 56 days after full bloom linked to poor pollen germination (Obayashi et al., 2009).

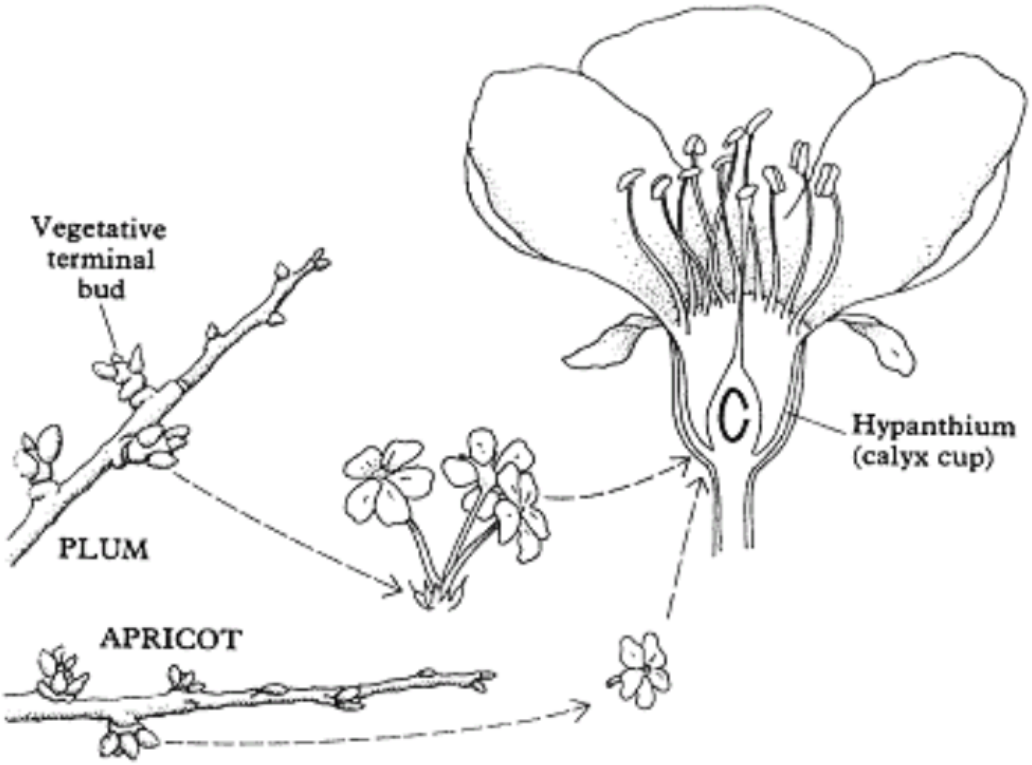


Figure 1.3 Plum flowering habits: lateral unmixed flower buds on two-year and older spurs; the inflorescence is a few-flowered fascicle. Apricot: fruit buds on one-year and older wood; flowers are solitary. Both species have perigynous flowers with a single pistil (Westwood 1978).

Objectives of this study

According to reviews, shoot bending and the joint tree training system are predicted to improve flower induction and result in increase fruit production in ‘Kiyo’ Japanese plum. Nevertheless, little is known on the changes in phytohormone levels and flower bud differentiation of ‘Kiyo’ plum, especially under a joint tree training system with shoot bending.

So, the objective of this study was to examine the effects of joint tree training system and bending on flowering, shoot growth, endogenous phytohormone levels and the expression of phytohormone related genes. We next characterized the dynamic changes of phytohormones, bud development and gene expression levels on flower induction period under joint tree training system.

Chapter 2

The effect of joint tree training system on endogenous phytohormone levels and flowering

2.1 Introduction

The joint tree training system was first introduced in Japan, this system is accompany between branch bending and three or more trees joined together. The advantage of these system is not only for saving labor cost but also for establishment of orchard in short duration with achieving a high yield in the early year and uniformity of fruiting distribution and fruit quality (Shibata et al. 2008). However, the changes of phytohormones level and flower induction had been studied only in bending effect. Ito et al. (1999) reported that shoots bending decreased IAA and GA_{4+7} but increased ABA and CK contents in lateral buds, resulting in enhanced flower induction, possible that GAs and IAA inhibit flowering, while CK and ABA promote in Japanese pear. There are several studies about the effect of GAs on flowering, it was found that GAs promote flowering in annual and long-day plants (Christiaens et al. 2012; Sumanasiri et al. 2013; Zhang et al. 2013), however GAs are considered as a flowering inhibitor in fruit trees such as apples, plums and loquat (Cao et al. 2001; Gonzalez-Rossia et al. 2006; Reig et al. 2011). In the view of this, GAs are very interesting phytohormone for investigation the effect of bending and joint tree training system on flowering. However under the joint tree training system which is a new technique of training system in Japan, the changes of endogenous phytohormone has not been clarified.

In this study, simultaneous analysis of phytohormones was accomplished by ultra-performance liquid chromatography-electrospray ionization-tamdem mass spectrometry (UPLC-ESI-MS/MS). This method was used for its high sensitivity, resolution and speed. No chemical derivatization of

analytes is required and interference from co-extracts is greatly reduced by using the selected-ion reaction (SIR). Hou et al. (2008) and Susawaengsup et al. (2011) also used UPLC-ESI-MS/MS in phytohormone analysis for these reasons.

'Kiyo' Japanese plum was used as the model, in which trees are suffered from low fruit set because of the clone's sterility linked with poor pollen germination (Jun et al. 2010). We hereby present evidence that the joint tree training system could improve the quantity and quality of flower induction in 'Kiyo' plum. Besides, the relationship between phytohormone levels and shoot growth and flowering would be verified. So, the objective of this chapter is to examine the effect of joint tree training system on flowering, shoot growth and endogenous phytohormone levels.

2.2 Materials and methods

2.2.1 Plant materials

Japanese plum 'Kiyo' (*Prunus salicina* Lindl.) were planted in pots (15 L) and maintained in the orchard of the Agricultural and Forestry Research Center of the University of Tsukuba, Japan. The main trunks were bent and joined in 2008, and allowed to establish for next 5 years. In 2012 and 2013, the joint group (lateral branch-bent, and main trunk-bent and joined 3 trees together) where characterized in terms of shoot growth, flowering rate and fruit setting rate vis-a-vis bent group (main trunk and lateral branch were bent, but not joint), and control group (upright trees, lateral branch not bent) (Figure 2.1). Young leaves of new shoot were collected from these groups in July and August 2012 for

phytohormone analysis.

2.2.2 Phytohormone extraction and analysis

Young leaves from new shoots were snap frozen and pulverized in liquid nitrogen and stored in -80^{o}C until used for analysis. Plant materials (≅200 mg FW) were extracted with 2 mL MeOH/H_2O/HCOOH (15:4:1 v/v/v) using alizarin as internal standard. After dispersion and mixing with a homogenizer (POLYTRON PT-MR2100, Kinematica AG, Littau/Lucerne, Switzerland) for 3 min, the mixture was centrifuged at 20,000 *x g* for 15 min (Suprema 21, TOMY, Tokyo, Japan). The resulting supernatant was then passed through a Sep-Pak plus C_{18} cartridges (Waters, Milford, MA, USA), evaporated and reconstituted with 1 M HCOOH to 5 mL. Phytohormones were extracted using an Oasis MCX column (Waters Corp., Tokyo). The column was washed with 5 mL of 1 M HCOOH followed by 2 mL of 80% MeOH to elute GAs and ABA. The eluent was filtered through a Millex-LG 0.2 μm PTFE filter and the filtrate was analyzed in UPLC-ESI-MS/MS. GA and ABA were separated in a BEH C18 column (1.7 μm, 2.1 mm X 50 mm) using a reverse phase Acquity UPLC, with 0.05% acetic acid in MeOH as solvent A and 0.05% acetic acid in water as solvent B in a linear gradient elution program: 0 min: 10% A; 0.97 min: 30% A; 8.26 min: 60% A; 9.7 min: 100% A, and held for 2.8 min at a flow rate of 0.62 mL min^{-1}. For data collection during UPLC MS/MS analyses, the capillary voltage was 2.2 kV, the source temperature was 120°C, the desolvation temperature was 450°C, the desolvation gas flow was 900 L h^{-1}, and the cone gas flow was 50 L h^{-1}. During each UPLC injection, the mass spectrometer was set to collect data in the SIR

mode using electrospray ionization in negative ion mode. Table 2.1 shows the mass products used and the retention times of the phytohormones analyzed.

2.2.3 Assessment of shoot growth, flowering, and fruit setting

The number of new shoots (long new shoots), and shoot elongation were recorded at the end of the growing seasons in 2012 and 2013. Flowering was assessed by counting the number of flower clusters per tree in 2013. Flowers were pollinated with the pollen of 'Hollywood' diploid plum. Fruit set was recorded on July in 2012 and 2013.

2.2.4 Statistical analysis

The computation and statistic analysis were done on Duncan's multiple range test (DMRT). Significant differences among groups were determined at $p < 0.05$. Data are presented as the mean ± standard error (SE).

2.3 Results

2.3.1 Phytohormone analysis

The levels of endogenous phytohormones hereby presented are from young leaves collected in summer 2012 (July and August). Bioactive (i.e. GA_1, GA_3, and GA_4) and the other GAs (i.e. GA_5, GA_6, GA_8, GA_{19}, and GA_{20}) were analyzed, but GA_5 content in the leaf samples appear to be lower than the detection limit.

In July, leaves from joint trees and bent trees were significantly lower concentration of bioactive GA_1 (0.53 and 0.33 ng/gFW) but higher level of ABA

(4.22 and 4.03 ng/gFW) compared to the control which revealed 1.16 ng/gFW of GA_1 and 3.24 ng/gFW of ABA contents. Bioactive GA_3 tended to show lower in joint trees and bent trees which about 10.86 and 10.91 ng/gFW, respectively. It was found that concentration of GA_4 of joint (14.14 ng/gFW) appeared significantly lower than bent tree (25.40 ng/gFW) and both of them were significantly lower than control (33.48 ng/gFW). Whereas the other GA_8, GA_{19}, and GA_{20} revealed the highest levels in bent-joint, in which GA_{20} was found the significant differences followed from joint trees, bent trees and control, respectively (Figure 2.2 A).

In August, the concentration of GA_3 and GA_4 in joint trees and bent trees were significantly lower than control. In which, GA_3 in joint trees, bent trees and control showed 3.42, 4.66 and 6.94 ng/gFW, and GA_4 showed 0.41, 0.61 and 1.49, respectively. GA_1 was not significant found but joint trees (5.67 ng/gFW) and bent trees (6.06 ng/gFW) tended to showed lower concentration than the control (6.93 na/gFW). The level of ABA in joint trees showed significantly higher than bent trees and the control. For GA_{19}, joint trees showed the highest level, followed by bent trees and the control, respectively. The level of GA_8 in bent trees revealed the highest followed by the control and joint trees, respectively. The other GA_6 and GA_{20} were not significantly found between treatment groups (Figure 2.2 B).

The comparison of the phytohormone contents on samples taken in July and August, the concentration of bioactive GA_1 was increased, while GA_3 and GA_4 were decreased in the August samples. ABA content was not changed. The inactive GA_8, GA_{19} and GA_{20} were increased, but GA_6 was decreased in August.

2.3.2 Flowering and fruit setting

Bending and joint tree training system appear to promote flower induction with the average number of flower clusters per tree two folds higher in joint (88 clusters/tree) and bent (87 clusters/tree) trees relative to the control (42 clusters/tree) (Figure 2.3A).

Average fruit set in 2012 was significantly higher in joint (0.44 fruits/tree) and bent (0.33 fruits/tree) (Figure 2.3B). These rates further increased to 2.67 and 1.00 fruits/tree in joint trees and bent trees, respectively albeit statistically insignificant (Figure 2.3C). Control trees bore no fruit in both seasons.

2.3.3 Number of new shoots and shoot length

The average number of new shoots per tree was not significantly different across treatment groups both in May 2012 and 2013 (Figure 2.4A and B). On the other hand, the average length of shoots in both bent and joint trees are significantly shorter than the control. The shoot lengths of bent trees and joint trees did not significantly differ in 2013, but in the control group shoot length was doubled of those in bent and joint groups (Figure 2.4C and D).

2.4 Discussion

The low level of bioactive GAs such as GA_1, GA_3, and GA_4, with high level of ABA in young leaves from new shoot of joint trees and bent trees in July and August (Figure 2.2 and Table 2.2) is consistent with the study of Wang et al. (2010) who also showed low level of GAs, and high ABA content in apple buds

after shoot bending compared to those from shoots that were not bent.

The decline in GA_3 and GA_4 levels but escalate of GA_1 in all treatment groups in August, Jiang et al. (2010) reported that the fluctuations in endogenous phytohormone levels depending on the stage of flower development suggesting their different roles at specific stages. In Satsuma mandarin, flower bud formation was inhibited by high $GA_{1/3}$ content in October, but the high IAA and ABA content in December increased the percentage of leafless inflorescence and the number of flower buds per node (Koshita et al., 1999). Consistent with our study that content of ABA in joint trees and bent tree were higher than control, may resulting in number of flower and fruit showed higher than control. A low endogenous GA concentration favors flowering over vegetative growth in fruit trees (Bangerth, 2009). Similarly, mechanical stress by shoot bending was reported in which influences to promoted early flower induction in *bougainvilla* (Liu and Chang, 2011), 'Boskoop' apples (Sanyal and Bangerth, 1998), and 'Le-Conte' pear (Sherif, 2012), with the latter also achieving a higher fruit set were indicated.

The vigorous vegetative growth of the control is not surprising due to its aforementioned high concentration of bioactive GAs. In this study, shoot length in joint group and bent group showed significantly lower than control. It related with the concentration of bioactive GAs of those two treatment groups that appeared lower than control. Similar in cotton, the elongation of fiber was low in GA-deficit phenotype (Zhou et al., 2014).

The concentration of GA_{20} showed the highest level compared to the other GAs in July and August. Dubois et al. (2011) reported that GA_{20} also

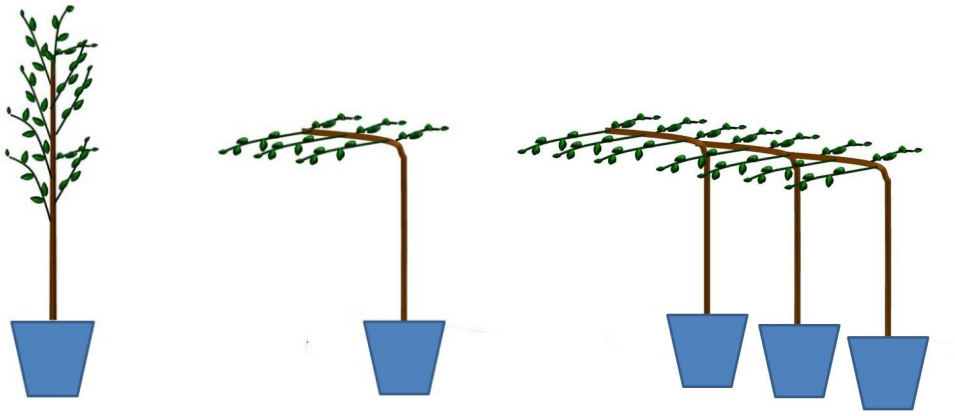
showed the highest concentration in rice compared to GA_1, GA_8, GA_{19}, GA_{29} and GA_{53}. In the report concentration of GA_{20} was about 4-fold higher than GA_{19}. Moreover, they also found that GA_8 and GA_9 tended to have high level in flooding stress condition. In our experiment, concentration of GA_8, GA_{19} and GA_{20} in joint and bent groups (mechanical stress) tended to be higher than that in control. However, GA_8, GA_{19}, and GA_{20} might be related to GA_1 content, since GA_{19} and GA_{20} are the precursors of GA_1, and GA_8 is a product of the deactivation from GA_1 (Yamaguchi 2008). Consequently, gene expression of encoding enzymes GA2ox and GA3ox in the GA biosynthetic pathway will be analyzed in next experiment. And also the differences between joint group and bent group was found in GA_8 and GA_{20} of joint showed higher level in July, but lower than bent trees in August may possible from regulation of GA3ox and GA2ox. Moreover, we found the differences of joint trees and bent trees in bioactive GA_4 in July. However, we could not exactly find the relationship that indicates the differences among joint trees and bent trees except the tendency of number of fruit per tree in joint that revealed higher than bent trees. Consistent with Hirai et al. (2013), who reported that under joint tree training system could produce yield per area higher than under stand-alone trees.

In the term of flowering and fruit set, bent trees and joint trees showed higher than control. Whereas, the average length of new shoots in both bent trees and joint trees showed shorter even the average number of new shoots per tree was not different among treatment group. It should be noted that the trees in the joint group and bent group of the present study both have bent trunks and horizontally bent branches. Rates of flower induction, fruit set, and

shoot elongation between these two groups were practically identical which may imply the possible major influence of bending, or the horizontal growth of branches, rather than joint. Nevertheless, joint tree showed significantly affect different from bent, stand-alone trees on the level of GA_4, GA_8 and GA_{20} that supposing the regulation under joint tree training system revealed some differences compared to stand-alone trees. However, only vertical branches of stand-alone non bent trees were observed in this experiment, the different of angle shoots on the same tree, and development of floral bud were not clarified yet. So, the development of floral bud and the contribution bending and branch growth orientation to flower needs further assessment.

Table 2.1 Mass products and retention times of phytohormones investigated in SIR mode

Endogenous hormone	Molecular weight	Mass product	Retention time (min)
GA_1	348.40	347.0	1.7
GA_3	346.38	345.0	1.6
GA_4	332.40	331.3	5.6
GA_5	330.38	329.0	3.2
GA_6	346.38	345.0	2.0
GA_8	364.40	363.0	1.0
GA_{19}	362.43	361.0	4.3
GA_{20}	332.40	331.0	3.4
ABA	264.00	263.2	2.3



Control (Non bent stand-alone tree)

Bent (Stand-alone bent tree)

Joint (Bent and 3 trees joined)

Treatment	Branches Bent	Main trunk Bent	Trees Joined
Control	X	X	X
Bent	O	O	X
Joint	O	O	O

Figure 2.1 Treatment of the 'Kiyo' plum trees (above). The detail on the differences of bending and jointing management of each treatment groups was described in table (below).

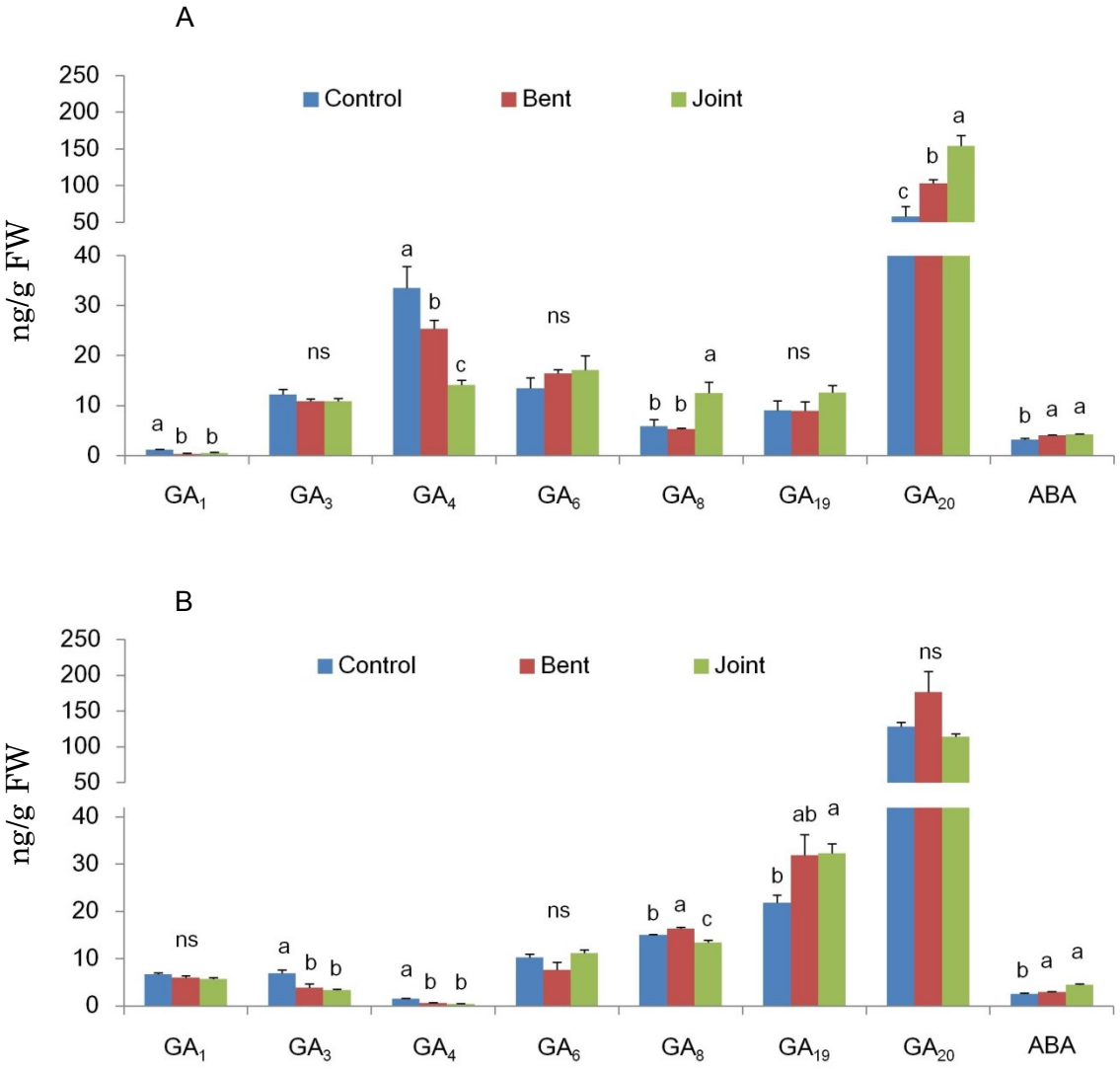


Figure 2.2 Phytohormone concentrations in young leaves of 'Kiyo' plum sampled in July (A) and August (B) 2012.

Vertical bars represent the mean ± SE (n = 3).

Different letters above the bars indicate a significant difference at 5% level (DMRT).

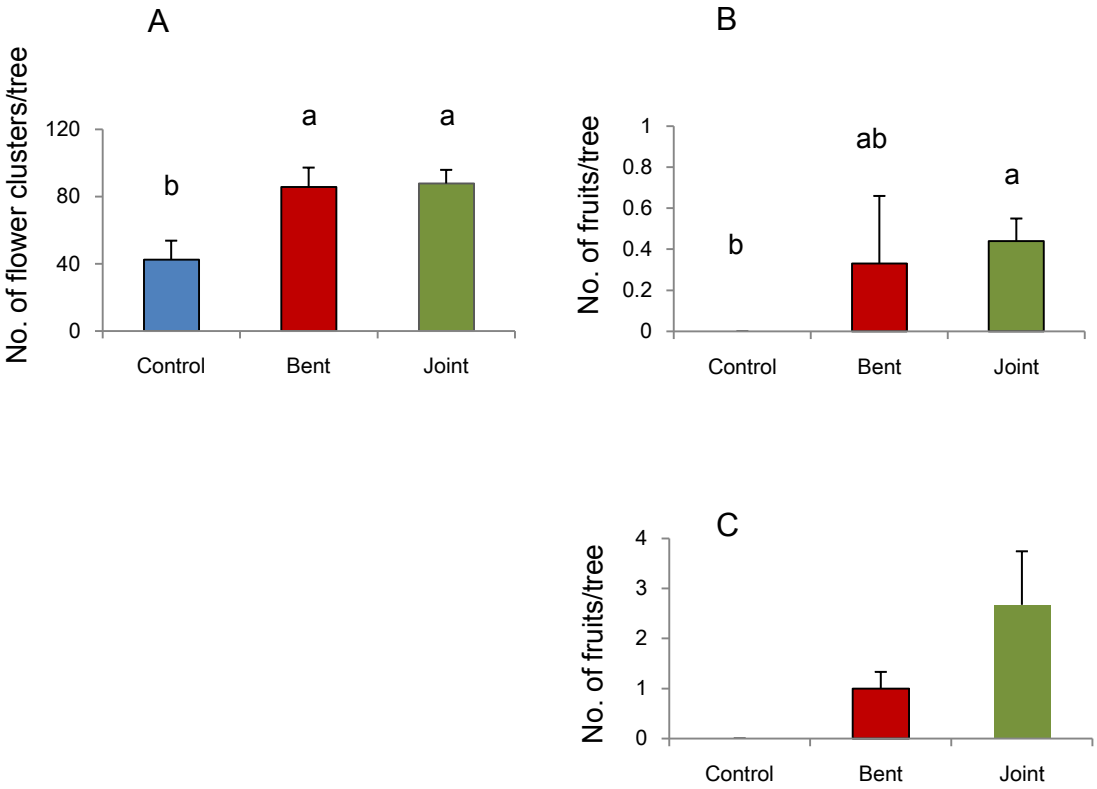


Figure 2.3 Number of flower clusters/tree in 2013 (A), and number of fruits/tree in 2012 (B) and 2013 (C).

Vertical bars represent the mean ± SE (n = 3).

Different letters above the bars indicate a significant difference at 5% level (DMRT).

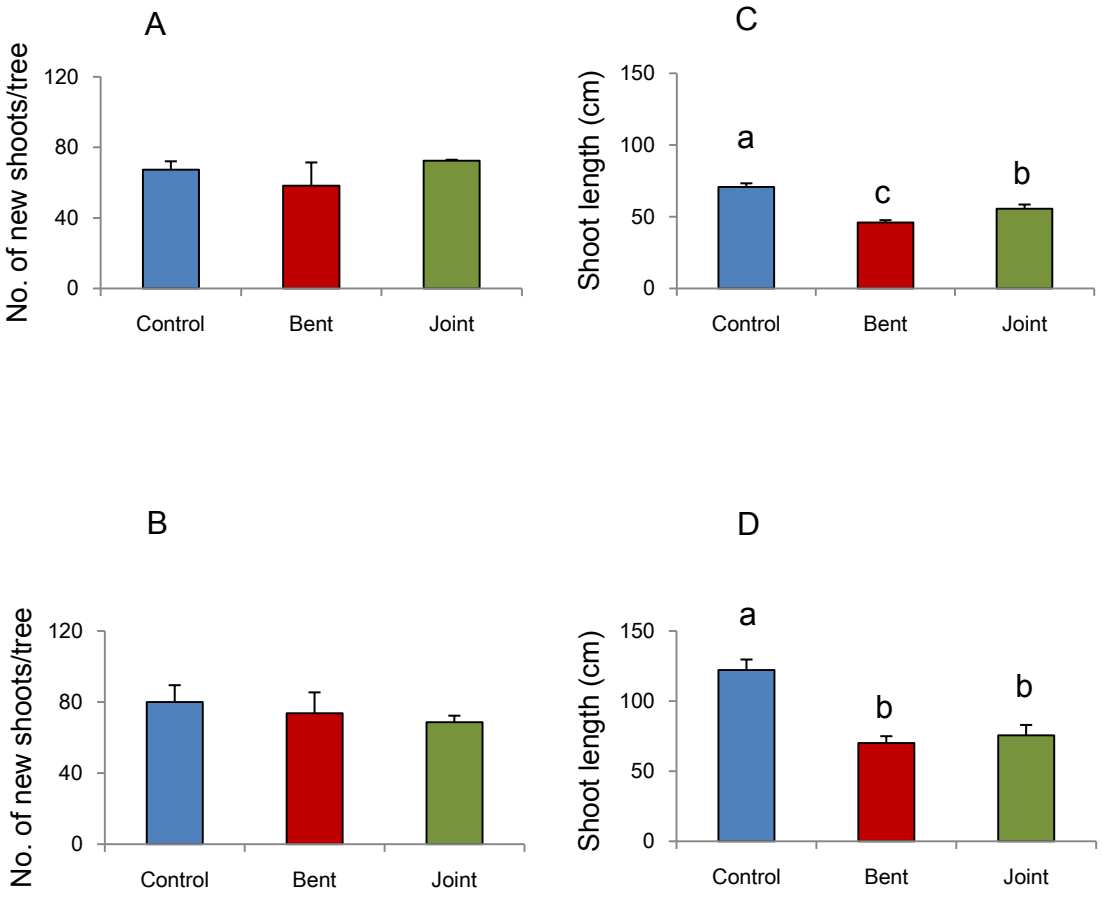


Figure 2.4 Number of new shoots/tree in 2012 (A) and 2013 (B) and average shoot length in 2012 (C) and 2013 (D).

Vertical bars represent the mean ± SE (n = 3).

Different letters above the bars indicate a significant difference at 5% level (DMRT).

Table 2.2 Summary of the results of bent and joint groups compared to control.

			Bent	**Joint**
GAs	GA_1	Jul/Aug	↓ / ↓	↓ / ↓
	GA_3	Jul/Aug	↓ / ↓	↓ / ↓
	GA_4	Jul/Aug	↓ / ↓	↓ / ↓
	GA_8	Jul/Aug	/ ↑	↑ / ↓
	GA_{19}	Jul/Aug	/ ↑	/ ↑
	GA_{20}	Jul/Aug	↑ / ↑	↑ /
ABA		Jul/Aug	↑ /	↑ / ↑
Shoot length		2012/2013	↓ / ↓	↓ / ↓
Flowering		2013	↑	↑
Fruiting		2012/2013	↑ / ↑	↑ / ↑

↑ = significantly higher ↓ = significantly lower

↑ = high tendency ↓ = low tendency

The big arrows indicated more effect.

Chapter 3

The changes of endogenous phytohormone, bud development and gene expression levels in flower induction period

3.1 Introduction

The development of flower buds and sufficient fruit are basic requirements for fruit growers to generate a marketable crop (Hanke et al. 2007). In apple, the seasonal changes of spur buds development illustrate that the flower induction period occurred on late of June with the swelling and broadening of the apex was observed by sectioning. Then, a doming of the apex was appeared on late of July and August which is defined to flower initiation period and after that the flower differentiation was defined (Figure 3.1). In Japanese plum, it has been reported that flower differentiation was found from July in the early cultivars such as 'Santa Rosa'. On the contrary, in many cultivars, it has been reported that it occurs in August (Yoshida, 2000).

The changes of endogenous phytohormones during flowering have been done in *Agapanthus*, it was found that the concentrations of GA_3 and GA_4 were decreased in the inflorescence bud and floral bud differentiation stages in leaf samples (Zhang et al. 2014). Likewise in stem tips, GA_3 and GA_4 was slightly increased in inflorescence bud stage after that active GAs decreased in floral bud differentiation, then increased again in floral bud development stage.

In this chapter, we analyzed abscisic acid (ABA). ABA is exhibit in all organs of higher plants (Preece and Read 2005). ABA biosynthesis apparently occurs in roots, stems, leaves, fruits, and seeds which is subject to complex regulation during plant development and in response to environmental stresses (Preece and Read 2005; Xiong and Zhu 2003). In apple, ABA seems to play no direct role in flowering, but it may participate in flower induction by antagonizing

GA (Zhang et al. 2015).

Moreover, we also analyzed the expression of *FLOWERING LOCUS T* (*FT*). *FT* serves as a florigen that regulates flowering in a variety of plant species (Galvao and Schmid 2014).The signaling of GA affects *FT* through DELLA. Since, DELLA proteins are important negative regulators of GA signaling (Galvao and Schmid 2014).

However, the seasonal changes of bud development and endogenous phytohormones were still unclear in ‘Kiyo’ Japanese plum. So in this study, buds development, endogenous hormone levels and the expression of *GA-oxidases* and *FT* genes were determined.

3.2 Materials and methods

3.2.1 Plant materials

7-year-old trees of Japanese plum ‘Kiyo’ same as the Chapter 2 that were planted in pots and maintained in the orchard of the Agricultural and Forestry Research Center of the University of Tsukuba, Japan was used. To investigate the changes of bud development, endogenous phytohormone levels and gene expression, the shoot tips from new shoots of joint trees and bent trees were used to compare with shoot tips of upright shoots and horizontal shoots of stand-alone non-bent trees (control trees) (Figure 3.2).

3.2.2 Histological observation

Development of buds were observed by collecting bud samples every month from August in 2013 to February in 2014 and May to November in 2014.

The tissues were fixed in FAA (3.7% formaldehyde, 5% acetic acid, and 50% ethanol), vacuum infiltrated twice for 15 min each, and left overnight. The fixed buds were dehydrated with *t*-butyl alcohol in a step-wise manner by serial incubation in increasing concentrations of *t*-butyl alcohol: 10%, 20%, 35%, 55%, 75%, and 100% unified with 40%, 50%, 50%, 45%, and 25% ethanol, respectively. Each step took around 1 day of incubation at room temperature, and the tissues were embedded in paraffin (Paraplast, Sigma-Aldirich, Missouri, USA). The embedded tissues were sliced into 5-10 μm sections. Paraplasts were removed with xylene, and the sections were hydrated, stained with 0.1% toluidine blue in sodium phosphate buffer, pH 7.0, and view under an Olympus BH2-RFCA microscope (Olympus Corporation, Tokyo, Japan). The micrographs were captured using an Olympus DP12 camera.

3.2.3 Phytohormone extraction

Endogenous phytohormones were extracted from shoot tips that sampling in May to September 2014.

Extraction of endogenous phytohormones was performed based on the procedures of Susawaengsup et al. (2011) and Kojima et al. (2009) with some modifications. Plant materials (about 200 mg FW) were soaked in 2 ml MeOH/H_2O/HCOOH (15:4:1, v/v/v), with $^2H_2$-labeled GAs, $^2H_5$-labeled IAA, and $^2H_6$-labeled ABA used as internal standards. After dispersion and mixing with a homogenizer (POLYTRON PT-MR2100, Kinematica AG, Littau/Lucerne, Switzerland) for 3 min, the homogenate was centrifuged (Suprema 21, TOMY, Tokyo, Japan) at 20,000 × *g* for 15 min. The supernatant was passed through a

Sep-Pak plus C18 cartridge (Waters, Milford, MA, USA) to remove plant pigments and lipids. The resulting extracts were evaporated *in vacuo* at 40 °C and reconstituted with 5 mL of 1 M HCOOH. The solvent was applied to an Oasis MCX 60 μm LP extraction cartridge (Waters). After washing with 5 mL of 1 M HCOOH, the solvent was eluted with 5 mL 80% MeOH. The eluent was evaporated *in vacuo* at 40 °C and reconstituted with 1 mL 80% MeOH. The solvent was filtered through a Millex-LG 0.20 μm PTFE filter.

3.2.4 Phytohormone analysis

Analysis of endogenous phytohormone contents in the eluent was performed using an UPLC system (Waters) with a Waters ACQUITY BEH C18 column (1.7 μm, 2.1 mm × 50 mm) at 30 °C. The analytes were eluted from the column with 0.05% acetic acid in MeOH as solvent A and 0.05% acetic acid in 10% MeOH as solvent B in a linear gradient elution program: 0 min: 10% A; 0.97 min: 30% A; 8.26 min: 80% A; 9.71 min: 100% A, and held for 2.8 min at a flow rate of 0.13 mL min^{-1}.

All quantifications were performed in the multiple reaction monitoring (MRM) mode. For MRM data collection during UPLC analyses, the capillary voltage was 2.2 kV, the source temperature was 120°C, the desolvation temperature was 450°C, the desolvation gas flow was 900 L h^{-1}, and the cone gas flow was 50 L h^{-1}. During each analysis, the mass spectrometer was set to collect data in the MRM mode using electrospray ionization in negative ion mode. The MRM transitions for the tested compound are listed in Table 3.1

3.2.5 Quantitative real-time PCR analysis

Shoot tip samples from May to September in 2014 were examined and quantify expression of *PslGA3ox*, *PslGA2ox*, and *PmFT* using quantitative real-time PCR (qRT-PCR). Total RNA was extracted from shoot tips using the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) in accordance with the supplier's instructions. Single-strand cDNA was synthesized from 100 ng total RNA using the SuperScript VILO cDNA Synthesis kit (Invitrogen, Tokyo, Japan). The amplification signal was measured with a Mx3000P™ Real-Time PCR System using SYBR Premix Ex Taq™ II (Takara, Shiga, Japan). Gene-specific forward (5′-CGGCTTGTTCAAGCTGGT-3′) and reverse (5′-GGGCCTGCCTTCTC TTTC-3′) primers for GA2ox [*PslGA2ox* (HM021156)] (El-Sharkawy et al. 2012) and forward (5′-CATGTGGTCTGAGGGCTTCACTAT-3′) and reverse (5′-CAGCCCATTTGACATCTTCCTTGG-3′) primers for GA3ox was designed using the Primer3web version 4.0.0 program (http://primer3.ut.ee/) based on the sequence of *PslGA3ox* (GeneBank: JX569806.1) (El-Sharkawy et al. 2014). Moreover, gene-specific forward (5'-AAGAGATTGTGTGTTATGAAAGCCC-3') and reverse (5'-GGAGCATACACTGTTTGCCTACCC-3') primers for *FT* gene [*PmFT* (FR729040)] (Xing et al. 2014). The thermocycler PCR program used was as follows: incubation at 95 °C for 30 s, followed by 40 cycles of 95°C for 5 s and 60 °C for 34 s. Transcript levels were calibrated against those for actin [*PslAct* (EF585293)] (El-Sharkawy et al. 2012, 2014). The Ct values between the *PslAct* gene and the specific genes and their standard deviation were calculated for each sample.

The quantification of genes expression was estimated with this equation:

$$\frac{\text{Unknown}}{\text{Control}} = \frac{(1 + E_{\text{target}}) - \Delta^{Ct}{}_{\text{target}}}{(1 + E_{\text{norm}}) - \Delta^{Ct}{}_{\text{norm}}}$$

Where *E* corresponded to the efficiency of ampliflication of the target gene (a value from 0 to 1 that represented the number of amplification products generated during each cycle of the reaction per molecule of target sequence, which derived from the standard curve) Ct = threshold cycle and 'control' represented the calibrator sample.

3.2.6 Assessment of shoot growth, flowering, and fruit setting

The number of new shoots, length of shoots, length of internodes, number of flower buds per tree before and after winter pruning, and fruits per tree were recorded in 2014. In 2015, number of flower buds per tree, flower buds per branch and flowering rate were investigated.

3.2.7 Statistical analysis

Each measurement was repeated three times. All computation and statistic analysis were done on Kruskal-Wallis test using GraphPad Prism 5 (GraphPad software, CA, USA). Significant differences among groups were determined using Dunn's test at $p < 0.05$. Data are presented as the mean ± standard error (SE).

3.3 Results

3.3.1 Bud development

In ‘Kiyo’ Japanese plum, flowers are formed on nodes of lateral branches and spur buds. At the leaf axil has three separate buds including one vegetative bud subtended with two flower buds (Figure 3.3). The stages of floral differentiation were distinguished according to Andreini and Bartolini (2008): stage A = undifferentiated meristematic apex; stage B = receptacle primordial arrangement; stage C = sepal primordial; stage D = petal primordial; stage E = stamen primordial (Figure 3.4).

In 2013, we observed the development of buds from August. It was found that the differentiation of receptacle (stage B) appeared in bent tree and joint tree groups. So in 2014 we started earlier by sampling from May. It was found that in May and June, the meristem of apices was broad and swollen or remained on undifferentiated bud (stage A) in all treatments, and sometimes leaf initiation still appeared (Figure 3.6 and 3.7). In July, the domed apex was observed, and the visible beginning of flower differentiation. In August, the differentiation on apices (stage B) of horizontal shoots of control tree, bent tree, and joint tree groups appeared as a high, dome-shaped peak (Figure 4.6). In September, the differentiation of sepal and petal primordial (stage C and D) occurred in horizontal shoots from control trees, bent trees, and joint trees. The differentiation of floral buds in August and September showed the same pattern in both 2013 and 2014. The minimum and maximum temperatures in both 2 years were not so different (Figure 3.5). In October, the upright shoots of control trees showed the receptacle primordial differentiation. The formation of flower

organs including sepals, petal, stamen and pistils was complete in all treatment groups by November (Figure 3.6 and 3.7). After November, flower organs inside the buds were enlarged (data not shown). Flower buds of all treatment groups opened at the same time at the end of March. Moreover, we found that there are one to three flowers per bud in 'Kiyo' Japanese plum.

3.3.2 Phytohormone analysis

In this experiment, shoot tips were collected from May to September in 2014 and endogenous phytohormone contents were analyzed by UPLC MS/MS in MRM mode. The concentrations of GA_1, GA_3, and GA_4 in the upright shoots of control trees showed a higher level compared to the other treatment groups from May to September. The concentration of GA_1+GA_3+GA_4 in both horizontal and upright shoots of control trees decreased from May to July. Nevertheless, the bent tree and joint tree groups revealed consistent levels from May to September. The expression level of GA_1+GA_3+GA_4 in the joint trees showed the lowest concentration in May and August (Figure 3.8).

The concentration of IAA in all treatment groups decreased from May to August 2014, and the upright shoots of control trees showed the highest level of expression. In contrast, the shoot tips from the joint tree training system trees showed the lowest concentrations in May, June and August (Figure 3.8).

The changes of ABA from May to September in 2014 were also analyzed. It was found that the concentration of *trans*-ABA of bent trees and joint tree groups showed higher level than control trees in both upright and horizontal shoots. The concentration of *trans*-ABA in all treatment groups remained

consistently level from May to September. For *cis*-ABA, it was found that the joint trees and bent trees groups showed higher level than those control trees. The concentration of *cis*-ABA in joint trees and bent trees decreased from May to July and stayed constantly from July to September. While, both of upright and horizontal shoots of control trees partially remained persistently level from May to September (Figure 3.9).

3.3.3 Expression of *GA3-oxidase*, *GA2-oxidase*, and *FT* genes

We determined the expression levels of the *GA3*- and *GA2-oxidase* genes in shoot tip samples from May to September 2014. The relative expression level of *PslGA3ox* in May was approximately 0.41 to 0.86. The expression gradually decreased in June and reached its lowest level in July at approximately 0.03 to 0.07. It then slightly increased to 0.10 to 0.15 in August and 0.11 to 0.20 in September. In May, the expression level of *PslGA3ox* in the joint tree group was significantly lower than in the upright shoots of control trees. In June, the expression level of *PslGA3ox* in the bent tree and joint tree groups was lower than in the upright shoots of control trees although statistically non-significant. In July, the relative expression level of *PslGA3ox* in the upright shoots of control trees was higher than in the other treatment groups. In August and September, the differences were not significant between the 4 treatment groups (Figure 3.10).

As for *GA2ox*, the relative expression level of *PslGA2ox* remained steady in May and June at approximately 0.68 to 1.50 and rapidly increased to 4.91-5.80 in July and then suddenly decreased to a level of 0.78 to 2.26 in

August and September. The differences between the 4 treatment groups were not significant from May to September (Figure 3.10).

The relative expression level of *FT* gene (*PmFT*) from May to September was evaluated. It was found that the expression level extremely increased about 200-300 times from June to July. Then, it was decreased in August and September. The relative expression of *FT* gene on upright shoots of control trees showed lower level than those horizontal shoots of control trees, bent trees, and joint trees groups from May to July (Figure 3.10).

3.3.4 Annual growth

In 2014, length of new shoots and internodes in control trees (regardless shoot orientation) showed significantly longer than bent trees and joint trees groups. Number of new shoots per tree was not significantly found (Figure 3.11 A-C).

3.3.5 Flowering and fruit setting

In 2014, number of flower buds per tree before and after winter pruning, and number of fruits per tree were not significantly found, however, bent trees and joint trees groups showed higher than control (Figure 3.11 D-F).

In 2015, it was found that number of flower buds per tree before and after winter pruning were not significant. The bent trees seem to have a higher number of flower buds per tree than joint trees and control trees groups. As a number of flower buds per branch, it was found that joint trees and bent trees groups revealed higher level than control trees. The upright shoots of control

trees showed lowest level in both before and after winter pruning (Figure 3.12 A-D). The percentage of blooming flowers per tree, it was found that the flowering of joint trees and bent trees groups reached to 50% and 100% per tree earlier than upright shoots of control trees about two to three days. The horizontal shoots showed earlier than those the upright shoots of control trees about one day (Figure 3.12 E).

3.4 Discussion

In ‘Kiyo’ Japanese plum, leaf axil generally has three separate buds including a single vegetative bud subtended by two flower buds. Similar to peach (*Prunus persica* L.), it has a vegetative bud that contains a shoot apical meristem that generates an annual shoot growth for the following season and flower buds that contain a floral meristem (Yamane et al. 2011). However in ‘Kiyo’ Japanese plum, each flower bud produces one to three flowers per bud, whereas peaches have a single flower within each flower bud (Larsen 2010). Based on the sections of buds, we were able to define the morphologically distinct stages from vegetative development to floral organ differentiation. The undifferentiated stage was observed in May and June by a broad and swollen shape of the bud’s meristem. The initiation stage was characterized by a doming of the apex in July. In the meristematic apices from joint trees, bent trees and the horizontal shoots of control trees, differentiation was observed by a change in the shape of receptacle (stage B) in August. Differentiation began and the formation of the flower occurred in August, but those from the upright shoots of control trees developed approximately 1-2 months later than those from the

other treatment groups. Flower organ differentiation in ‘Kiyo’ Japanese plums completed in November, then the enlargement stage lasted until February. These developments were the same as in ‘Shimizu Hakuto’ and ‘Tsukuba Ichigo’ peaches (Yamane et al. 2011). The induction of flowers in other fruit trees, particularly apple (*Malus domestica* Borkh.), has been reported to occur in late June, with the initiation stage beginning from the end of July to August, followed by the flower differentiation stage (Hanke et al. 2007). In sweet cherry (*Prunus avium* L.) and peach, the apex signified the initial change from the vegetative to the reproductive stage in July (Engin and Unal 2007). In this study, we found that differentiation clearly showed in August while the beginning of differentiation appeared in July as initiation stage in ‘Kiyo’ Japanese plum. So, it is possible that the induction stage would be May and June.

GAs are important plant hormones that regulate floral induction and inhibit the development of flower buds in grape vines (*Vitis vinifera* L.) and apple trees (Palma and Jackson 1989; Zhang et al. 2008; Kittikorn et al. 2010). Thus, we analyzed the gene expression levels of *GA3ox* and *GA2ox*, which are key enzymes in the GA biosynthetic pathway. The relative expression of *PslGA3ox* from May to September in the shoot tips of ‘Kiyo’ Japanese plum in 2014 showed the highest level in May, which is an undifferentiated primordial apex stage. Afterwards, it rapidly decreased in June and July and then slightly increased in August and September (receptacle and sepal primordial arrangement stages). The relative expression levels of *PslGA2ox* showed consistently low levels from May to June with a sudden increase in July then dramatically decreased back to the same levels as in June for August and September. In July (flower initiation

stage), there appears to be a critical stage in ‘Kiyo’ Japanese plum that shows the lowest level of expression for *PslGA3ox* and the highest level of expression for *PslGA2ox*. The relative expression level of *PslGA3ox* from the upright shoots of control trees is higher from May to July. This might affect the development of flower buds and cause them to differentiate later than those from the other treatment groups. Moreover, we found that the levels of active GA_1, GA_3 and GA_4 in joint trees, bent trees and the horizontal shoots of control trees were lower than in the upright shoots of control trees (Table 3.2). It is possible that low levels of *PslGA3ox* expression result in the reduced concentration of active GA_1, GA_3 and GA_4 in those 3 treatment groups. Many studies have reported on the effects of bioactive GAs on growth and flowering in plants. For instance, an increasing bending angle favored the low concentration of GA during the flower induction stage (Koshita et al. 1999; Jiang et al. 2010), resulting in an increased flower bud number in apples (Zhang et al. 2015). Lenahan and Whiting (2006) have reported that GA_3 and GA_{4+7} inhibited cherry flower bud induction, resulting in reduced blossom density and yield in the following season. In Japanese plum and loquat (*Eriobotrya japonica* Lindl.), flowering was significantly reduced following the application of GA_3 during flower bud induction (Gonzalez-Rossia et al. 2006; Reig et al. 2011). *PslGA3ox* and *PslGA2ox* encode key enzymes that promote GA biosynthesis and deactivate GAs respectively, in *Prunus salicina* ‘Early Golden’ (El-Sharkawy et al. 2012, 2014). It is possible that these genes are also important for flower bud development in ‘Kiyo’ Japanese plum.

In addition, our results showed that high levels of IAA from May to July are related to higher levels of *PslGA3ox* expression in the upright shoots of

control trees when compared to the other three treatment groups. This evidence supports the finding that IAA elevates *GA3ox* transcription activity (Van Huizen et al. 1995; de Jong et al. 2009). The decreasing expression levels of *PslGA3ox* from May to July may be an effect from the continuously decreasing IAA level, resulting in a reduction of bioactive GAs levels in the same period. The correlation between IAA content, the expression level of *PslGA3ox* and GAs content were also found from July to September. From July to August, most of the treatment groups remained consistent in the levels of relative expression of *PslGA3ox*, IAA and GAs, and then partially increased in September. *GA2ox* is an important gene for deactivating bioactive GAs; however in this study, the differences of levels of *PslGA2ox* from May to September were not significant between the treatment groups. Therefore, *PslGA3ox* may influence flower bud development when using bending and the joint tree training system. From July to August, the development of flower buds grown in the joint tree training system, bent trees, and the horizontal shoots of control trees progressed from initiation stage to early differentiation stage faster than in the upright shoots of control trees, which progressed to early differentiation stage in October. This advance may be a result from the relatively low level of *PslGA3ox* expression from May to July. From these results, we suggest that the possible time for pruning and bending could be done at the usual time in winter and prolong to before July (the beginning of flower differentiation stage) in 'Kiyo' Japanese plum.

Moreover, in this experiment we also analyzed the concentration of ABA. It has been reported that ABA mediates the response to stresses such as drought and drought accelerates flowering (Finkelstein and Gibson 2002; Levy

and Dean 1998). The (*R*)-(-)-2-*cis*-ABA, a generally termed (-)-ABA, was identified as bioactive compounds that induce abscission or bud dormancy. (*S*)-(+)-2-*trans*-ABA (*trans*-ABA) has been detected from plant tissues but did not exhibit the activity of ABA in *Arabidopsis* (RIKEN 2015). In this experiment, we found that *cis*-ABA was high in joint trees and bent trees groups, which showed higher flower buds than the control trees. Similar with the report of Koshita et al. (1999), they found that high content of ABA related with high flower buds in Satsuma mandarin.

FT is a phloem-mobile florigen that is produced in leaves and then transferred to shoot apical meristems (Turnbull 2011). It has been reported that the expression of *MdFT1* showed the highest level in transition phase from vegetative to reproductive in apple (Mimida et al. 2013). Moreover, *MdFT* could function upstream of *SOC1* and *AP1* genes to regulate flowering (Kotoda et al. 2010). In this study, we analyzed the expression of *FT* gene in 'Kiyo' Japanese plum by using *PmFT* primers. We also found that the expression level was high in July and August that is a transition phase.

In this study, it appears that the crucial technique to induce flower development in 'Kiyo' Japanese plum is bending, either in stand-alone trees or using the joint tree training system. We observed that the joint tree training system, stand-alone bent trees, and the horizontal shoots of control trees showed an inhibition of the expression level of *PslGA3ox* resulting in a decrease of endogenous hormone levels and subsequently the earlier development of flower buds. The joint tree training system significantly differed from the upright

shoots of control trees but did not significantly differ from stand-alone bent trees or the horizontal shoots of control trees. Moreover, the joint tree training system may show effects on fruit set and fruit development. In a previous study, the number of flowers per tree and the percentage of fruits per flower exhibited no significant differences between the bent shoots on stand-alone trees and the bent shoots on joint trees. However, fruit set was greater in joint trees (Chapter 2). These results are consistent with Hirai et al. (2013), who found that the joint tree training system induced fruit production early in the year and enhanced the yield per area when compared to traditional training systems. Consequently, the effects of the joint tree training system following the flower induction stage and particularly on fruit set and fruit development should be investigated further. Moreover, in this chapter we did not clarify on each position among joint tree training system, so in the next chapter the effects on phytohomone levels, gene expression and flowering under this system will focus on.

Table 3.1 Optimized conditions for mass spectrometric analysis and retention time of each phytohormones and internal standards in the multiple reaction monitoring (MRM) mode

Endogenous hormone	Parent (*m/z*)	Daughter (*m/z*)	Cone voltage (V)	Collision energy (eV)	Retention time (min)
GA_1	347.0	273.0	40	26	3.74
GA_3	345.0	239.0	31	16	3.60
GA_4	330.9	257.0	40	23	7.69
IAA	173.8	129.8	25	10	4.31
trans- ABA	263.0	219.0	33	15	4.75
cis- ABA	263.0	152.8	41	11	5.27
d2-GA_1	349.2	275.3	40	20	3.74
d2-GA_3	347.0	241.0	34	12	3.56
d2-GA_4	333.0	259.0	50	10	7.72
d5-IAA	178.8	135.1	25	10	4.27
d6-ABA	268.9	158.8	25	10	5.20

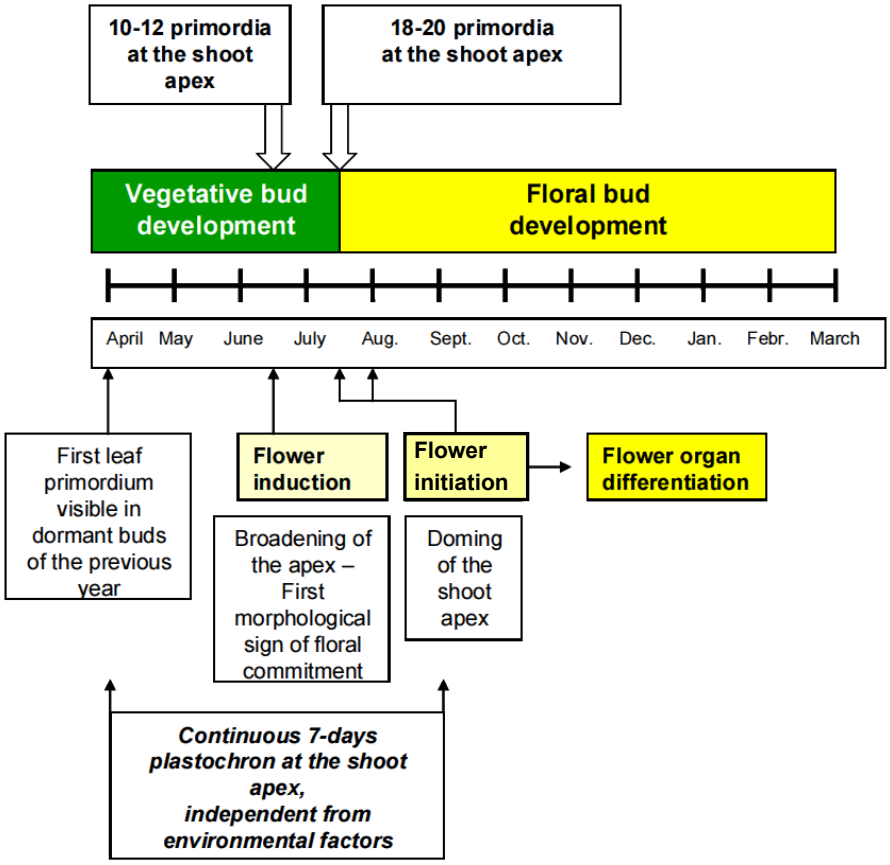
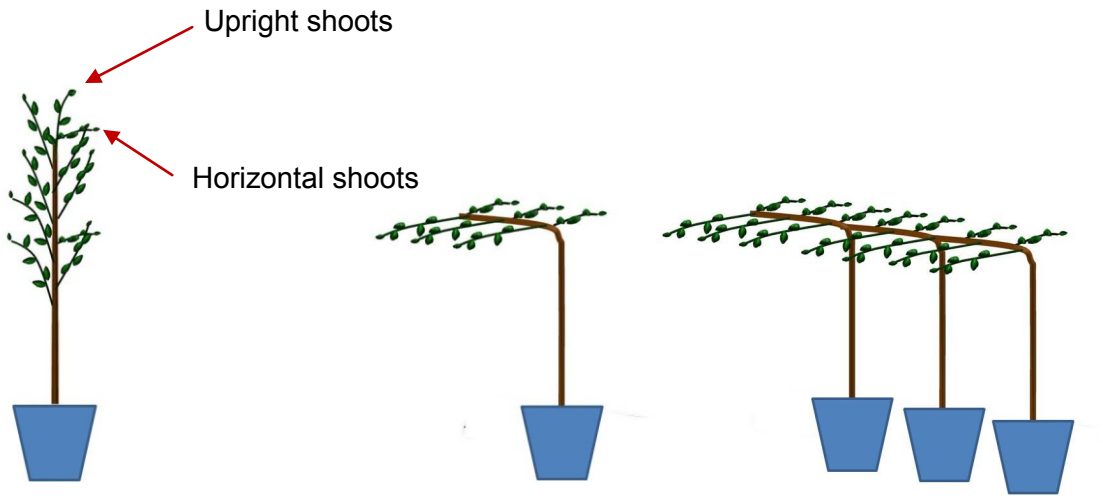


Figure 3.1 Schematic illustration of seasonal changes in the development of terminal spur buds in apple (Hanke et al. 2007).



Control (Non-bent stand-alone tree)

Bent (Stand-alone bent tree)

Joint

Treatment	New shoots Bent	Main trunk Bent	Trees Joined
Control upright	X	X	X
Control horizontal	O	X	X
Bent	O	O	X
Joint	O	O	O

Figure 3.2 Treatment of the 'Kiyo' plum trees (above). The detail on the differences of bending and jointing management of each treatment groups was described in table (below).

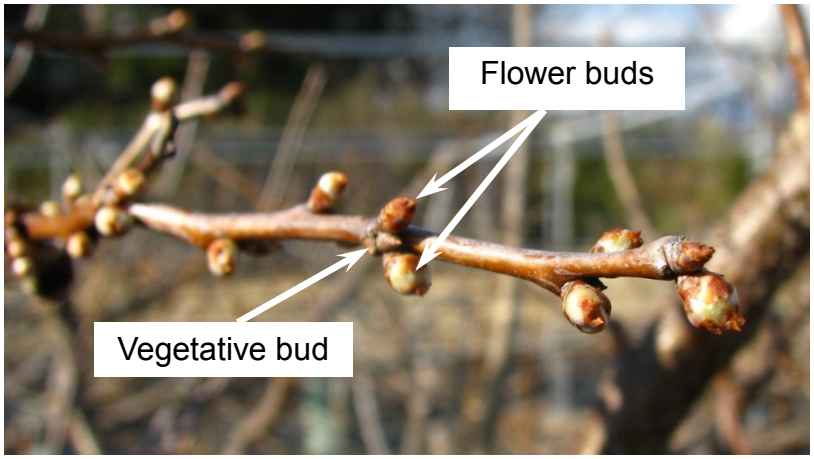


Figure 3.3 Flower buds of ‘Kiyo’ Japanese plum that develop laterally on 1-year old (above) and spurs (below).

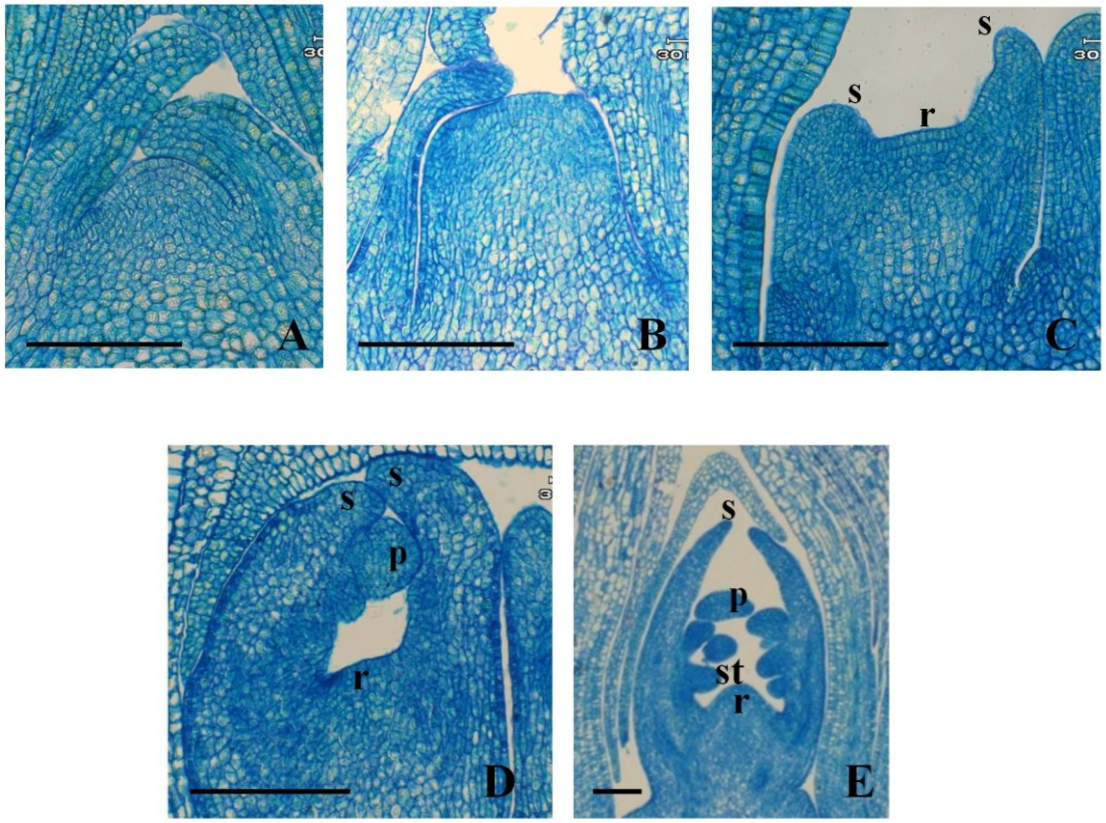


Figure 3.4 Developmental stages observed in meristematic apices of 'Kiyo' Japanese plum.

Stage A = undifferentiated meristematic apex;

Stage B = receptacle primordium;

Stage C = sepal primodia;

Stage D = petal primodia;

Stage E = stamen primordial

(r = receptacle; s = sepal; p = petal; st = stamen). Bars = 100 µm.

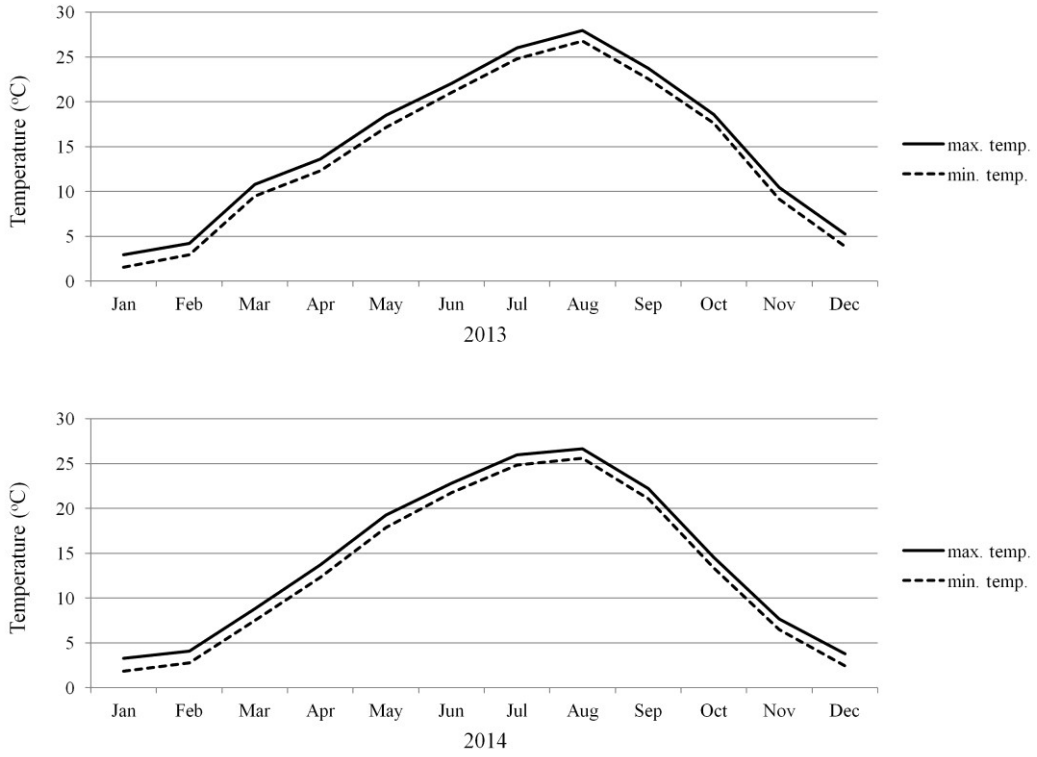


Figure 3.5 Minimum and maximum temperatures recorded at Tsukuba Experiment Forest, University of Tsukuba during 2013 and 2014.

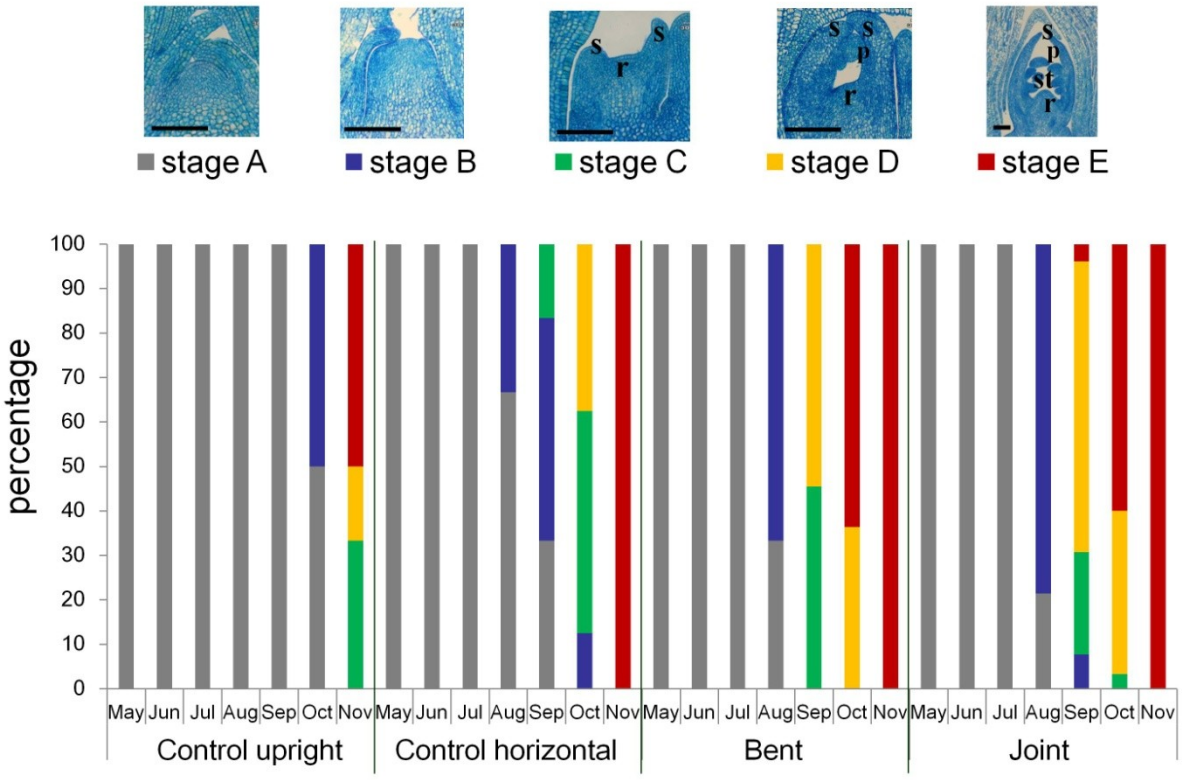
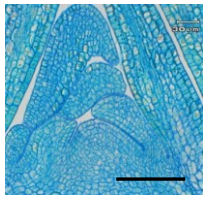
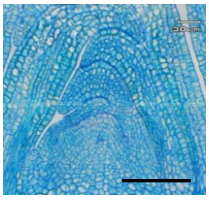
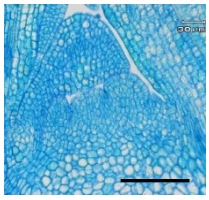
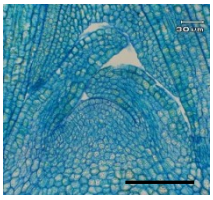
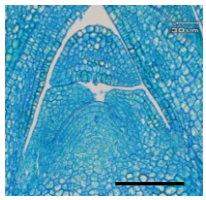
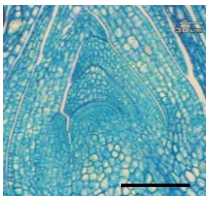
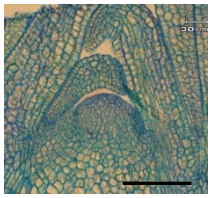
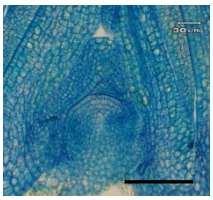
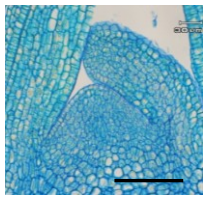
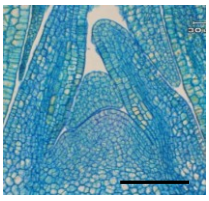
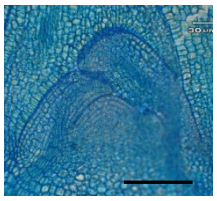
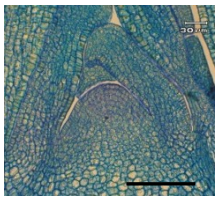
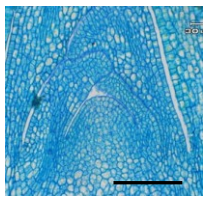
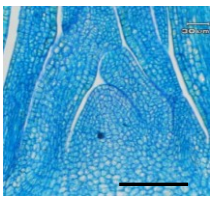
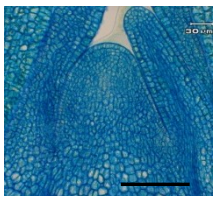
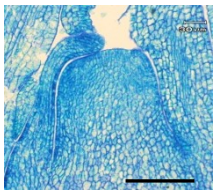
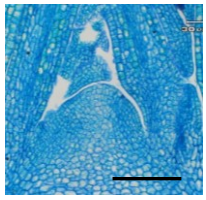
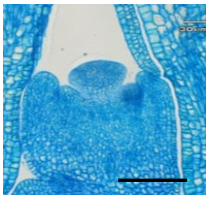
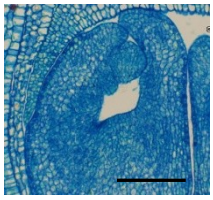
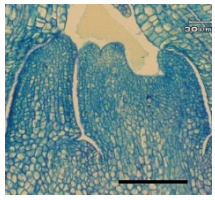
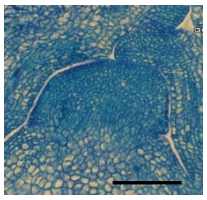
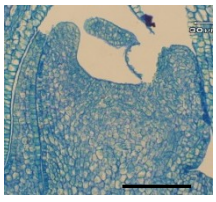
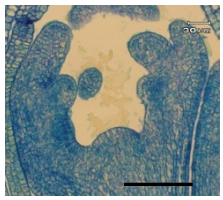
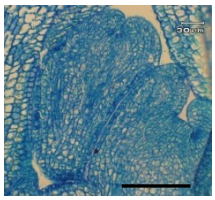
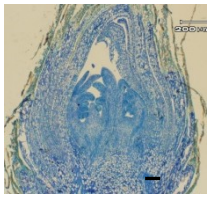
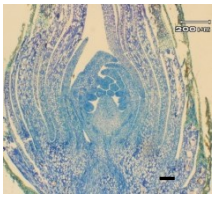
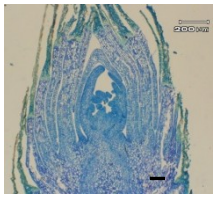
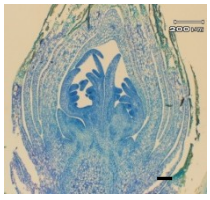


Figure 3.6 Distribution of the percentage of floral bud development from stage A to E in upright and horizontal bent shoots of control trees compared to bent trees and joint trees from May to November.

Figure 3.7 Development of flower meristems and organs in the flower buds of the ‘Kiyo’ Japanese plum from August 2013 to November 2014 and from May to September 2014. Bars = 100 μm.

	Control upright	Control horizontal	Bent	Joint
May				
June				
July				
August				
September				
October				
November				

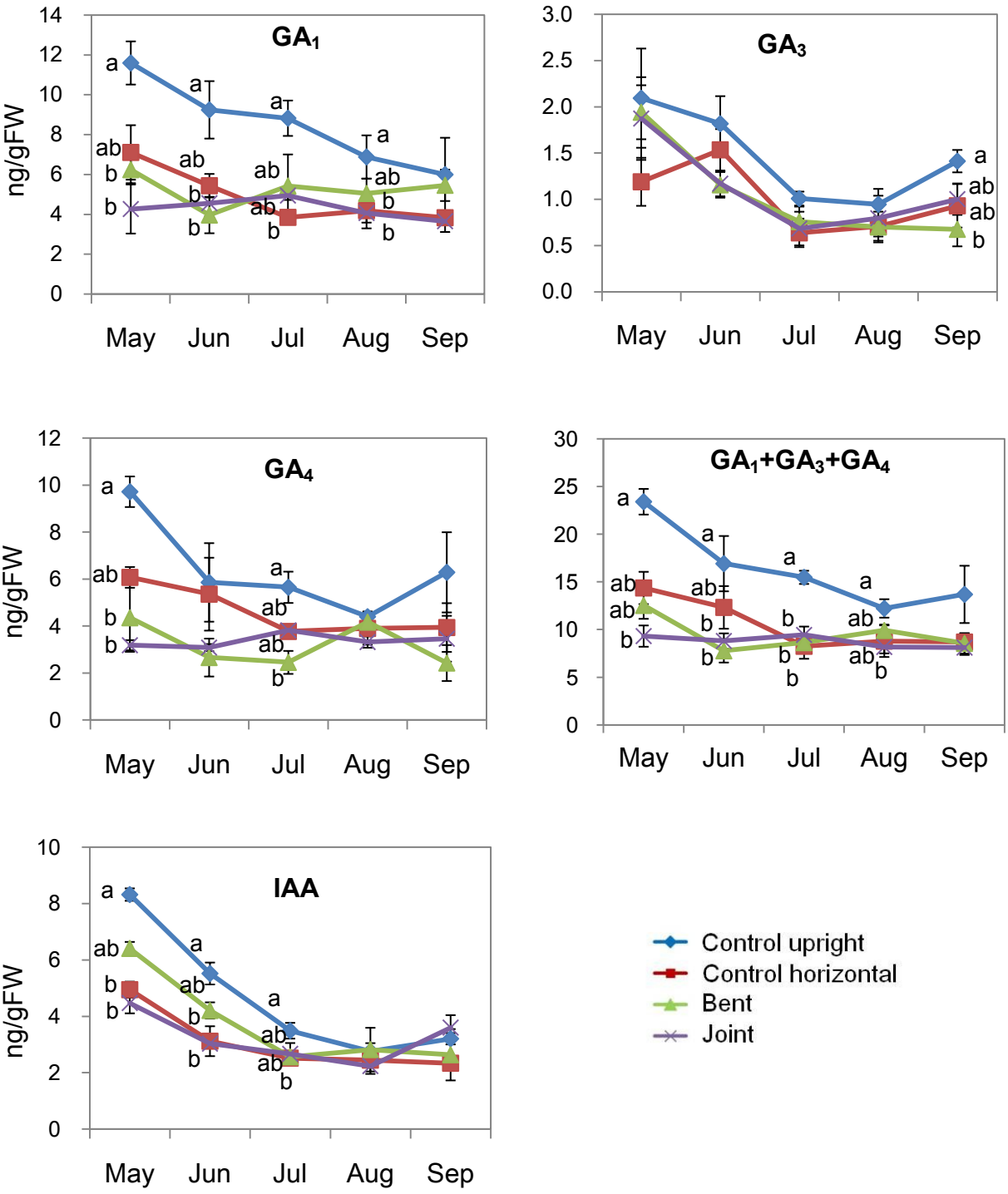


Figure 3.8 Endogenous GA_1, GA_3, GA_4, GA_1+GA_3+GA_4 and IAA concentrations of shoot tips of 'Kiyo' Japanese plum sampled in May to September 2014. Vertical bars represent the mean ± SE (n = 3). Different letters above the bars indicate a significant difference at $p < 0.05$ using Dunn's test.

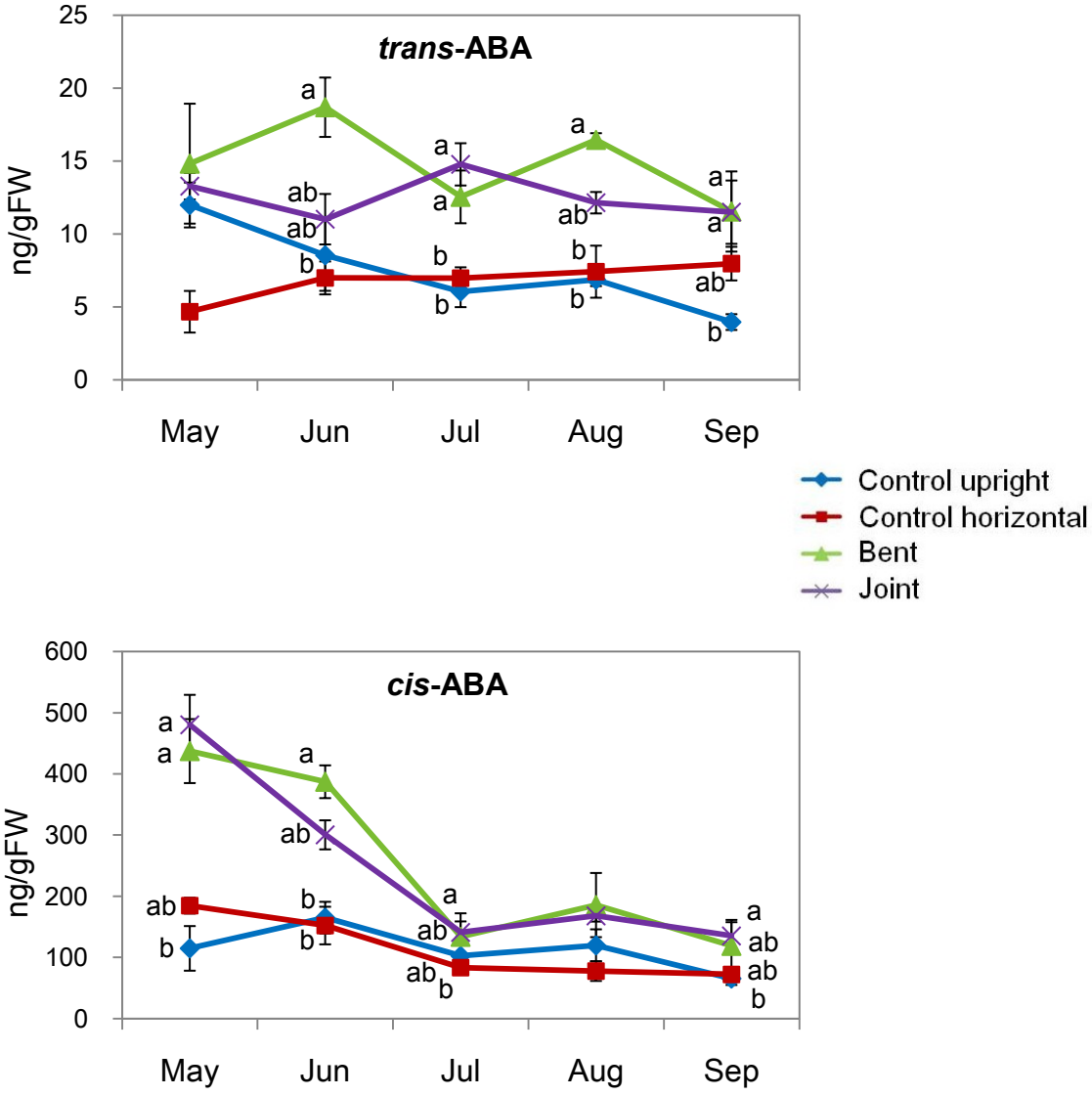


Figure 3.9 Endogenous *trans*- and *cis*-ABA concentrations of shoot tips of 'Kiyo' Japanese plum sampled in May to September 2014. Vertical bars represent the mean ± SE (n = 3). Different letters above the bars indicate a significant difference at $p < 0.05$ using Dunn's test.

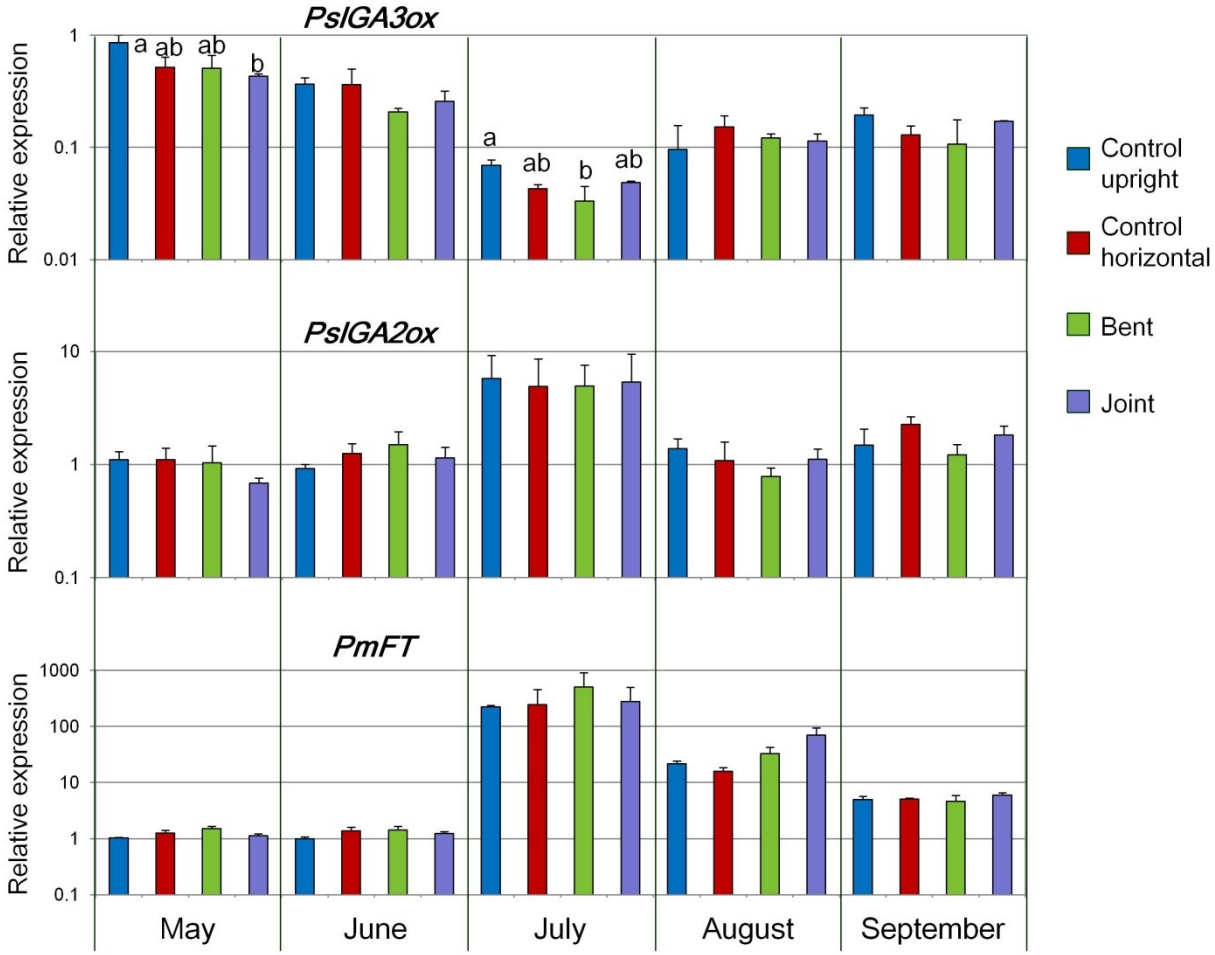


Figure 3.10 Expression level of *PslGA3ox*, *PslGA2ox*, and *PmFT* by qRT-PCR in upright and horizontal shoots of control trees compared to bent trees, and joint tree training system. Total RNA was extracted from shoot tips sampled from May to September 2014. Transcript levels were normalized relative to *PslAct* expression. Vertical bars represent the mean ± SE (n = 3). Different letters above the bars indicate a significant difference at $p < 0.05$ using Dunn's test.

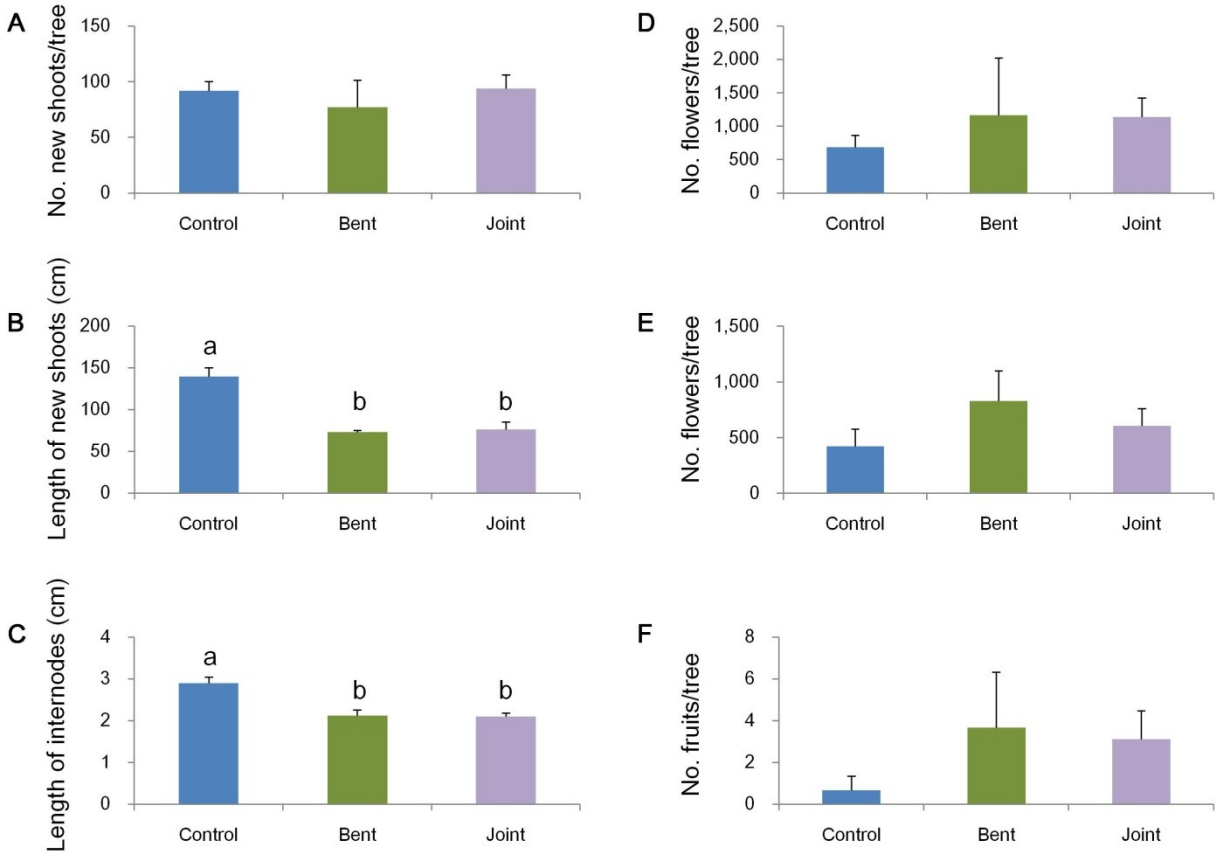


Figure 3.11 Number of new shoots per tree (A), new shoots length (B), internodes length (C), number of flower buds per tree before winter pruning (D), number of flower buds per tree after winter pruning (E), and number of fruits per tree (F) of control tree, bent tree, and joint tree training system in 2014. Vertical bars represent the mean ± SE (n = 3). Different letters above the bars indicate a significant difference at $p < 0.05$ using Dunn's test.

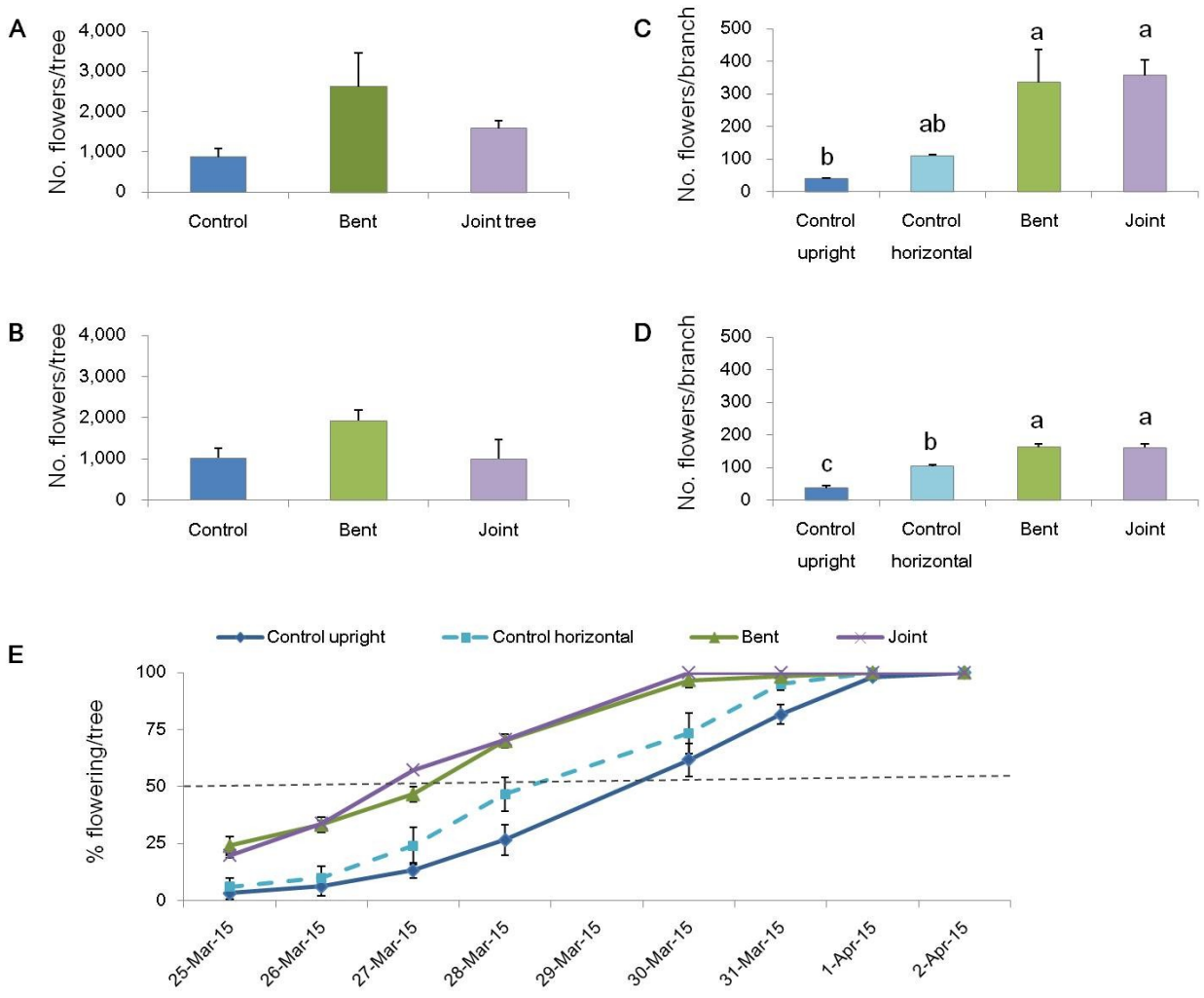


Figure 3.12 Number of flower buds per tree before winter pruning (A), number of flower buds per tree after winter pruning (B), number of flower buds per branch before pruning (C), number of flower buds per branch after pruning (D), and percentage of flowering per tree (E) of control tree, bent tree, and joint tree training system in 2015. Vertical bars represent the mean ± SE (n = 3). Different letters above the bars indicate a significant difference at $p < 0.05$ using Dunn's test.

Table 3.2 Summary of the results of horizontal shoot of control trees, bent trees, and joint trees compared to upright shoot of control trees.

			Control horizontal	**Bent**	**Joint**
Buds development					
GAs	GA_1	May/Jun/Jul/Aug/Sep			
	GA_3	May/Jun/Jul/Aug/Sep			
	GA_4	May/Jun/Jul/Aug/Sep			
IAA		May/Jun/Jul/Aug/Sep			
ABA		May/Jun/Jul/Aug/Sep			
PslGA3ox		May/Jun/Jul/Aug/Sep			
PslGA2ox		May/Jun/Jul/Aug/Sep			
Shoot length		2014	-		
Flowering		2014/2015	/	/	/
Fruiting		2014/2015	/	/	/

= significantly higher

= significantly lower

= high tendency

= low tendency

The big arrows indicated more effect.

Chapter 4

The effect of different position among three-tree joined on endogenous phytohormone, gene expression levels and flowering

4.1 Introduction

The joint tree training system has been invented to solve the problem of vigor imbalances in a scaffold branch trained horizontally onto a flat trellis, which showed too much vigor at the bottom, less vigor in the distal part and a shortage of lateral branches (Seki et al. 2009). The other advantages of this system has been reported that could achieve a high yield in early year for new orchard, bring about early return, early maturation and labor saving for growers (Shibata et al. 2008). Moreover, Shibata et al. (2008) reported that joint tree training system could uniform fruiting distributions in Japanese pear. However, in preliminary observation of three-tree joined of ‘Fuji’ and ‘Shinano Gold’ apples we found that top position in both cultivar produced number of fruit per tree higher than middle and base positions from 2011 to 2013. However, there is no report about these criteria yet. In chapter 2 to chapter 4 the effects of joint tree training system were average regardless different position among this system. So, in this chapter we would like to present the effects on endogenous phytohormones, gene expression levels and flowering among the different position of joint tree training system in case of three-tree joined together.

4.2 Materials and methods

4.2.1 Plant materials

7-year-old three-tree joined of Japanese plum ‘Kiyo’ same as the Chapter 4 was observed. To investigate the changes of endogenous phytohormones and gene expression under joint tree group by comparing each

tree in the top, middle and base position (Figure 4.1), the shoot tips from new shoots were used to compare.

4.2.2 Phytohormone extraction and analysis

Endogenous phytohormones were extracted from shoot tips that sampling from May to September in 2014. The extraction procedure and endogenous phytohormones analysis using UPLC MS/MS in MRM mode were followed same as the Chapter 3.

4.2.3 Quantitative real-time PCR analysis

Shoot tip samples in July 2014 were examined and quantify expression of *PslGA3ox*, *PslGA2ox* and *PmFT* same as the Chapter 3.

4.2.4 Assessment of shoot growth, flowering, and fruit setting

The number of new shoots, length of shoots, length of internodes and the number of nodes, number of flowers per tree before and after winter pruning, and fruits per tree were recorded in 2014. In 2015, number of flower buds per tree, flower buds per branch and flowering rate were investigated.

4.2.5 Statistical analysis

Each measurement was repeated three times. All computation and statistic analysis were done on Kruskal-Wallis test using GraphPad Prism 5 (GraphPad software, CA, USA). Significant differences among groups were determined using Dunn's test at $p < 0.05$. Data are presented as the mean ±

standard error (SE).

4.3 Results

4.3.1 Phytohormone analysis

Shoot tips from top, middle and base position of tree under joint tree training system (Figure 5.1) were evaluated from May to September in 2014. The concentration of active GA_1, GA_3, and GA_4 in top position showed lower than middle and base positions especially from May to July. The concentration of IAA, it was found that top position bared the lowest level from May to September, while base position partially showed the highest level. On the other hand, top and middle positions showed higher level of *cis*-ABA than base position from June to August (Figure 4.2).

4.3.2 Expression of *GA3-oxidase*, *GA2-oxidase*, and *FT* genes

Under the different position of joint trees group, the expression of *PslGA3ox* and *PslGA2ox* were not significant. However, the tendency of *PslGA3ox* level in top position was lower than the middle and base positions. The relative expression level of *PslGA2ox* showed the highest in Top position compared to middle and base positions (Figure 4.3 A and B).

For *FT* gene, the relative expression in top position exhibited higher level than middle and base positions (Figure 4.3 C).

4.3.3 Annual growth

In 2014, it was found that number of new shoots per tree in top position

was higher than middle and base positions. Length of new shoots showed the longest in base followed by middle and top positions. Length of internodes was not significant (Figure 4.4 A-C).

4.3.4 Flowering and fruit setting

In 2014, number of flower buds and fruits per tree revealed the highest levels in top position followed by base and middle positions, respectively (Figure 4.4 D-F). In 2015, number of flower buds per tree and flower buds per branch in top position showed the highest level, followed by base and middle positions, respectively (Figure 4.5 A-D). For the flowering rate, it was found that top position achieved to 50% earlier than base and middle positions, respectively (Figure 4.5 E).

4.4 Discussion

Under the different position of three trees of joint tree training system, we found that the top position tended to have lower active GAs and IAA content than the other position of the series. It related with low tendency in expression level of *PslGA3ox* but high level of *PslGA2ox* and *PmFT* genes, may resulting to inhibit the length of new shoots in top position (Table 4.1). These evidence confirmed the results in chapter 3 and 4, in which low level of IAA content influence to induce *GA2ox* but depress *GA3ox* resulting in decrease active GAs content. Otherwise, number on new shoots in top position showed higher than base and middle position, respectively. It is possible that the un-equal of distance between each tree of three tree joined, which the middle tree was block in the limited

space in the joined pattern. Top position has more space to growth forward, while base position has space in backward of each set.

In citrus, the increasing in $GA_{1/3}$ level and reduction of flowering may relate with down-regulation of *CiFT* gene (Koshita et al. 1999; Munoz-Fambuena et al. 2011). Nakagawa et al. (2012) reported that GA_3 application inhibits flowering by repressing *MiFT* expression in mango. In our study, Top position revealed number of flowers and fruits per tree higher than middle and base, possible from high tendency on the expression of *PmFT* gene.

However this finding was conflict with Shibata et al. (2008), who indicated that joint tree training system could uniform fruiting distributions. In our case only three trees were joined, it was found that top position produced more fruit than the other position. The un-uniformity of flowering and fruit set in this study which the three trees of joint tree not only was effected from phytohormones and gene expression levels, but also possible from the tree was grown in the pot and the distance among tree less than one meter which un-uniform branching. Whereas the joint tree training system in Japanese pear in the study of Shibata et al. (2008) was joined more than three trees and the distance between trees about 2 meters. So, the effect of more than three trees joined should be clarified further.

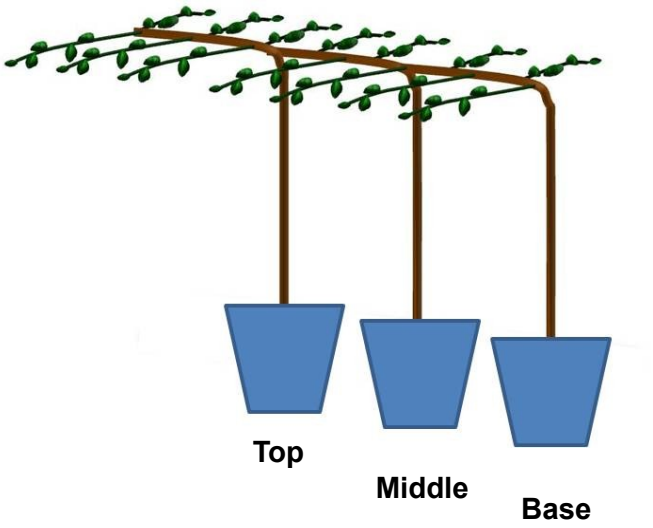


Figure 4.1 Treatment among three-tree joined of the ‘Kiyo’plum trees.

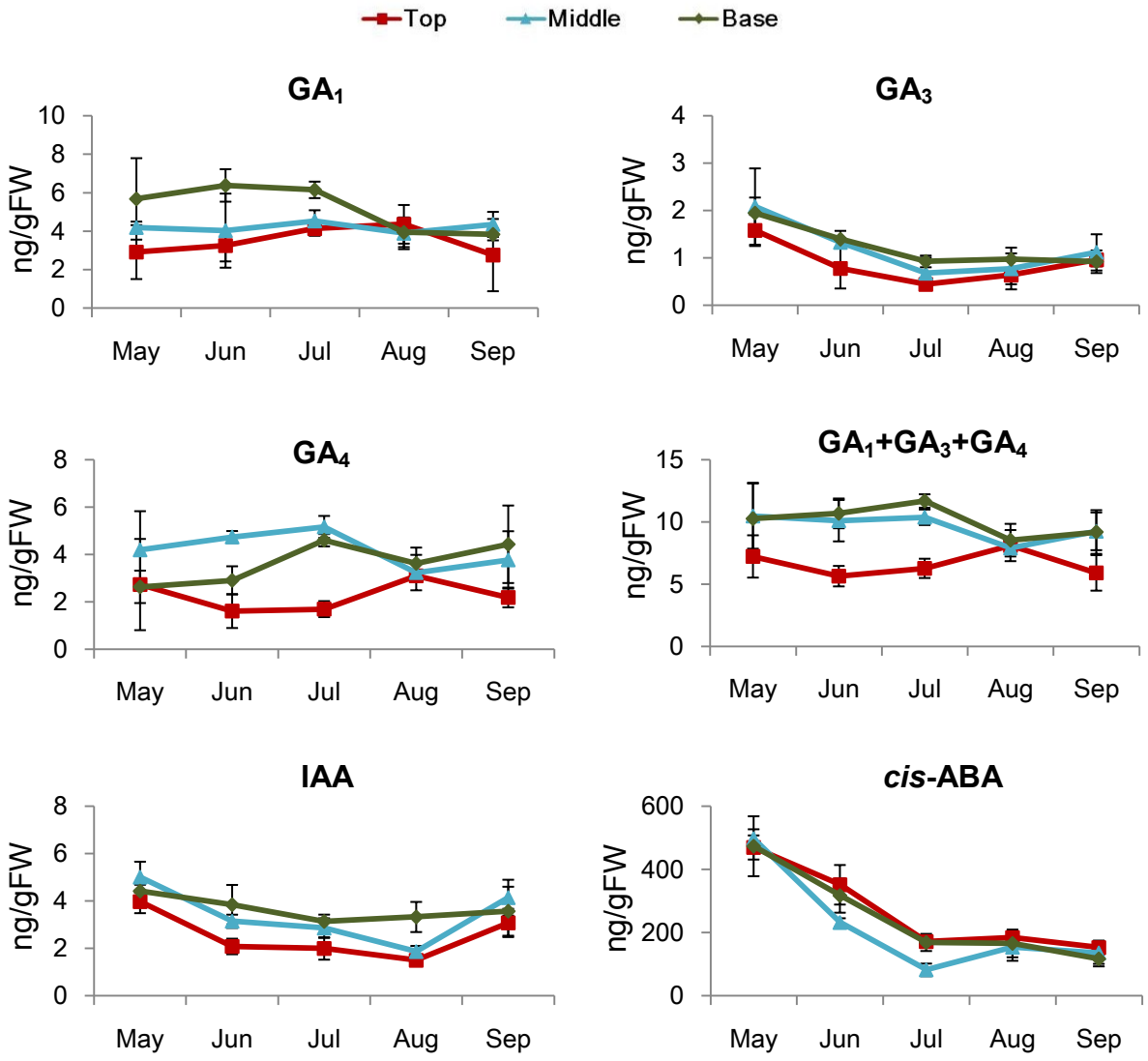


Figure 4.2 Concentration of endogenous GAs, IAA, and *cis*-ABA in shoot tips of 'Kiyo' Japanese plum among joint tree group sampled from May to September 2014.

Vertical bars represent the mean ± SE (n = 3).

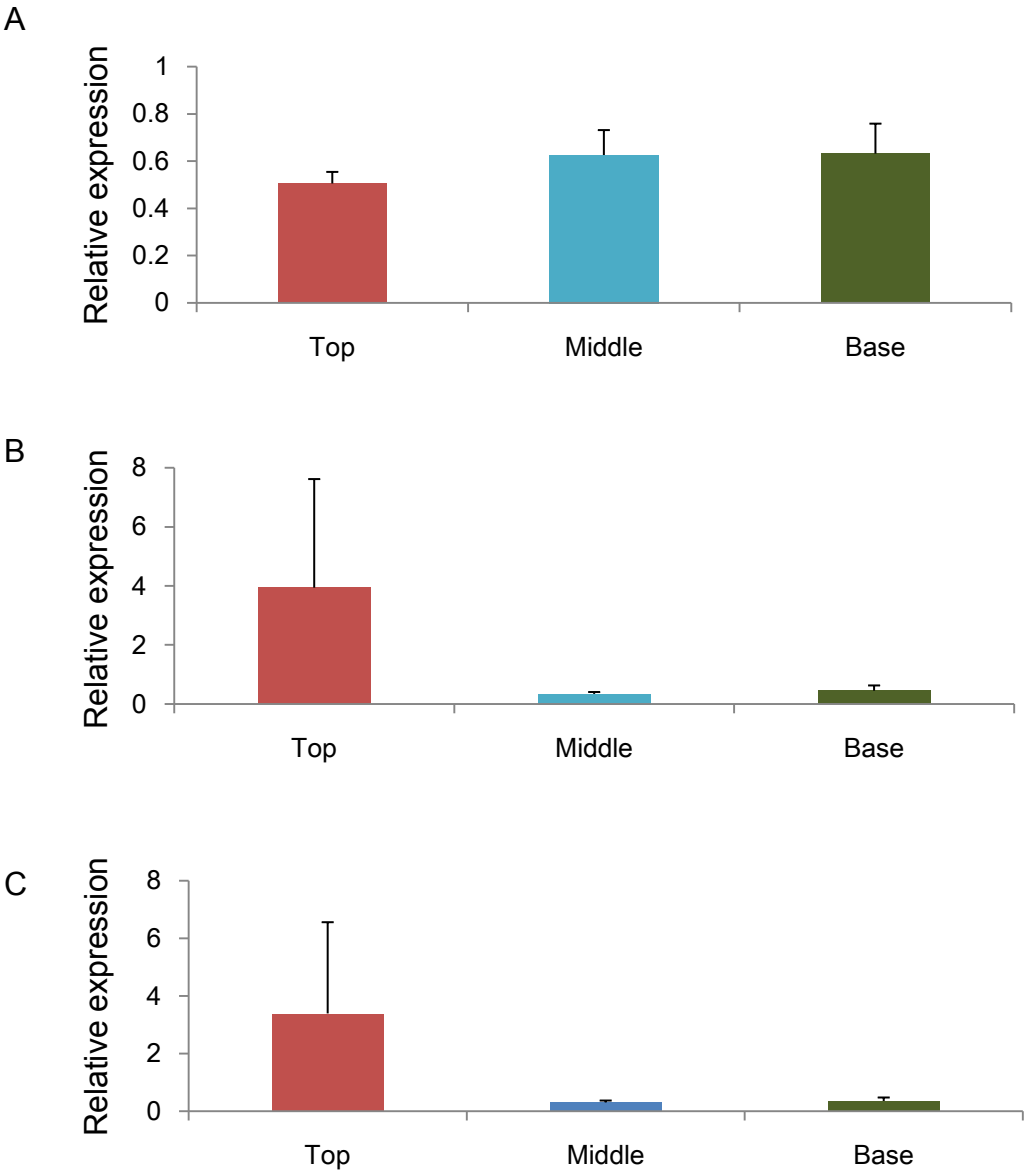


Figure 4.3 Expression level of *PslGA3ox* (A), *PslGA2ox* (B), and *PmFT* (C) by qRT-PCR in Top, Middle and Base positions of Joint tree training system. Total RNA was extracted from shoot tips sampled in July 2014. Transcript levels were normalized relative to *PslAct* expression.

Vertical bars represent the mean ± SE (n = 3).

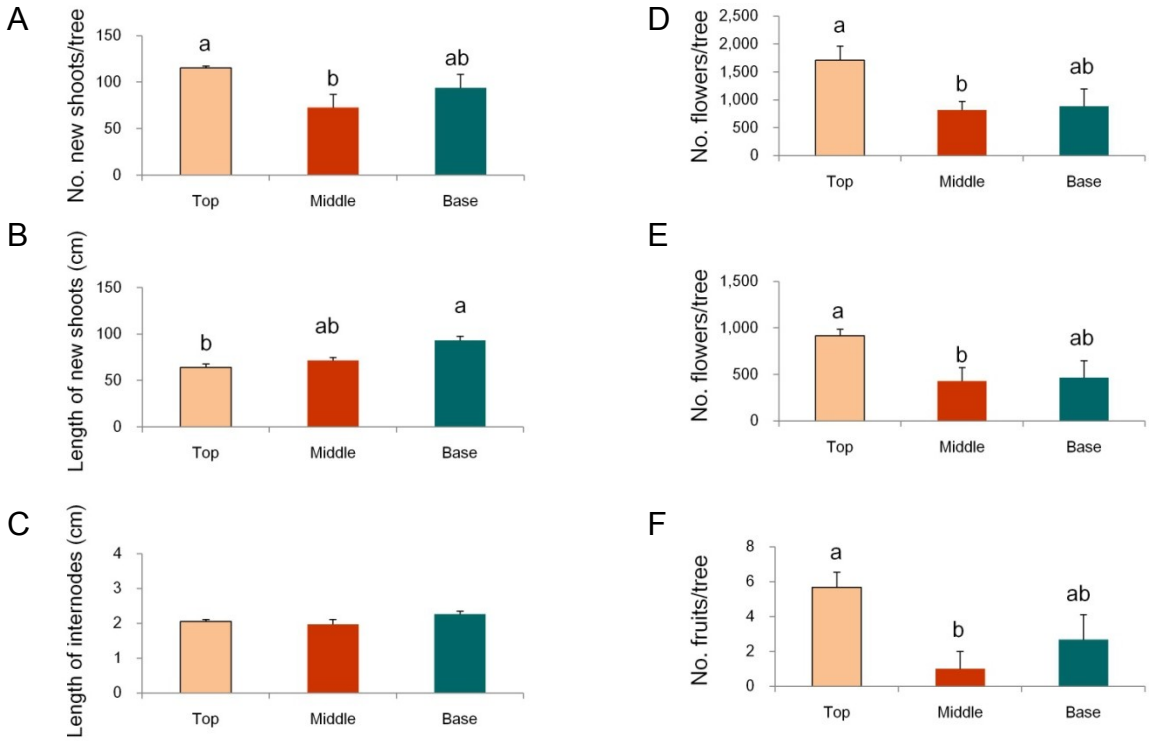


Figure 4.4 Number of new shoots per tree (A), new shoots length (B), internodes length (C), number of flower buds per tree before winter pruning (D), number of flower buds per tree after winter pruning (E), and number of fruits per tree (F) among joint tree group in 2014. Vertical bars represent the mean ± SE (n = 3). Different letters above the bars indicate a significant difference at $p < 0.05$ using Dunn's test.

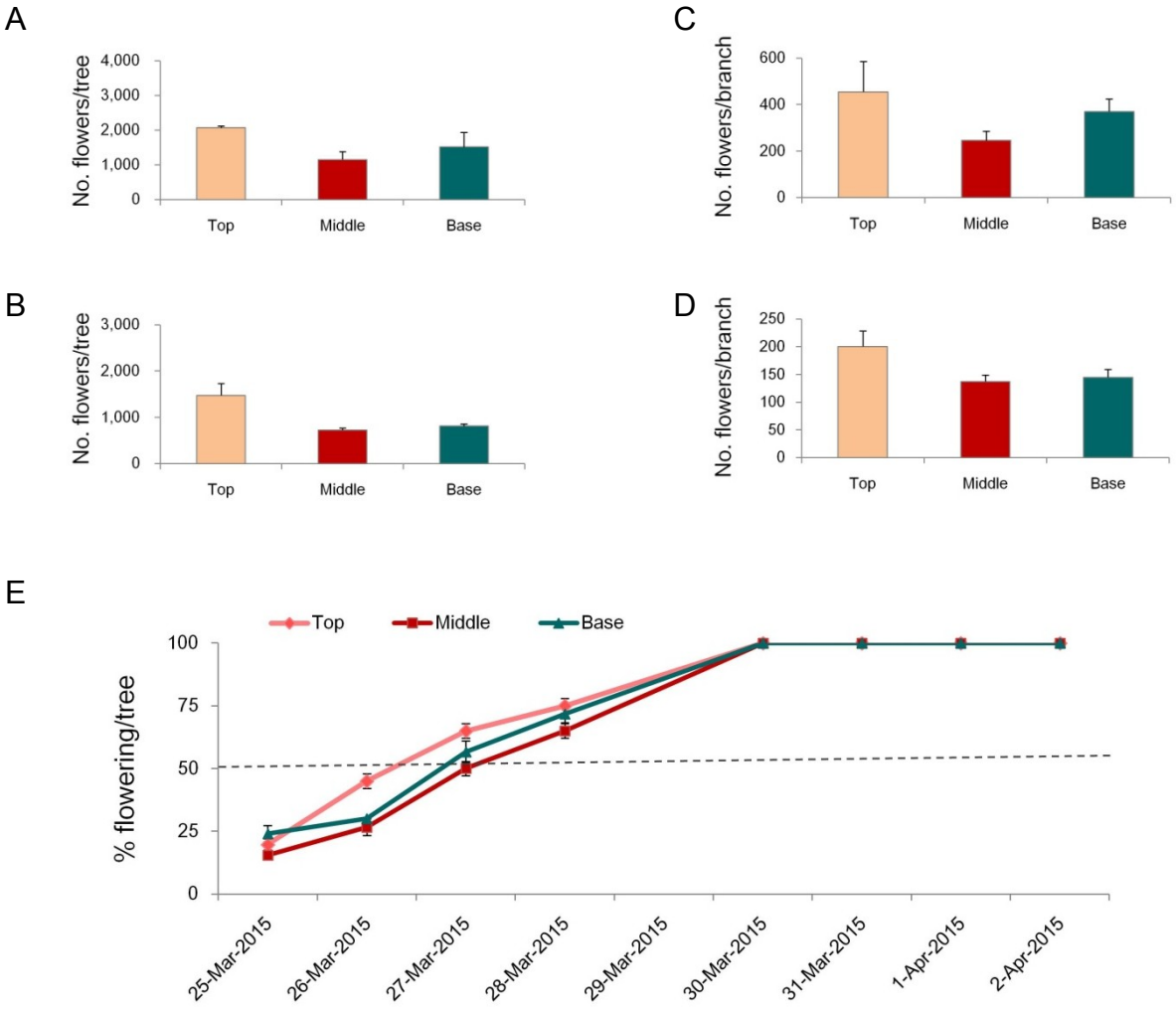


Figure 4.5 Number of flower buds per tree before winter pruning (A), number of flower buds per tree after winter pruning (B) in 2014, number of flower buds per branch before pruning (C), number of flower buds per branch after pruning (D), and percentage of flowering per tree (E) among joint tree group in 2015. Vertical bars represent the mean ± SE (n = 3). Different letters above the bars indicate a significant difference at $p < 0.05$ using Dunn's test.

Table 4.1 Summary of the results under joint tree training system, comparison between top, middle, and base positions.

		Top	Middle	Base
GAs, IAA		⇩		⇧
ABA		⇧	⇩	
PsIGA3ox		⇩		
PsIGA2ox		⇧		
New shoots/tree		⇧	⇩	
Shoot length		⇩		⇧
Flowering	2014/2015	⇧ / ⇧	⇩ / ⇩	/
Fruiting	2014/2015	⇧ / ⇧	⇩ / ⇩	/

⇧ = significantly higher ⇩ = significantly lower

⇧ = high tendency ⇩ = low tendency

Chapter 5

General discussion and conclusion

The joint tree training system is a unique and novel practical technique that has been invented in Japan. This system was first studied in Japanese pear by joined the main trunk that were bent onto a neighboring tree in a row of three trees or more trees with grafting. The changes of endogenous phytohormone levels and the expression of *GA2-oxidase* and *GA3-oxidase* levels were evaluated under commonly technique for induce flowering, branch or shoot bending. A triploid non-fertile 'Kiyo' Japanese plum was used as a model in this study. Shoot bending and the joint tree training system are predicted to improve flower induction resulting in increase fruit production of this cultivar.

Endogenous phytohormones and flowering

Endogenous GA signaling is linked to pathway that regulates flowering in response to environmental stimuli (Galvao and Schmid 2014). Florigen is a mobile long-distance signal produced in leaves, which produces flowering at the shoot apical meristem. In a variety of plant species, the small globular protein *FLOWERING LOCUS T* (*FT*) serves as a florigen. The transition to flowering is mainly controlled in the leaf phloem companion cells, and the shoot meristem. The expression of *FT* in the leaf vasculature is controlled by a complex regulatory network that involves the *DELLA* protein, the MADS-box transcription factor *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), and *LEAFY* (*LFY*) etc. GA is an important signal in the transcription of *DELLA*, *SOC1*, and *LFY* (Galvao and Schmid 2014; Jiang and Fu 2007)). In the view of this, the level of endogenous GA is a main phytohormone that we analyzed using

UPLC-ESI-MS/MS. It was found that GA_4 is the major active GA in 'Kiyo' Japanese plum, concomitant with the concentration of GA_{4+7}, which was much higher than that of GA_{1+3} in Japanese pear (Ito et al. 1999).

In our experiments, bending and joint tree training system appeared to have low active GAs (GA_1, GA_3, and GA_4) contents compared to upright shoots of stand-alone trees, resulting in decreased vegetative growth including shoot and internodes length that were shorter than upright shoots of stand-alone trees. Moreover, the other study about branch bending such as in pear and apples, its had been indicated that bending stimulate flowering (Tromp 1972; Lin et al. 1990; Goldchmigt-Reischel 1997), possibly by low content of active GAs. There are many reports about the effect of GAs on growth and flowering in plants in which a high level of GA_3 or GA_{1+3} had an inhibitory effect on floral formation during the induction and initiation stages in olive (Ulger et al. 2004) and satsuma mandarin (Koshita and Takahara 2004). Evans et al. (1990) reported that application of GA_1 and GA_4 to *Lolium temulentum* during vegetative growth caused poor or no flowering but the stems elongated substantially in a similar response to GA_3 application. In rice and lentil, GA_3 application increased internodes length and plant height, respectively (Azuma et al. 1997; Giannakoula et al. 2012). In Japanese plum and loquat, flowering significantly reduced after application of GA_3 during flower bud induction (Gonzalez-Rossia et al. 2006; Reig et al. 2011). In cucumbers, dwarf cultivar remained lower levels of GA_3 and IAA compared to vine-type (Ming et al. 2014). Stem elongation is positively regulated by both bioactive GAs and IAA (Yamaguchi 2008). Similar to Bangerth (2009), we observed that a low endogenous GA content promoted

flowering over vegetative growth.

Auxin plays an important role in plant development. In *Dendrobium*, lentil, and pharbitis, IAA showed beneficial effect on flower induction (de Melo Ferreira et al. 2006; Khalil et al. 2006; Koshio et al. 2015). However in this study, the concentration of IAA in upright shoots of stand-alone trees was higher than that in horizontal bent shoots and joint trees, consistent with reports by Ito et al. (1999, 2004). The latter authors indicated that IAA concentration in bent shoots was lower than that of non-bent shoots in 'Kosui' Japanese pear and demonstrated that the diffusible amount of IAA in bent shoots was less than that in vertical shoots (Ito et al. 2001). The result of high IAA in vertical shoots compared to bending shoots affected to increase of vegetative growth and suppress of flower induction in 'Kiyo' Japanese plum. It is possible that, a high concentration of IAA may increase GA content, because IAA promotes the activation of transcription (*GA3ox*) which activates inactive GAs to an active form, increasing of enzyme activity of GA20ox which catalyzes among inactive GAs (Van Huizen et al. 1995; O'Neill and Ross 2002; de Jong et al. 2009) and decrease of transcription of *GA2ox* which deactivates an active GAs into inactive form (Ozga et al. 2009).

ABA plays an important role and acts as a signal that regulates plant growth and development (Qin et al. 2013). In *Arabidopsis*, ABA is a regulating hormone in the floral transition that was initially proposed base on the early-flowering phenotype of an ABA-deficient mutant (Martinez-Zapater et al. 1994). Koshita et al. (1999) reported that endogenous ABA influences the pattern of inflorescence and might affect flower bud development in satsuma

mandarin. Domagalska et al. (2010) concluded that the effects of GA and ABA on timing of floral induction are only in partially coordinated action. In our experiment, the content of ABA related with number of flower buds.

Not only GAs, IAA, and ABA, CK also influences to plant growth, while the function was different in various plant. It has been reported that CK is a key component to promote new shoot and flowering of *Scoparia dulcis* L. (Premkumar et al. 2011). In *Dendrobium*, high content of CK affected to increase flower induction (de Melo Ferreira et al. 2006). However, the other reported that CKs play a role correlate with GAs and IAA such as after treated *Ammi visnaga* L. with some bioregulators, the increment of plant height was associated with elevated contents of CKs, GA_3, and IAA (Talaat et al. 2014). In *Catharanthus reseus*, during the process of the first flower bud differentiation, the contents of IAA, GA_3, and zeatin, one type of CK, in leaves decreased evidently (Ma and Zu 2009). Therefore, the effect of bending and joint tree training system on contents of CKs should be studied further.

Expression of *GA3ox*, *GA2ox*, and *FT* genes

GA3ox and *GA2ox* encode key enzymes involved in biosynthesis and inactivation of GA, respectively (Yamaguchi 2008). In the present study of 'Kiyo' Japanese plum, it was shown that, a high level of *PslGA3ox* expression resulted in high concentrations of bioactive GAs in upright shoots of stand-alone trees compared with the other treatments in shoot tip samples collected from May to September 2014 (Chapter 3). It is concomitant with the study on Gunma prefecture, we found that a low level of *PslGA2ox* expression and high level of

PslGA3ox expression resulted in high concentrations of GA_1 and GA_4 in upright shoots of stand-alone trees compared horizontal shoots of stand-alone trees and joint tree training system in young leaf samples collected on July 2012 (Data not shown). Likewise in *Prunus salicina* 'Early Golden', *PslGA2ox* and *PslGA3ox* promoted GA biosynthesis and deactivated GAs (El-Sharkawy et al. 2012, 2014).

GA3ox generates the physiologically active GAs of the GA biosynthetic pathway. In many plants, overexpression of *GA3ox* resulted in elevated levels of bioactive GA_4 and/or GA_1 as well as increased stem length or plant height (Radi et al. 2006; Gallego-Giraldo et al. 2008; Reinecke et al. 2013). In rice, *OsGA3ox2* was important in controlling bioactive GA synthesis for shoot elongation, but not for reproductive development (Sakamoto et al. 2003). In this study, the expression level of *PslGA3ox* was high in upright shoots of non-bent stand-alone trees, which may lead to the high concentration of active GA_1 and GA_4 and result in increasing of lateral branch length, and decreasing the number of flowers per branch in 'Kiyo' Japanese plum.

GA2ox gene exhibits reduced plant height and shoot length by deactivates bioactive GAs. Ectopic expression of 'Early Golden' plum *PslGA2ox* in wild-type *Arabidopsis* resulted in a typical dwarf phenotype owing to a significant decline in the length of all stem growth-related characters, resulting in about 60% reduction in overall plant height, which was associated with considerably increased *PslGA2ox* expression (El-Sharkawy et al. 2012). In *Lolium temulentum*, bioactive GA_1 and GA_4 are deactivated by *LtGA2ox5*, of which reduced expression allows GA_1 and GA_4 to promote stem elongation

(Evans et al. 1990). In this study, the differences were not significantly found, however under joint tree training system a high level of *PslGA2ox* expression was indicated to affect the content of active GA_1, GA_3, and GA_4 that showed a lower concentration in top position.

In addition to *GA3ox* and *GA2ox*, *GA20ox* would play an important role in the GA biosynthesis pathway. The *GA20ox* removes a carbon molecule by successive oxidation of GA_{12} to GA_9 and GA_{53} to GA_{20} (Yamaguchi 2008). Expression of *GA20ox* leads to an increase in bioactive GA levels and subsequently increased growth and tree height (Coles et al. 1999; Eriksson et al. 2000; Vidal et al. 2001; Israelsson et al. 2004). GA20ox is a key enzyme in the penultimate catalyzes steps in GA biosynthesis. In longan, up-regurated *GA20ox* might be involved in the floral reversion process (You et al. 2012). In rice, the increasing of endogenous GA level due to the increasing expression level of *OsGA20ox1* gene (Tetsuo et al. 2004). Therefore, further studies of *GA20ox* function are needed to understand the regulatory mechanisms of GA biosynthesis in plum under the joint tree training system.

It has been reported that gibberellin pathway is one of six major pathways which control flowering time in *Arabidopsis* (Fornara et al. 2010). All pathways converge to regulate a small number of 'flower integrator genes' which including *FT*, *SOC1*, and *LFY*. Among these, *FT* gene is a well-known floral integrator gene that plays a crucial role in controlling flowering time (Kardailsky et al. 1999; Kobayashi et al. 1999). However, in this experiment the expression level of FT was not significantly found between treatment groups.

Bud development and dynamic changes of phytohormone levels and genes expression on flower induction period

Flower bud is an organ which represents the changes on the transition from vegetative growth to reproductive development in flower induction period of plant. Flower induction in other fruit tree, especially in apple was reported that occurs in late of June, initiation stage started from end of July and August, after that is a flower differentiation stage (Hanke et al., 2007). Based on sections of buds in this study (Chapter 3), the morphologically distinct stages from vegetative development to floral organ differentiation were defined. The anthesis time of flower of 'Kiyo' Japanese plum is at the end of March, so we started to examine the development from May. We observed pronounced doming of the apex in the majority of individuals sampled that were designated induction stage, thus the vegetative growth performed before May. In 'Kiyo' Japanese plum, flower induction stage was found in May and June with the broad and swollen shape of spur buds, then initiation stage was characterized by doming of the apex in July, and the differentiation was started and the form of flower was occurred from August onwards (Figure 5.1).

Shoot bending and joint tree training system inhibited the expression level of *PslGA3ox* resulting in lessen endogenous hormone levels possibly induced flower bud development earlier than upright shoot of stand-alone trees. *PslGA2ox* is also an important gene for deactivated bioactive GAs in biosynthesis pathway, however in Chapter 3, the differences from May to September were not significant among treatment groups. *PslGA3ox* is possible a

major gene influence in flower buds development under bending and joint tree training system. The development of flower buds in joint tree training system, stand-alone bent trees, and horizontal shoot of control trees that changed from initiation stage to early differentiation stage in July to August faster than in upright shoot of control trees that has been changed to early differentiation stage in October may effected from low level of relative expression of *PslGA3ox* in May to July. The expression of *PmFT* gene also showed high level in July. Fu et al. (2014) also found that *ClFT* has a conserved function in regulating the transition from vegetative phase to the reproductive phase and acts as a flowering promoter in *Chrysanthemum*. Hanke et al. (2007) reported on the relationship between changes of metabolism and flower bud differentiation in apple. It was shown that the transition from vegetative to reproductive stage is accompanied directly with an increasing of RNA synthesis, in which flower differentiation is based on synthesis of a specific ribonucleic acid. It is possible that the transition phase of 'Kiyo' is July according to the initiation of bud development, the decreasing of GAs and IAA contents, and regulation level of *GA2ox*, *GA3ox*, and *FT* genes.

From these results, we suggest that possible time for pruning and bending could be done at the usual time in winter and prolong to before July (the beginning of flower differentiation stage) in 'Kiyo' Japanese plum.

The effects of position in the joint tree training system

Among the joint tree training system, influences of endogenous phytohormones and gene expression to flower induction are also interesting.

Shibata et al. (2008) reported that joint tree training system could accomplish uniform fruiting and fruit quality. However, we found the inconsistent tendency of phytohormone contents and relative expression level of genes along with flowering and fruit setting under three trees of joined series. The top position had higher number of flowers and fruits, concomitant with low content of active GAs. Moreover, the tendency of *PslGA3ox* expression level in top position seems to be low, but high level of *PslGA2ox* and *FT* genes compared to the other positions. Consequently, in case of more than 3 trees joined in joint tree training system should be clarified further.

In summary, under joint tree training system and bending effected on the changes of endogenous phytohormone level in 'Kiyo' Japanese plum. Branch or tree bending and naturally bent shoots to the horizontal influence to partially depressed active GA_1, GA_3, and GA_4 levels in flower induction period, indicated from low relative expression of *PslGA3ox*. IAA level is also suppressed by bending, while ABA level is expressed. The influence of those evidence resulting to decreased vegetative growth such as length of new shoots or lateral branch, that may lead to induce flowering. Even the benefit of joint tree training system in 'Kiyo' Japanese plum was not significantly different shown comparing with individual bent trees. The tendency of joint tree training system seems to increase fruit setting.

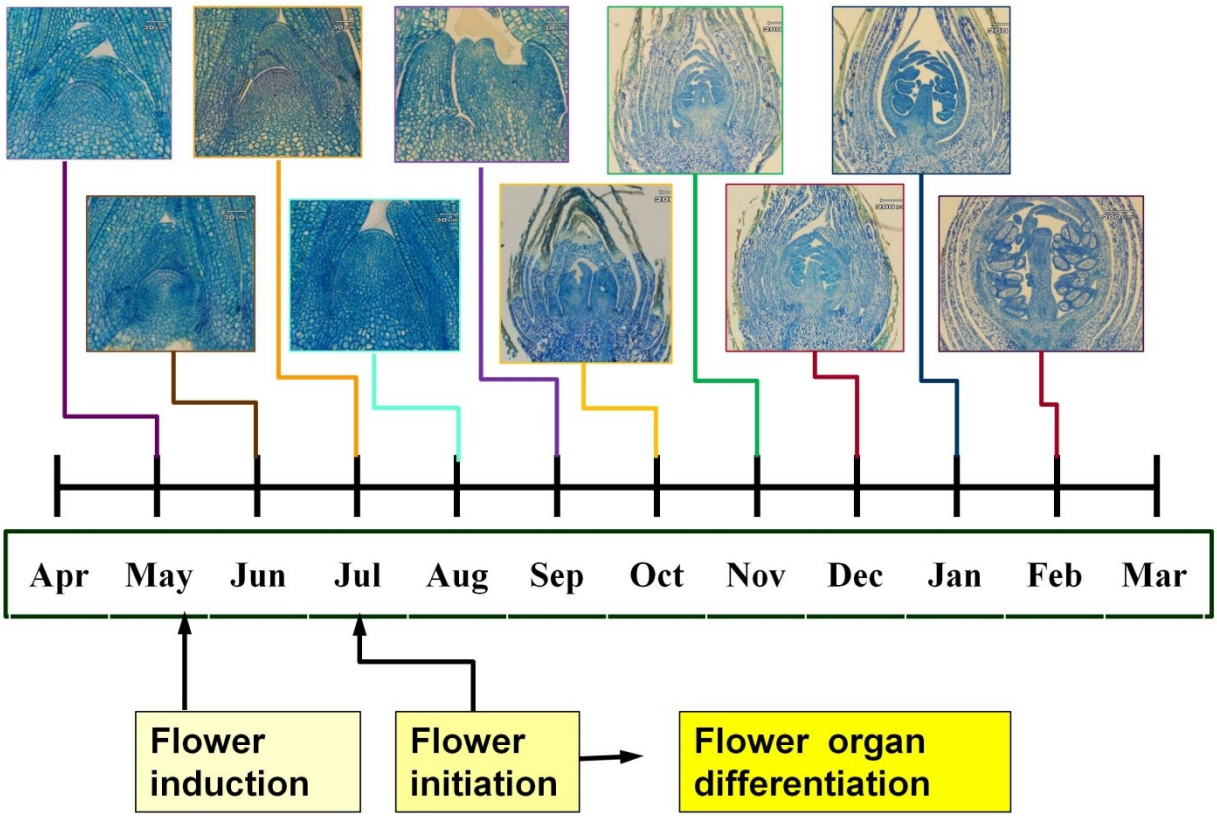


Figure 5.1 Schematic illustration of seasonal changes in the development of ‘Kiyo’ Japanese plum’s buds. In ‘Kiyo’ Japanese plum, the domed apex was observed in July, then the differentiation on apices as a high, dome-shaped peak in August. It is possible that the undifferentiated apice meristem in May and June are supposing as flower induction stage.

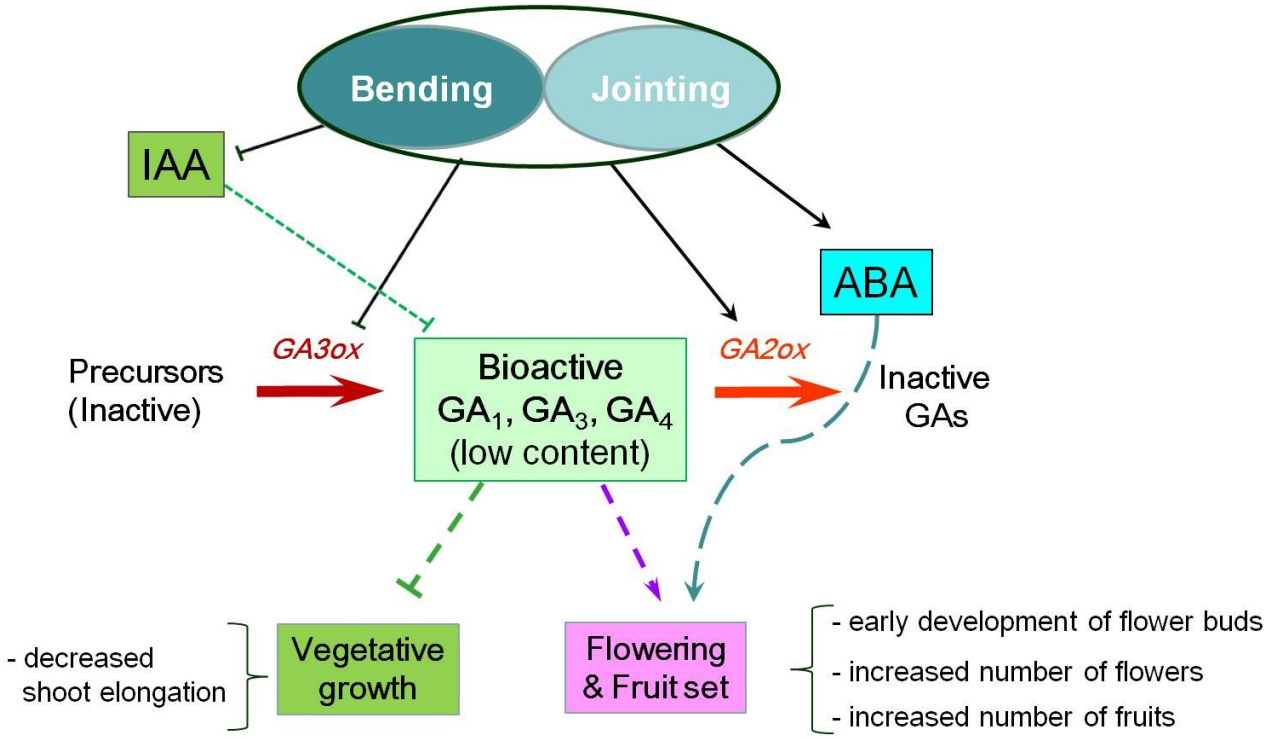


Figure 5.2 Schematic of the influence of bending and jointing on phytohormones and flowering in 'Kiyo' Japanese plum. Bending and Jointing decreased expression level of *GA3ox*, while increased expression level of *GA2ox*, influenced to decreased bioactive GAs content. Bending and jointing affected to decrease IAA but increase ABA contents. Low level of bioactive GAs and IAA, while high level of ABA contents from bending and jointing may result in inhibited vegetative growth with the decreasing of shoots length, and enhanced flower induction and fruit set.

——⊣ decreased —→ increased

– – – – –⊣ may result in decrease – – – –→ may result in increase

Summary

Important stages in the flowering process are flower initiation, followed by flower differentiation, and fertilization leading to fruit set and development. The overall mechanism remains unclear, but strong evidence suggests that endogenous phytohormones play a central role in flower induction and differentiation. The joint tree training system utilized in this study to evaluate the changes of endogenous hormones using UPLC-ESI-MS/MS.

Bending and joint tree training system appeared to have low active GAs contents and may play role in decreasing of shoot length and increasing of flowering. Low content of active GAs in bending and joint tree involved with a low level of *PslGA3ox* expression and high level of *PslGA2ox* expression, since *GA3ox* catalyzes the last step of GA biosynthesis pathway to generate active GAs, while *GA2ox* encodes key enzyme results in inactivation of GAs.

IAA also showed low content in bent shoot, and joint tree training system, affecting inhibited shoot growth and induced flowering. IAA may associate GAs content as a reported that IAA promotes activity of GA20ox, and GA3ox for the conversion between inactive GAs, and activating inactive to an active form of GAs, respectively, but decreased transcription of *GA2ox* that deactivated active to inactive GAs.

The content of ABA related the number of flower bud especially *cis*-ABA that revealed higher level in stand-alone bent trees and joint trees compared to control trees.

The expression of *FT* gene showed the highest level in the transition

phase from vegetative to reproductive in July.

The development stages of ‘Kiyo’ plum buds were clarified as the vegetative growth was continue until May and June that induction stage was observed. The initiation stage was defined in July, and then the floral organ differentiation stage performed from August. Concomitant with the *PslGA3ox* was exhibited high in May, afterward significantly decreased until July, then slightly increased in August and September.

Under the joint tree training system of 3 trees joined, top position revealed the shorter of new shoot length and higher number of flower per tree. Since active GAs contents showed lower, but tendency on expression levels of *GA2ox* and *FT* higher than the Middle and Base position.

In ‘Kiyo’ Japanese plum, joint tree training system would enhance flowering and fruit set, in which the joint tree training system decreased bioactive GAs, IAA contents and expression level of *GA3ox*, while increased ABA contents and expression level of *GA2ox*. It is possible that, the joint tree training system could apply to enhance flower induction in the other fruit trees. However, regulatory mechanism of phytohormones on flowering especially *FT*, *SOC1*, DELLA etc. has not been clarified. So, more research about joint tree training system needs to be done.

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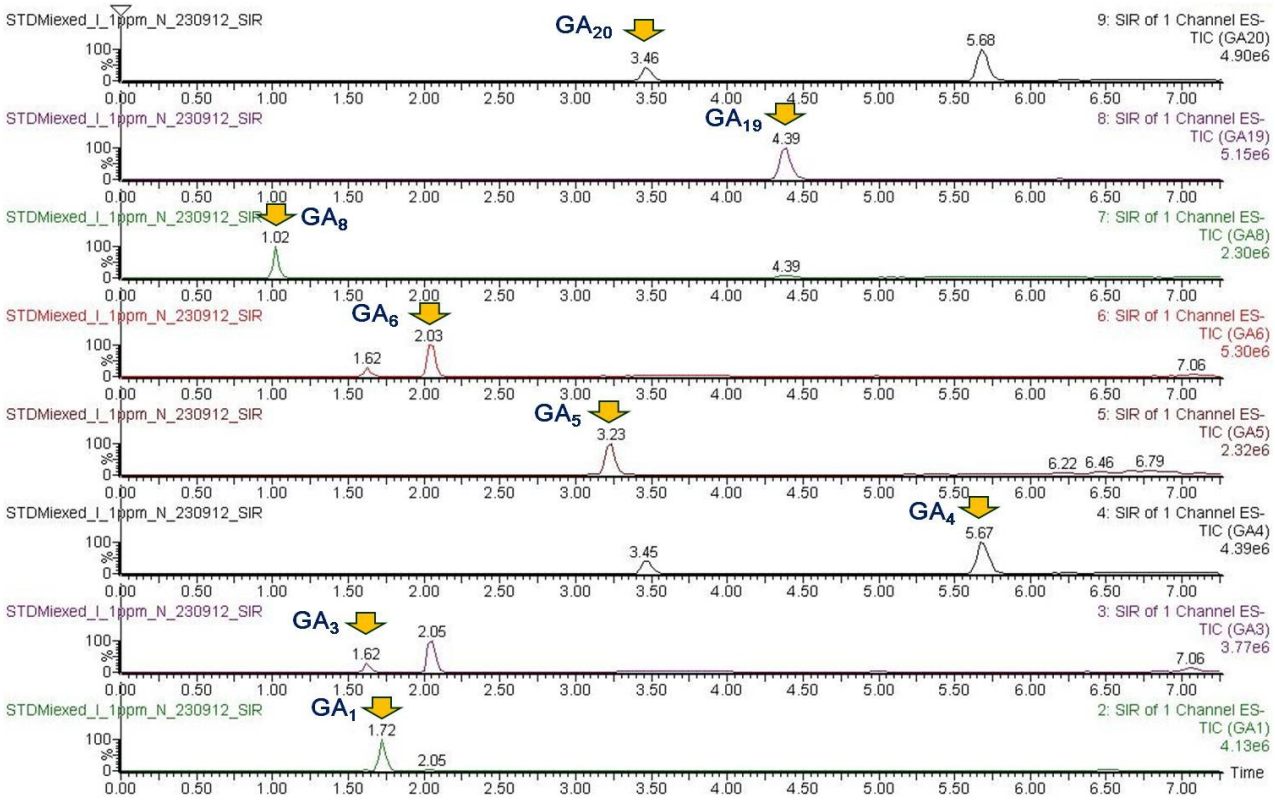
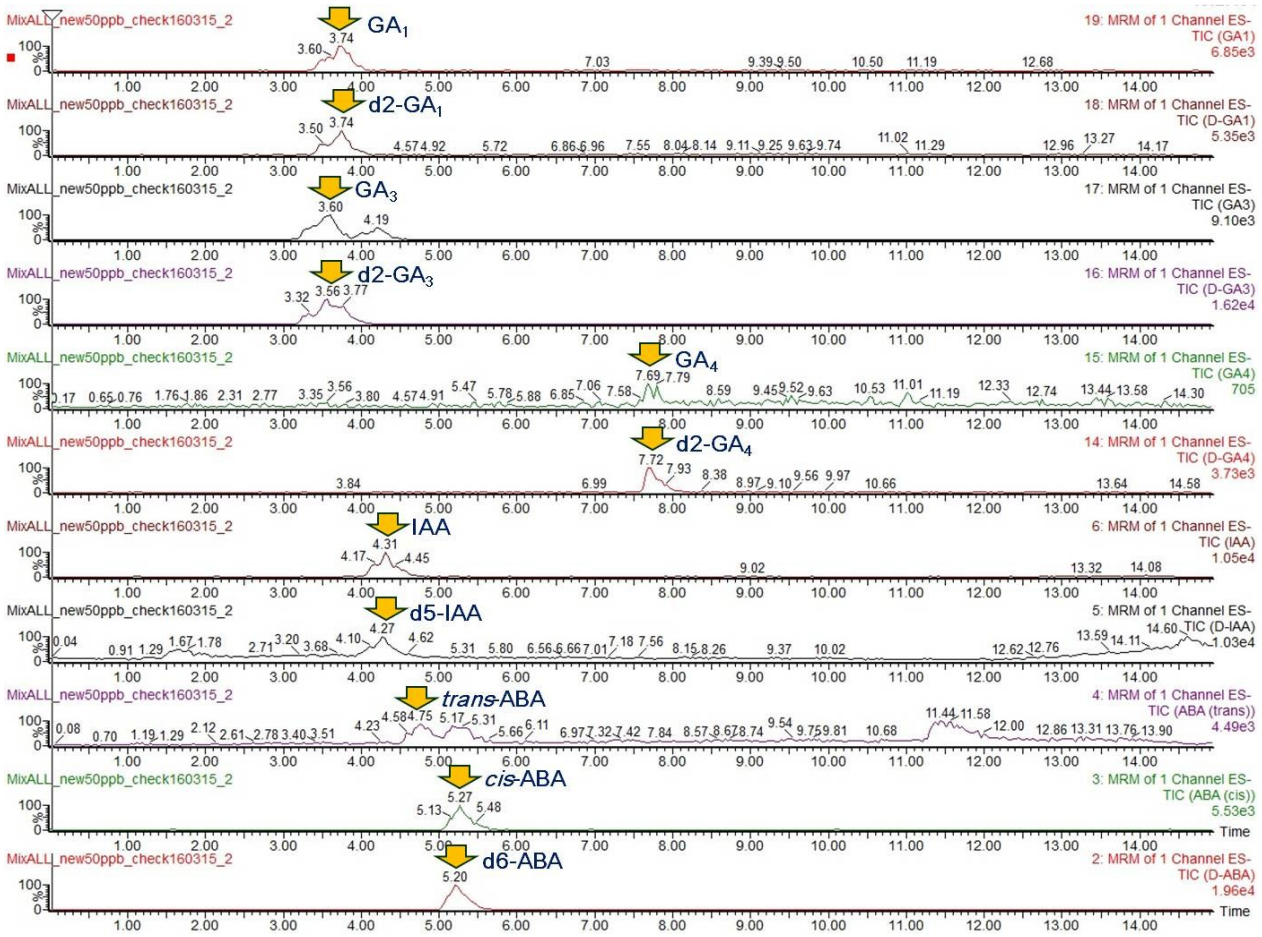
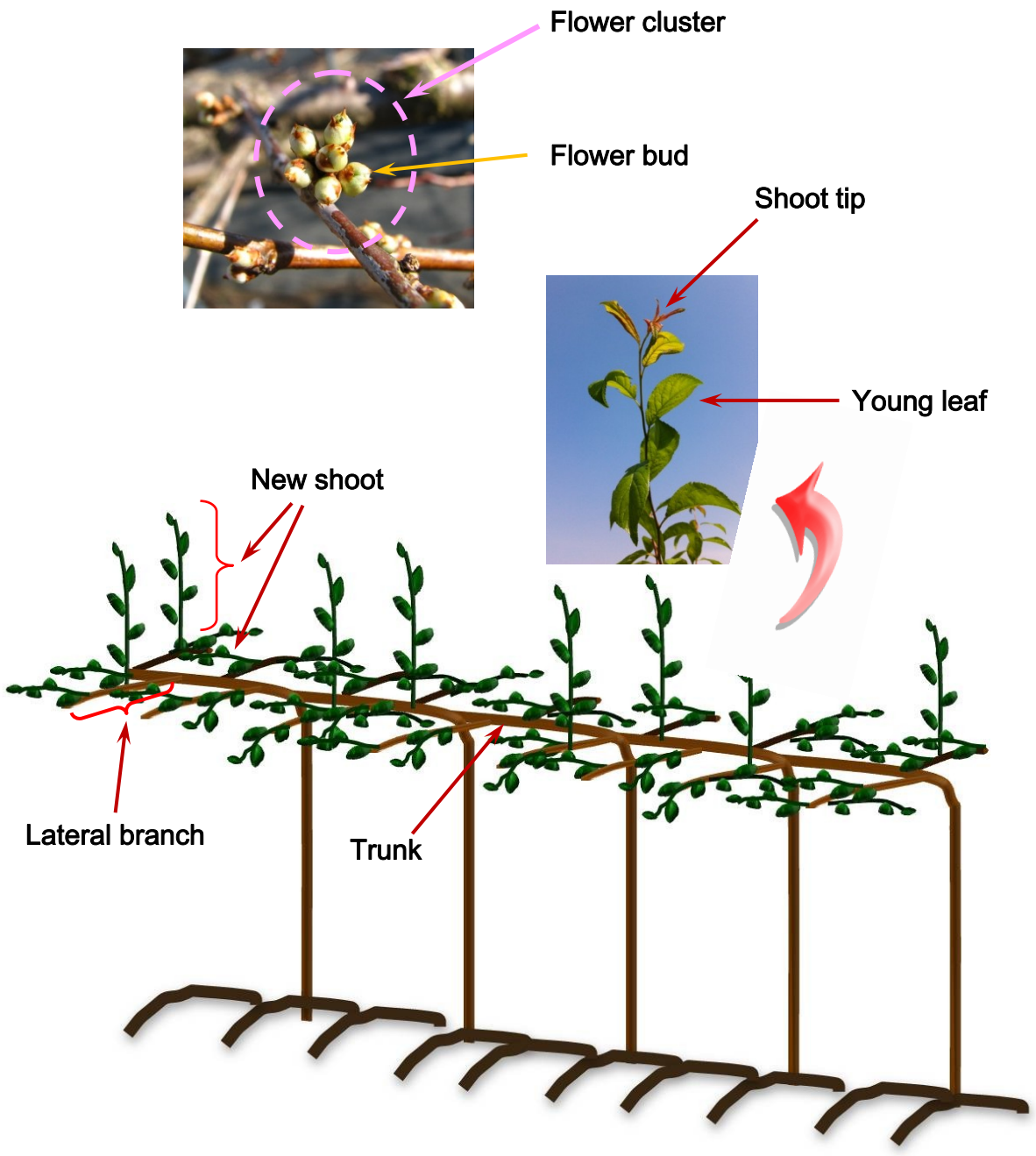
Appendix



Appendix 1 Chromatogram of phytohormones investigated in SIR mode.



Appendix 2 Chromatogram of phytohormones investigated in MRM mode.



Appendix 3 Plant model and detail on sampling and measurement parts

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