

**Relationship between Cell Wall Characteristics and Fruit Texture in
Japanese Pear Cultivars**

(ニホンナシ果実の肉質の品種間差異と果肉の細胞壁組成との関係)

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Chapter 1

General Introduction

Pear, belonging to the genus *Pyrus* (Rosaceae, Pyrinae), is one of the oldest fruit crops and is cultivated worldwide in 85 countries or regions (Wu et al., 2013). Production ranks after just the apple and grape within the perennial deciduous fruit crops (Li et al., 2015). Pear cultivation can be traced back 3,000 years. Within the past 100 years, breeders and cultivators have been selecting and breeding new varieties in order to improve fruit quality and meet consumer demands (Lombard and Westwood, 1987). The Japanese pear (*Pyrus pyrifolia* Nakai), European pear (*Pyrus communis* L.), and Chinese pear (*Pyrus bretschneideri* Rehd., *Pyrus ussuriensis* Maxim.) are the major edible species that are commercially grown for fruit production. The Japanese pear and Chinese pear are cultivated in East Asia, and the European pear is grown in Europe, North America, and temperate regions of the southern hemisphere (Terakami et al., 2006).

The Japanese pear, one of the most important fruit trees in Japan, is also widely distributed in China and Korea (Islam et al., 2013), where more than a thousand cultivars are grown naturally or commercially (Nakajima et al., 2012). The fruits vary in size from very small to very large and are sweet, juicy, and gritty. Although the texture of the Japanese pear is influenced by the cultivation environment, including edaphic factors and climatic conditions (Hayashi and Maclachlan, 1984), it also depends on the cultivar. For example, ‘Kosui’ and ‘Hosui’ have smooth and exquisite flesh, whereas ‘Chojuro’ and ‘Imamuraki’ are rough (Machida and Kozaki, 1975).

The current Japanese pear breeding program began in 1909 (Kajiura and Sato, 1990) and aims to develop new cultivars that ripen at various times and have high productivity, excellent fruit quality, low production costs, high disease resistance, self-compatibility, and freedom from

physiological disorders. To date, no single Japanese pear cultivar possesses all these characteristics. Cultivars with excellent texture, such as ‘Kosui’ pears, have poor productivity and storage potential (Fumuro, 2000; Itai et al., 1999). In contrast, ‘Chojuro’ pears have high productivity but poor texture and storage potential (Machida and Kozaki, 1975).

Black spot disease, which is caused by the Japanese pear pathotype of the filamentous fungus *Alternaria alternata* (Fries) Keissler, is one of the most harmful diseases in Japanese pear cultivation (Terakami et al., 2016). Cultivars that are resistant to black spot disease include ‘Hosui’ and ‘Kosui’. However, the disease is still considered a serious threat to cultivation because all the commercially important cultivars are susceptible (Iketani et al., 2001). Abe and Kurihara (1993) investigated the differences in resistance to this disease among pear species and cultivars and observed that ‘Kinchaku’ is the only Japanese pear cultivar that is highly resistant. Some Chinese pear cultivars and all the European pear cultivars investigated were also highly resistant. Breeding programs to introduce resistance from ‘Kinchaku’ into commercially leading cultivars have been initiated. Still, several generations may be required in order to produce resistance in these cultivars, due to the low fruit quality of the native cultivar (Gonai et al., 2009).

In pear breeding programs, improvement of fruit quality is a main objective. A few reports on the inheritance of fruit characters have dealt with pears (Machida and Kozaki, 1976). It is very important to elucidate the mode of the main characteristics that influence fruit quality in order to increase breeding efficiency. These characteristics are the fruit weight, flesh hardness, soluble solid content, organic acid content, ripening time, and storage potential. In addition, improvement of fruit texture is desirable; the Japanese pear is also called the “sand pear” because of the gritty texture of the traditional cultivars.

The texture of Japanese pear fruit is affected by the flesh hardness (Machida and Maeda, 1966; Machida and Tashiro, 1968) and the density of stone cells (Tao et al., 2009), and the texture is certainly poor in fruit with high flesh hardness (Machida and Maeda, 1966). In addition, the percentage of cell wall materials, which are a major component of dry matter (McNeil et al., 1984; Rose et al., 2003), influences the texture of fruit (Agoda et al., 2012; Cybulska et al., 2010). The ethanol-insoluble solid (EIS) extracted from fruit is usually used to determine the percentage of cell wall materials (Prinzivalli et al., 2006; Zhang et al., 2007).

It is well known that fruit ripening is a complex, genetically programmed process involving marked changes in the color, flavor, aroma, texture, and nutritional content of the fruit (Giovannoni, 2004; Seymour et al., 2002, 2013). Studies have shown that fruit softening involves three sequential steps: loosening of the cell wall mediated by expansions, depolymerization of hemicellulose, and finally polyuronide depolymerization by polygalacturonase or other hydrolytic enzymes (Brummell et al., 1999).

It is necessary to breed new Japanese pear cultivars to meet the demands of the market and of the cultivation environment. However, breeding new cultivars is time-consuming and costly, as seen in other deciduous fruit trees. Therefore, it is important to understand the genetic control of texture differences in Japanese pear cultivars. In the present study, we selected leading Japanese pear cultivars that have different textures in order to determine the changes in EIS content and cell wall composition content during fruit (see Chapter 2). Based on our results, we selected two representative cultivars, rough 'Chojuro' and soft 'Kosui', to observe the accumulation of photosynthetic products (see Chapter 3). We determined the development of stone cell clusters, phenylalanine ammonia lyase (PAL) activity, and peroxidase (POD) activity during fruit growth to

clarify the correlation between the development of stone cells and fruit texture (Chapters 4 and 5).

Chapter 2

Cell Wall Composition during Fruit Growth

2.1 Introduction

Plant cell walls are approximately 30% cellulose (McNeil et al., 1984), 20%-30% hemicellulose (Hayashi, 1989), 35% pectin (Albersheim et al., 1996), and 5% protein (Fry, 1988) in dicotyledonous plants. In fruit cell walls, pectin content is higher and protein content lower than in other plants (Knee and Bartley, 1981). Cellulose microfibrils are coated with and crosslinked together with hemicellulose and the spaces in these networks are filled with pectin, which also forms a network (Brummell and Harpster, 2001; Carpita and Gibeaut, 1993; Talbott and Ray, 1992). During fruit softening, pectin (Fischer and Bennett, 1991) and hemicellulose (Wakabayashi, 2000) typically undergo solubilization and depolymerization that are thought to contribute to cell wall loosening and disintegration. Cell wall polysaccharide breakdown causes ripening-associated softening (Vicente et al., 2007).

Previous studies have demonstrated that the percentage of plant cell wall material influences the texture of fruits and vegetables (Agoda et al., 2012; Cybulska et al., 2010). It is necessary to clarify whether the content or structure of cell walls influences the texture of Japanese pear. Studies have shown that the texture of different Japanese pear cultivars differs. For example, ‘Kosui’ and ‘Hosui’ have soft and exquisite flesh, whereas ‘Chojuro’ and ‘Imamuraki’ are rough (Machida and Kozaki, 1975).

In the present study, we selected six Japanese pear cultivars that have different fruit textures. Our objective was to compare the EIS content and cell wall polysaccharide content during fruit growth in Japanese pear cultivars, in order to characterize the changes in cell walls during the

ripening process in Japanese pear.

2.2 Materials and Methods

2.2.1 Fruit Materials

Fresh Japanese pear fruits harvested from ‘Kosui’, ‘Gold-Nijisseiki’, ‘Chojuro’, ‘Hosui’, ‘Zuisyu’, and ‘Ousyu’ trees grown at the farm of Tottori University, Tottori, Japan (35.5°N, 134.2°E) were used as experimental materials from early May (30 days after onset of flowering) until harvest periods in 2012 and 2013.

2.2.2 Treatment for Determination of Cell Wall Composition

Hardness was measured with Rheometer (RT-3010D, Rheotech, Tokyo, Japan). The skin was removed from the equator of fruit and the hardness was measured with a flat-tipped probe (0.5 cm diameter). Values were expressed as Newton (N).

EIS were prepared according to the method of Huber (1984) by homogenizing (Ultra Turrax, Tokyo, Japan) flesh samples in ethanol to a final concentration of 80% (v/v). The homogenate was boiled at 90°C for 20 min and filtered through Miracloth (Calbiochem), treated with chloroform:methanol (1:1, v/v) for 30 min, and then filtered with 80% (v/v) cold ethanol and 100% (v/v) acetone. The residual ethanol was evaporated and the EIS was oven dried at 37°C (Tokyo Rikakikai Co. LTD, Tokyo, Japan), and weighed using an electronic balance (Mettler Toledo, Switzerland). And then the residues were stored in a desiccator at 25°C.

Soluble pectin fractions and 4 and 24% (w/v) KOH hemicellulose fractions of EIS were isolated as described by Cheng and Huber (1996) and Maclachlan and Brady (1994), respectively. Briefly, the EIS were sequentially extracted with deionized water in 0.02% (w/v) sodium-azide for

4 h at 25°C , 50 mM ethylenediaminetetraacetic acid (EDTA) in 50 mM sodium acetate (pH 6.5) for 4 h at 25°C, 50 mM Na₂CO₃ containing 20 mM Na-borohydride for 4 h at 4°C, 90% (v/v) dimethyl sulphoxide (DMSO) for 4 h at 25°C, 4% (w/v) KOH containing 0.1% (w/v) Na-borohydride for 24 h at 25°C, and 24% (w/v) KOH containing 0.1% (w/v) Na-borohydride for 24 h at 25°C, respectively. All isolations were conducted with continuous stirring. At the end of each extraction, the mixture was separated by centrifuge (15,000 ×g, 30 min) and the supernatant filtered through glass microfibers (GF/C, Whatman, Middlesex, England). Lignin was obtained after the 24% (w/v) KOH treatment with 72% (v/v) sulfuric acid, and the residues oven dried at 37°C (Tokyo Rikakikai Co. LTD, Tokyo, Japan) and weighed. The cellulose content was determined by subtracting the weight of lignin from the residue post 24% (w/v) KOH treatment.

Total sugar content in the EIS was determined by the phenol-sulfuric acid method (Dubois et al., 1956), with slight modifications. Briefly, 500 µL specimens were incubated with 500 µL of 5% (w/v) phenol and 2.5 mL of 98% (v/v) H₂SO₄. Total sugar was then determined using a spectrophotometer (Hitachi, Tokyo, Japan) at an absorbance of 490 nm.

2.2.3 Statistical Analysis

Statistical analysis of the data was carried out by one-way analysis of variance, and the means were separated by Tukey's test using the SPSS software package for Windows version 17.0. A probability of p>0.05 was considered non-significant.

2.3 Results and Discussion

Table 2-1 Flesh hardness of six Japanese pear cultivars at the harvest periods.

Tested cultivars	Flesh hardness (N)	
	2012	2013
Chojuro	26.3 a ^z	31.6 a
Gold-Nijisseiki	22.8 b	27.0 b
Zuisyu	23.5 b	18.9 d
Hosui	15.0 c	18.0 d
Kosui	14.3 c	21.7 c
Ousyu	12.2 d	14.4 e

^z Different letters indicate significantly difference ($P < 0.05$, Tukey's test; $n = 10$).

In this study, flesh hardness differed based on cultivars (Table 2-1), and was significantly higher in the rough 'Chojuro' and 'Gold-Nijisseiki' than in the soft 'Kosui' and 'Hosui' pears. Although 'Kosui' and 'Hosui' have soft and exquisite flesh, understanding the relationship between cultivar effect and seasonal changes in cell wall structure is needed for developing a new variety. Because the percentage of plant cell wall material influences the texture of fruit (Hamauzu and Mizuno, 2011).

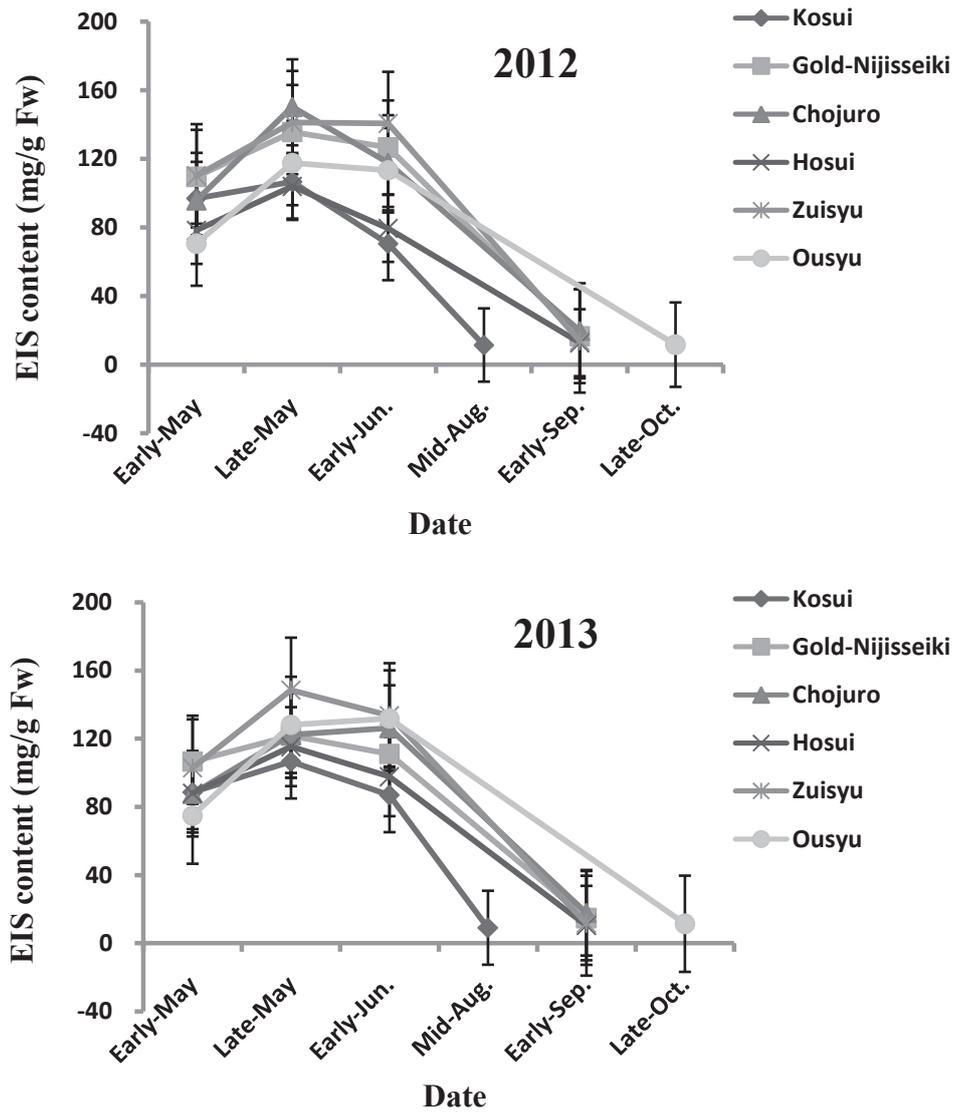


Fig. 2-1 EIS content in six Japanese pear cultivars during fruit growth.

(Vertical bar indicates standard error, n = 5).

Table 2-2 EIS content, cellulose content and lignin content in late-May, early-June, and harvest periods in 2012 and 2013.

Tested Cultivars	Late-May				Early-Jun.				Harvest periods ^z			
	EIS (mg/g Fw)	Cellulose (mg/g Fw)	Lignin (mg/g Fw)		EIS (mg/g Fw)	Cellulose (mg/g Fw)	Lignin (mg/g Fw)		EIS (mg/g Fw)	Cellulose (mg/g Fw)	Lignin (mg/g Fw)	
2012	Chojuro	150.2 a ^y	66.9 a	31.9 b	117.7 b	69.3 b	22.2 b		19.8 a	8.1 a	3.7 a	
	Gold-Nijisseiki	135.6 b	23.1 c	35.3 a	126.6 b	60.1 c	32.2 a		16.7 b	5.1 bc	1.6 b	
	Zuisyu	141.1 ab	45.4 b	22.4 d	140.6 a	84.6 a	13.4 d		13.8 c	5.8 bc	1.0 cd	
	Hosui	103.9 c	10.4 e	24.9 cd	79.4 c	24.7 e	18.0 c		12.8 cd	4.6 c	0.5 e	
	Kosui	106.5 c	14.3 d	21.8 e	70.6 c	13.1 f	14.8 d		11.5 d	6.2 b	1.1 c	
2013	Ousyu	117.5 c	24.7 c	26.7 c	113.3 b	49.6 d	19.5 bc		11.7 d	3.0 d	0.9 d	
	Chojuro	122.2 b	59.2 a	19.7 d	126.2 a	57.4 b	41.5 a		17.9 a	7.7 a	3.1 a	
	Gold-Nijisseiki	121.8 b	37.6 c	28.9 a	111.1 b	48.1 c	27.2 b		14.8 b	8.2 a	2.7 b	
	Zuisyu	148.5 a	58.1 a	25.7 b	133.6 a	40.8 e	38.8 a		11.7 c	4.0 e	1.6 c	
	Hosui	115.3 c	49.4 b	13.7 e	97.7 c	44.4 d	23.9 c		10.5 c	5.4 c	1.3 d	
2013	Kosui	106.6 c	35.6 c	22.8 c	86.8 c	25.6 f	24.9 bc		9.1 d	4.2 de	1.0 e	
	Ousyu	128.1 b	57.6 a	29.1 a	131.8 a	73.0 a	35.0 a		11.4 c	6.6 b	1.4 cd	

^z Kosui---Mid-August; Chojuro, Gold-Nijisseiki, Zuisyu, and Hosui---Early-September; Ousyu---Late-October.

^y Different letters indicate significantly difference (P < 0.05, Tukey's test; n = 5).

In this study, EIS content was highest during late May and early June in all cultivars (Fig. 2-1 and Table 2-2), consistent with the findings of previous studies where alcohol-insoluble solid contents were higher at the immature stage rather than at the ripe stage (Hamauze and Mizuno, 2011). Redgwell *et al* (1997) also showed that cell wall content of fruit was higher during the unripe period than when ripe. In the present study, EIS content varied significantly between cultivars from early May to early June especially in both late May and early June (Table 2-2) which occurred during the pre-enlargement period (Yamaki *et al.*, 1979), was significantly higher in ‘Chojuro’, ‘Gold-Nijisseiki’, ‘Zuisyu’, than in soft ‘Hosui’ and ‘Kosui’ pears. At harvest periods, the content was higher in rough ‘Chojuro’ and ‘Gold-Nijisseiki’ than in soft ‘Hosui’ and ‘Kosui’ pears.

The composition of plant primary cell wall varies significantly from one cell type to another, but the cell wall is basically a biphasic structure consisting of cellulose microfibrils embedded in a matrix of non-cellulosic polysaccharides (i.e., hemicellulose) and pectin (Carpita and Gibeaut, 1993). The cell wall also contains polymer, such as lignin, cutin, and suberin, depending on the plant organ, location within the organ, and stage of development (Doblin *et al.*, 2010). Further, changes in the components and structure of the cell wall, particularly due to solubilization and depolymerization of pectin and hemicellulose (Brummell and Harpster, 2001), partial disassembly of hemicellulose and the cellulose network in the cell wall, cause changes in fruit texture during the ripening process (Voragen *et al.*, 1995).

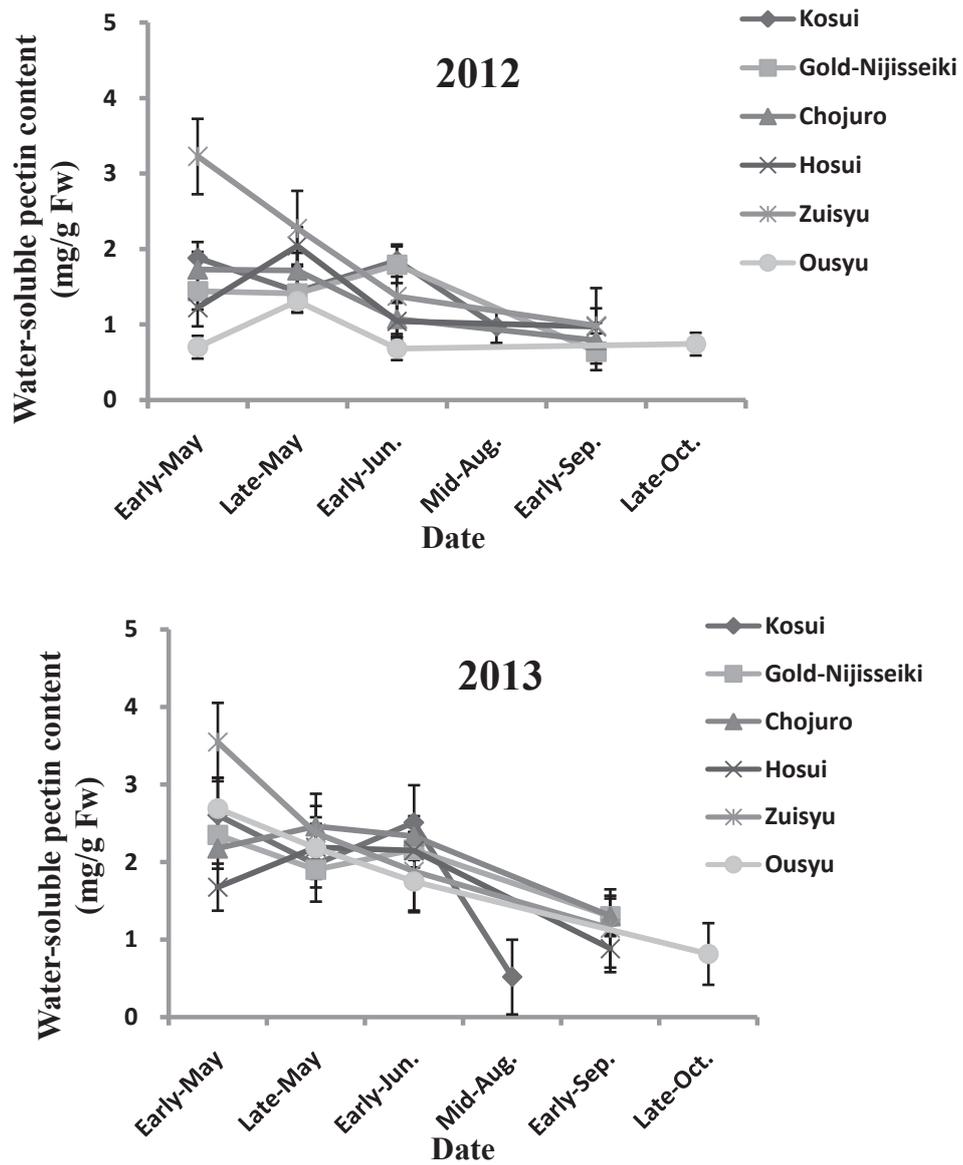


Fig. 2-2 Water-soluble pectin content in six Japanese pear cultivars during fruit growth.
 (Vertical bar indicates standard error, n = 5).

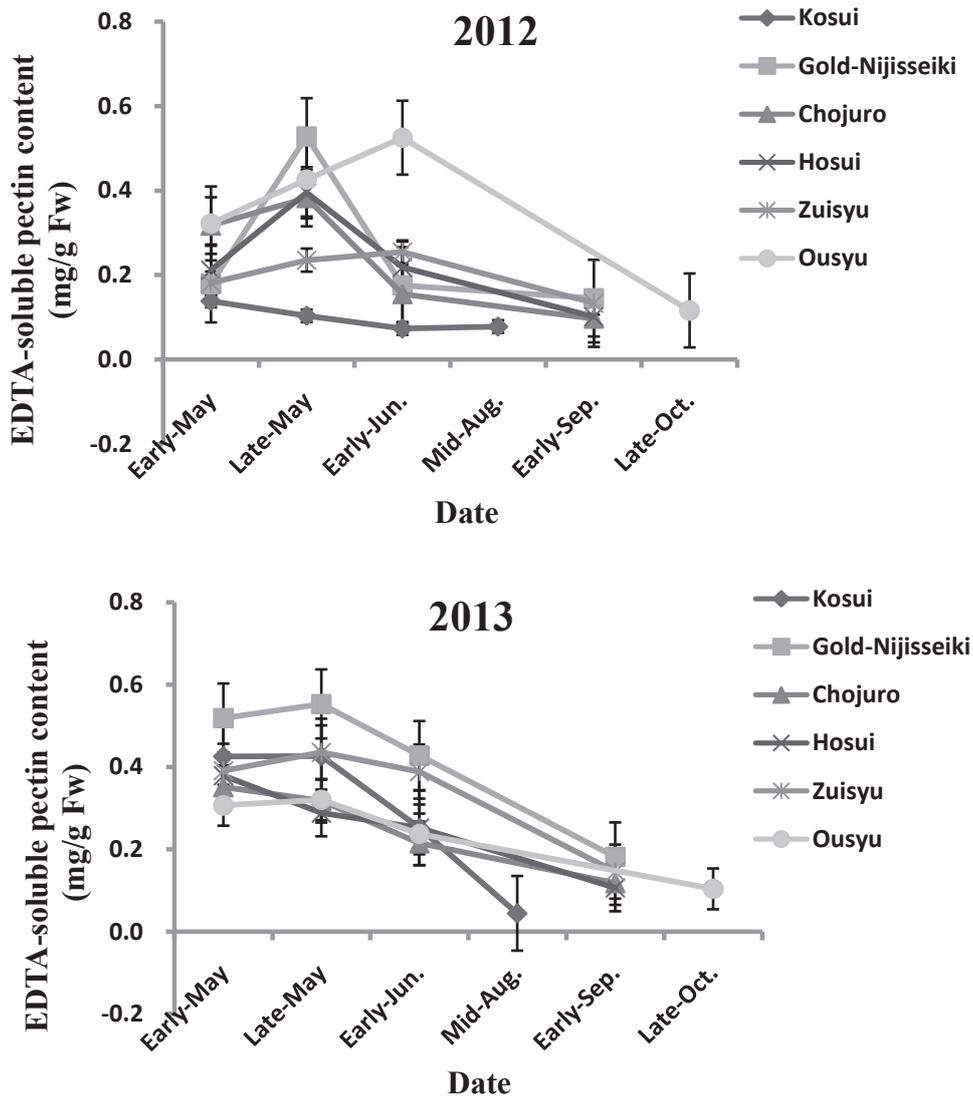


Fig. 2-3 EDTA-soluble pectin content in six Japanese pear cultivars during fruit growth.
 (Vertical bar indicates standard error, n = 5).

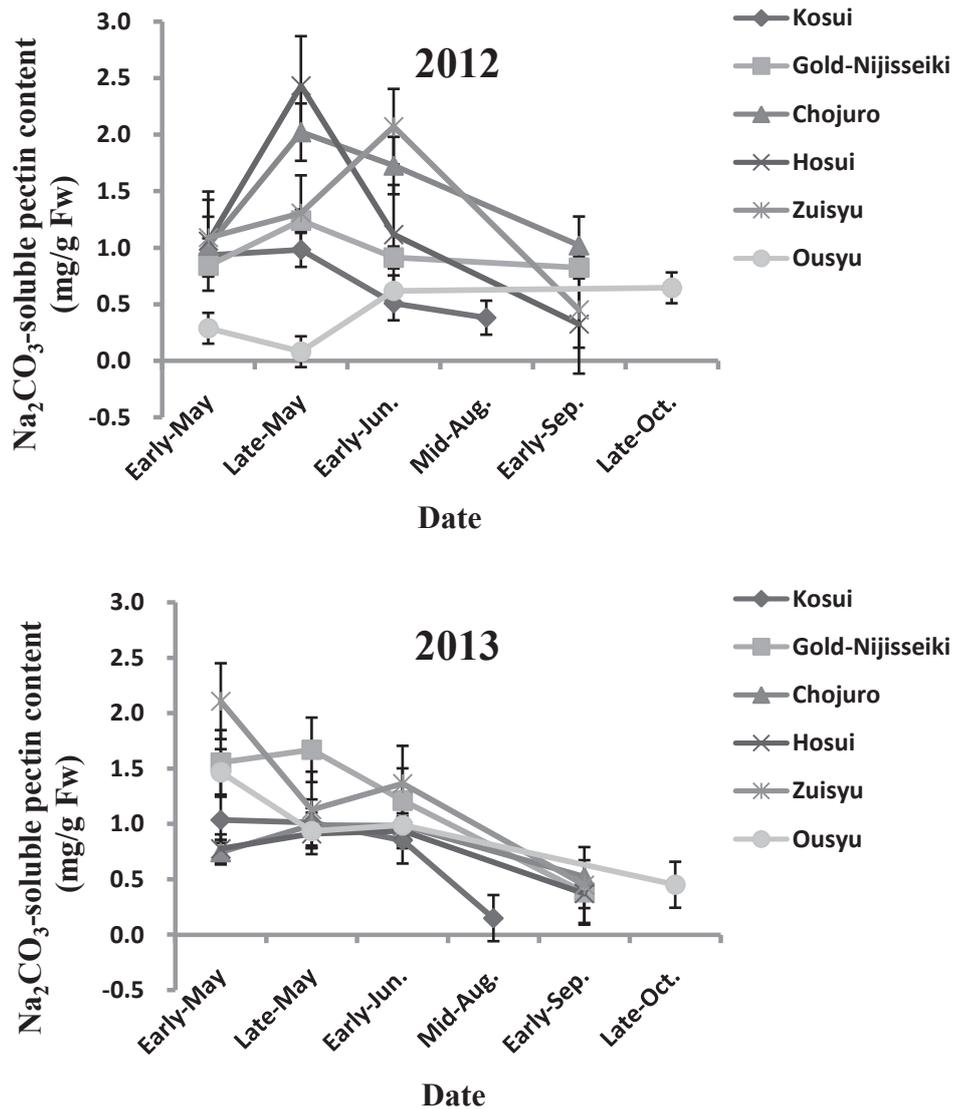


Fig. 2-4 Na_2CO_3 -soluble pectin content in six Japanese pear cultivars during fruit growth.
(Vertical bar indicates standard error, $n = 5$).

In this study, soluble pectin content (water, EDTA, and Na_2CO_3) in the cultivars generally (but many exceptions in Fig. 2, especially see 2012) decreased during fruit growth (Fig. 2-2, Fig. 2-3, and Fig. 2-4). No differences in soluble pectin contents were observed among the cultivars in the whole growth periods. We inferred that although pectin is the cell wall component that changes most during fruit softening, but their role in fruit hardness is considered controversial. The studies in Paniagua *et al* (2014) also provided foundation for our conclusion.

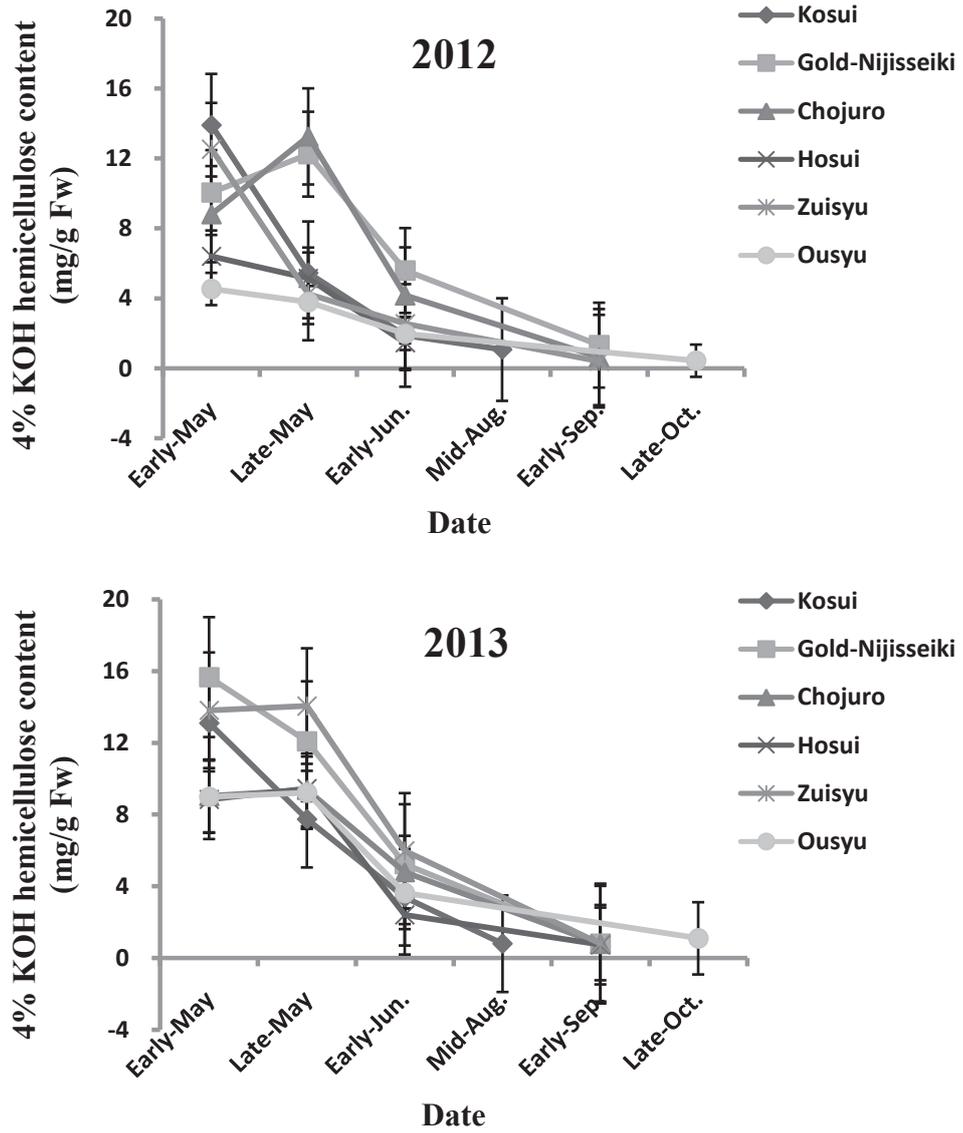


Fig. 2-5 4% KOH hemicellulose content in six Japanese pear cultivars during fruit growth. (Vertical bar indicates standard error, n = 5).

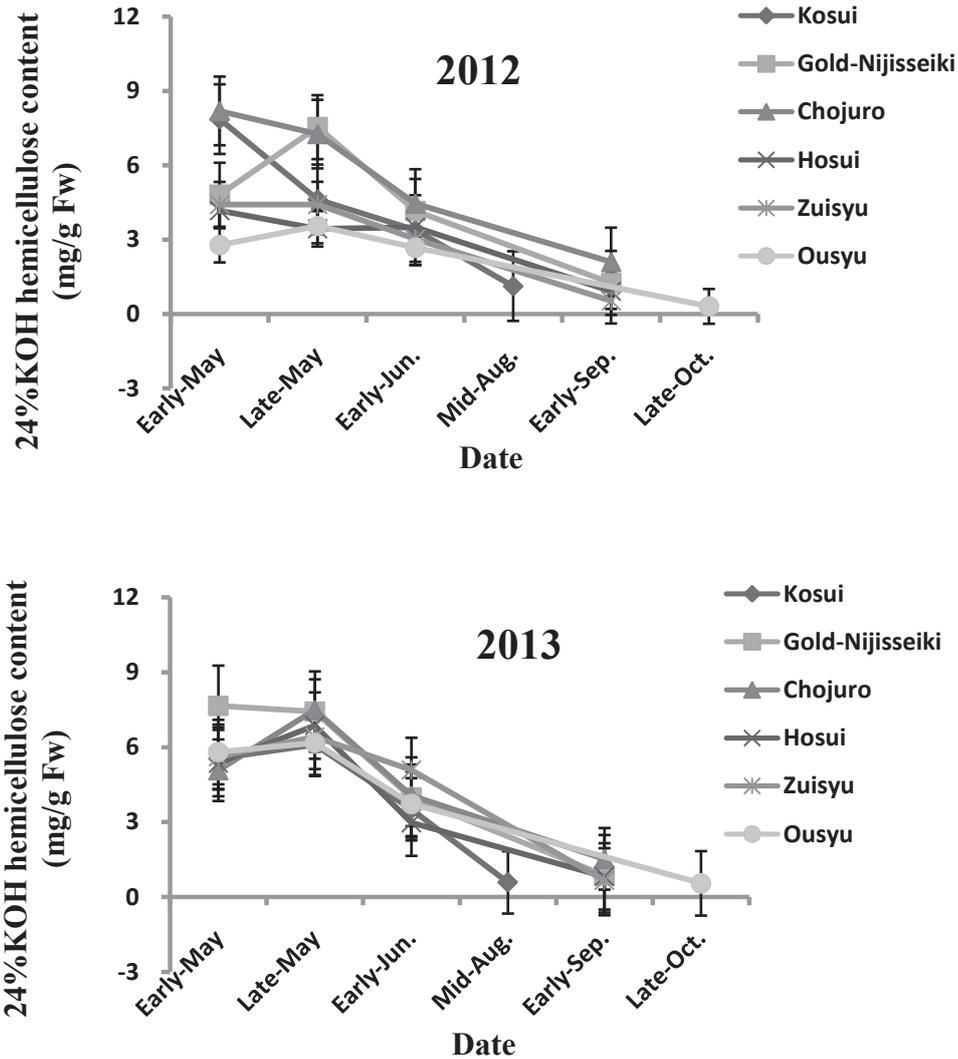


Fig. 2-6 24% KOH hemicellulose content in six Japanese pear cultivars during fruit growth.

(Vertical bar indicates standard error, n = 5).

In this study, the 4% (w/v) KOH hemicellulose and 24% (w/v) KOH hemicellulose content decreased from late May until to harvest periods (Fig. 2-5 and Fig. 2-6). 4% (w/v) KOH hemicellulose content was higher in rough ‘Chojuro’ and ‘Gold-Nijisseiki’ than in soft ‘Hosui’ and ‘Kosui’ pears during late May and early June. For 24% (w/v) KOH hemicellulose content, we couldn’t find differences in 2013 although it was higher in rough ‘Chojuro’ and ‘Gold-Nijisseiki’ than in the others in late May of 2012. Differences were not noted at harvest periods consistent with

the findings of Murayama *et al* (2002) who suggest no significant difference in hemi-cellulosic polysaccharide between rough and soft pears.

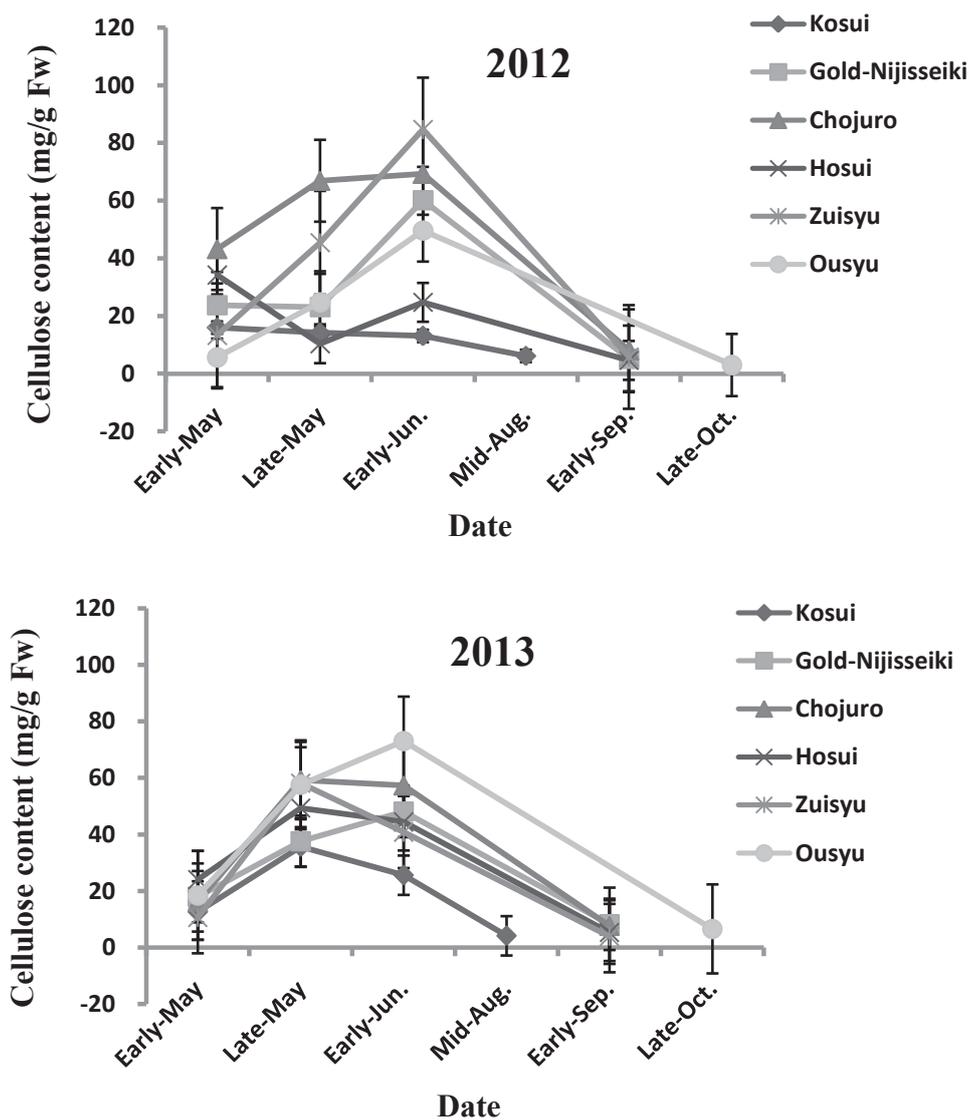


Fig. 2-7 Cellulose content in six Japanese pear cultivars during fruit growth.

(Vertical bar indicates standard error, n = 5).

In this study, cellulose content was highest during late May and early June (Fig. 2-7 and Table 2-2), the significant differences between the cultivars were almost noted during the whole growth stages. Take harvest periods as example, the content was higher in ‘Chojuro’ than in ‘Zuisyu’, ‘Kosui’, ‘Hosui’, and ‘Ousyu’ pears. Cellulose content was higher in rough ‘Chojuro’ than in soft ‘Hosui’ and ‘Kosui’ pears (Table 2-2).

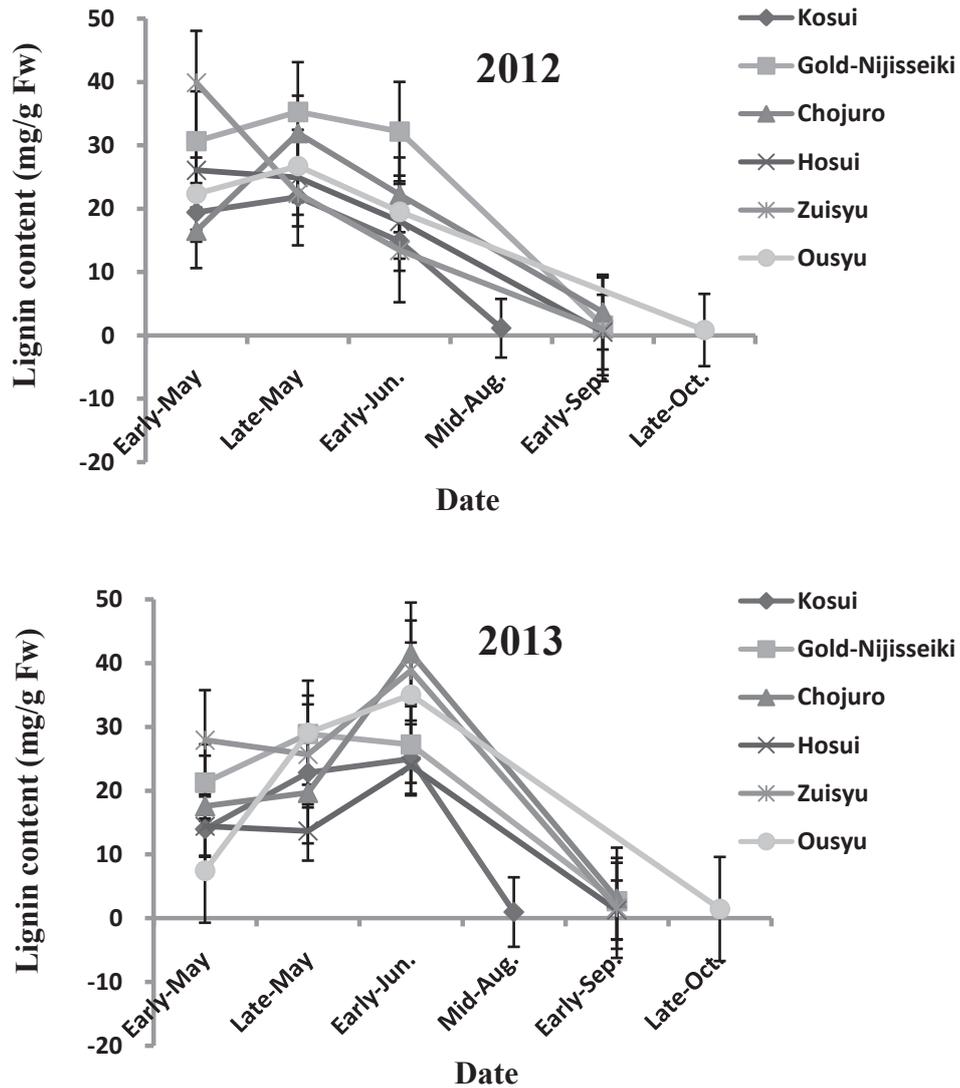


Fig. 2-8 Lignin content in six Japanese pear cultivars during fruit growth.
(Vertical bar indicates standard error, n = 5).

In this study, lignin content was highest during late May and early June (Fig. 2-8 and Table 2-2), and significant differences between cultivars were noted. Consistent with the previous studies which showed that fruit lignin content varies according to the stages of plant maturity and species (Whetten and Sederoff, 1995). At harvest periods, the content was higher in rough ‘Chojuro’ and ‘Gold-Nijisseiki’ than in soft ‘Hosui’ and ‘Kosui’ pears (Table 2-2). Further, the content was higher in rough ‘Gold-Nijisseiki’ than in soft ‘Hosui’ and ‘Kosui’ pears almost in the whole growth stages,

consistent with the conclusion that tissue lignifications may result in greater flesh firmness, and toughness of the texture in fruit (Shan et al., 2008).

2.4 Summary

Our data showed that the flesh hardness of Japanese pear fruit at harvest differed according to cultivars. It was significantly higher in the rough ‘Chojuro’ and ‘Gold-Nijisseiki’ than in the soft ‘Kosui’ and ‘Hosui’ pears. Further, we found that EIS content was significantly higher in rough texture cultivars than in soft texture cultivars and the content in late May and early June influenced the content at harvest and influenced flesh hardness. Cellulose content and lignin content strongly influenced EIS content, especially during late May and early June.

Chapter 3

Studies on Accumulation of Photosynthetic Products

3.1 Introduction

EIS content is a major component of dry matter in Japanese pear (McNeil et al., 1984; Rose et al., 2003). Previous studies showed that the amount of EIS during late May to early June indirectly influenced the flesh hardness at harvest times. The EIS content was higher in Japanese pear cultivars with higher flesh hardness. It is well known that fruit growth is a process of cell division and enlargement, accompanied by dry matter accumulation and an increase in water content in fruit. Approximately 40% of a plant's dry matter consists of carbon, fixed in photosynthesis, which is the most fundamental biological process in plants (Feng et al., 2015). This process is vital for the growth and survival of virtually all plants during the major part of their growth cycle. The accumulation of dry matter in fruit is a result of the integrated processes of photosynthetic carbon assimilation and subsequent partitioning and utilization of photo-assimilates throughout the entire growth period. The efficient translocation of photosynthetic products to growing fruit is one of the key factors affecting yield.

Using ^{13}C or ^{14}C carbon labeling, it has been shown that in annual crops such as soybean (Yamagata et al., 1987), wheat (Austin et al., 1977), and barley (Bidinger et al., 1977), different proportions of carbon assimilated at different growth stages contributed to seed production. Currently assimilated carbon during the seed-filling stage is the major source of carbon for seed production. However, little information is available on the contribution of photosynthates fixed at different growing stages to fruit in fruit trees. However, it is possible to trace the fate of photoassimilates in fruit at particular phases of fruit growth.

In the present chapter, we determined the accumulation of photosynthetic products in late May in the rough 'Chojuro' and soft 'Kosui' cultivars. Our aim is to understand the effect of the accumulation of photosynthetic products on EIS content during early growth periods in Japanese pear fruit.

3.2 Materials and Methods

3.2.1 Fruit Materials

Fresh Japanese pear fruits and leaves harvested from ‘Kosui’ and ‘Chojuro’ trees grown at the farm of Tottori University, Tottori, Japan (35.5°N, 134.2°E) were used as experimental materials in mid-May.

3.2.2 Treatment for Determination of Photosynthetic Products

¹³C labeling was conducted according to Teng (1998), with slight modifications. Briefly, healthy, uniform fruiting spurs without bourse shoots on the lateral branch were selected for ¹³C labeling in mid-May. Individual spurs were exposed to ¹³CO₂ by enclosing them in a polyethylene bag that contained a 20 mL glass vial fixed on the frame of the bag. The ¹³CO₂ was generated by injecting 1mL of 80% (v/v) lactic acid onto 1 g Ba¹³CO₃, which had an abundance of 99% ¹³C. To ensure uniform labeling among the spurs, 2 h after the start of ¹³C labeling, unlabeled CO₂ was produced by injecting lactic acid into another vial containing 1 g BaCO₃. Each labeling experiment occurred under ambient field conditions with clear skies and lasted for 3 h between 07:00 and 10:00 h. Spurs were harvested 1 week after ¹³C labeling, and separated into leaves and fruits, then stored on ice and brought to the laboratory. Fruits were divided into flesh and cores. The flesh was subjected to EIS treatment as previously described (Zhang et al., 2007). The cores and leaves were oven dried at 90°C for 3 days. The dried materials were finely ground in a coffee mill and stored in glass vials for ¹³C analyses.

The ¹³C and total carbon content of ‘Kosui’ and ‘Chojuro’ were determined using an infrared

$^{13}\text{CO}_2$ analyzer (Flash EA1110 and DELTA plus Advantage, Thermo Fisher Scientific K.K., Tokyo, Japan) according to Zhang *et al* (2005). The absolute amounts (mg) of labeled ^{13}C recovered in each organ were calculated as total carbon in each organ \times ^{13}C atom%. The total amount of ^{13}C taken up by spur leaves in 2 h was calculated by the sum of the amount of ^{13}C recovered in individual organs. The rate of ^{13}C accumulation in fruit was expressed as $\text{mg fruit}^{-1}\text{h}^{-1}$.

3.2.3 Statistical Analysis

Statistical analysis of the data was carried out by one-way analysis of variance, and the means were separated by Tukey's test using the SPSS software package for Windows version 17.0. A probability of $p > 0.05$ was considered non-significant.

3.3 Results and Discussion

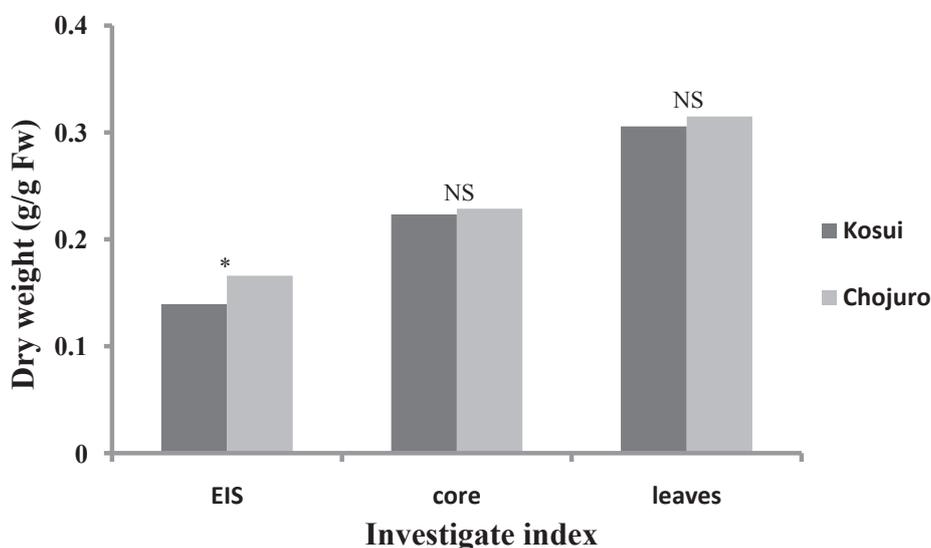


Fig. 3-1 Dry weight of three investigate indices from 'Kosui' and 'Chojuro' in mid-May.

(*and NS: indicate significant difference and no significant difference, respectively, $P < 0.05$, t-test; $n = 4$).

In this study, EIS content was higher in the rough 'Chojuro' than in the soft 'Kosui' cultivars, but no difference was noted in dry weight of cores or leaves (Fig. 3-1). Previous studies have shown that photosynthesis products of Japanese pear begin to accumulate in early May, when fruit growth is not highly active (Teng, 1998). Importantly, photosynthesis is the basis of fruit production and quality. Moreover, some reports have shown that photosynthetic rate, light saturation point, and light compensation point of pear fruits vary by cultivar (Fan and Li, 2006).

Table 3-1 Amount of ^{13}C a week later after labeling in mid-May.

Tested cultivars	Amount of ^{13}C (mg)		
	Residue	Starch	Core
Kosui	0.83	0.25	0.65
Chojuro	2.61	0.13	1.34
Significance	*	NS	*

* Significant difference ($P < 0.05$, t-test; $n = 4$).

NS: no significant difference ($P < 0.05$, t-test; $n = 4$).

In this study, in order to study whether there is effect of accumulation of photosynthetic products on pear texture or not, we did the studies by ^{13}C labeling. The results showed that ^{13}C content in the residue and cores was higher in rough ‘Chojuro’ pears than in soft ‘Kosui’ pears (Table 3-1). ‘Chojuro’ pears showed higher accumulation of photosynthetic products compared with ‘Kosui’. We concluded that accumulation of photosynthetic products during the early growing season was one of important factors for EIS content differences.

3.4 Summary

We determined that both ^{13}C content and EIS content were higher in 'Chojuro' than in 'Kosui' in late May. We inferred that the accumulation of photosynthetic products during the early growing season strongly influences the EIS content differences.

Chapter 4

Studies on Development of Stone Cell

4.1 Introduction

Stone cells are a type of sclerenchyma cell formed by the secondary deposition of lignin on the primary walls of parenchyma cells (Rogers and Campbell, 2004). They are a crucial factor influencing the internal quality of pear fruit (Cai et al., 2010), including not only sucrose content, but also flesh hardness, adhesiveness, and chewiness (Choi et al., 2007; Dibuz, 1998; Yan et al., 2014). Ranadive and Haard (1973) concluded that stone cells are lignocellulosic, containing approximately 18% lignin and 82% of a material principally composed of carbohydrates. They are present in pulp in both isolated and aggregated forms (Boerjan et al., 2003; Chang et al., 2006; Donaldson, 2001).

Smith (1935) established that the quantity of stone cells in a pear depends primarily on the extent to which stone cell formation occurs during the early stages of fruit development. In addition, Nii (1980) suggested that the density of stone cells in fruit flesh tends to be highest after cell division has ceased. A stone cell derives from a parenchyma cell with lignification, called a stone cell primordium, which appears in the flesh of the fruit about 15 days after full bloom (DAFB). Stone cells form clusters within 60 DAFB and remain at a constant level by two months before maturity (Liu et al., 2005; Qiao et al., 2005). Other studies have shown that the number of stone cells was highest at 47 DAFB (Wang et al., 2013). The early growth stages are thus clearly important for the development of stone cells.

Studies have examined the development of stone cell clusters in pear fruits (Choi and Lee, 2013; Tian et al., 2011). However, no studies have explored the effect of stone cell clusters on

different Japanese pear cultivars. In addition, there have been no previous studies of the relationship between stone cell clusters and EIS content; we found that EIS content in early growth periods indirectly played an important role in flesh hardness (Chapter 3). Studies exploring whether or how stone cell clusters play a role in EIS content during the early growth stages are essential for understanding Japanese pear fruit development.

In the previous study, we examined the pear cultivars ‘Chojuro’, ‘Zuisyu’, ‘Gold-Nijisseiki’, ‘Hosui’, and ‘Kosui’. We previously determined that the EIS content in these cultivars during early growth periods influences flesh hardness at harvest (see Chapter 3). We determined the development of stone cell clusters at 30, 45, and 60 DAFB (In Tottori, nearly all the tested cultivars reached the full-bloom stage in mid to late April, and ‘Kosui’ reached the harvest stage earlier, in mid-August, compared with late August to early September for the other cultivars).

4.2 Materials and Methods

4.2.1 Fruit Materials

The rough cultivars ‘Chojuro’, ‘Zuisyu’, ‘Gold-Nijisseiki’, smooth cultivars ‘Kosui’, and ‘Hosui’, flesh hardness is 26.3 N, 23.5 N, 22.8 N, 14.3 N, and 15.0 N, respectively, in which the EIS content during the early growth period influences the EIS content at harvest periods and determines flesh hardness (Chapter 3), were employed in this study. Ten fruits were collected from three trees of each cultivar at development stages of 30 DAFB, 45 DAFB, and 60 DAFB. All fruit were obtained from the orchard in Tottori University, Japan (35.5°N, 134.2°E). After selecting for uniformity of size and freedom from defects, the harvested fruits were transported to the laboratory in plastic boxes packed with ice.

4.2.2 Treatment for Morphological Observation

Small cubes of tissue were cut from the flesh of Japanese pears and fixed with FAA fixative solution [70% (v/v) ethanol, formaldehyde, and acetic acid (8:1:1, v/v/v)]. Pulp treatment was carried out as described by Fukuda (2009), with slight modifications. Briefly, the cubes were washed using running water for 24 h. After dehydration in a graded series of ethanol [30% (v/v), 50% (v/v), 70% (v/v), 80% (v/v), and 96% (v/v)] for 2 h, and in 100% (v/v) ethanol for 1 h. Tissues were embedded in Technovit 7100 resin (Kulzer and Co., Wehrheim, Germany) by placing them in a solution of ethanol and Technovit 7100 resin (1:1, v/v) for 1 h, and then in Technovit 7100 resin (1:1, v/v) for 12 h. Subsequently, the tissues were infiltrated and embedded in an embedding medium [0.9 mL of Technovit hardener II, 15 mL of Technovit 7100 resin, and Technovit

hardener I (100:1, v/w)] at 25°C and then oven dried at 37°C (Tokyo Rikakikai Co. LTD, Tokyo, Japan) for 1 h. For biological microscopy, the samples were cut to a thickness of 2 µm using a microtome (NS-31, Japan) and stained with 0.03% (v/v) toluidine blue. The stained sections were observed under a microscope to confirm the existence of stone cells in the flesh. The formation and development of cell walls were also examined and photographed using a biological microscope (ECLIPSE E200, Nikon, Japan). The images were analyzed using Image J software (National Institute of Health, United States).

4.2.3 Statistical Analysis

Statistical analysis of the data was carried out by one-way analysis of variance, and the means were separated by Tukey's test using the SPSS software package for Windows version 17.0. A probability of $p > 0.05$ was considered non-significant.

4.3 Results and Discussion

Appendix Date of 30, 45, and 60 days after full bloom for the tested cultivars.
(Horticultural research center in Tottori, Japan. 1997-2004).

Tested Cultivars		Days after full bloom (DAFB)		
		30	45	60
Rough	Chojuro	5月14日	5月29日	6月13日
	Zuisyu	5月15日	5月30日	6月14日
	Gold-Nijisseiki	5月13日	5月28日	6月12日
Smooth	Kosui	5月16日	5月31日	6月15日
	Hosui	5月12日	5月27日	6月11日

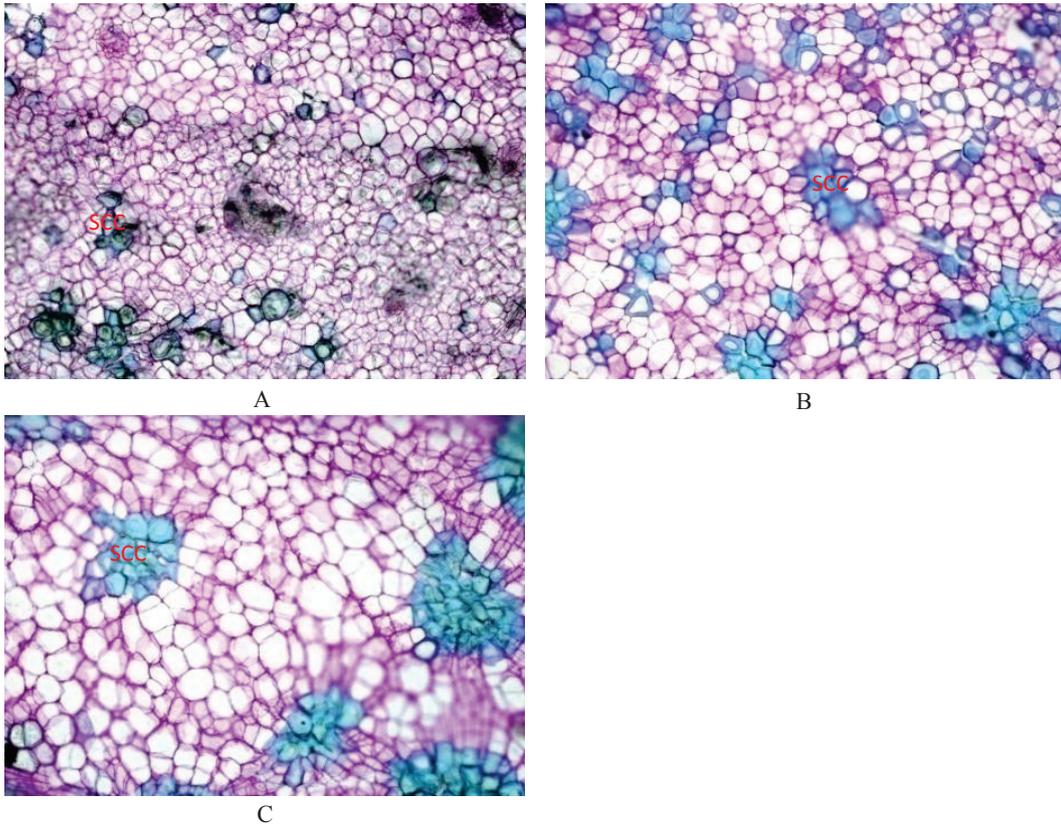


Fig. 4-1 Changes in flesh tissue observed by biological microscopy (100×) at 30 DAFB (A), 45 DAFB (B), and 60 DAFB (C) in the 'Zuisyu' pear cultivar. (DAFB: days after full bloom. SCC: stone cell clusters.)

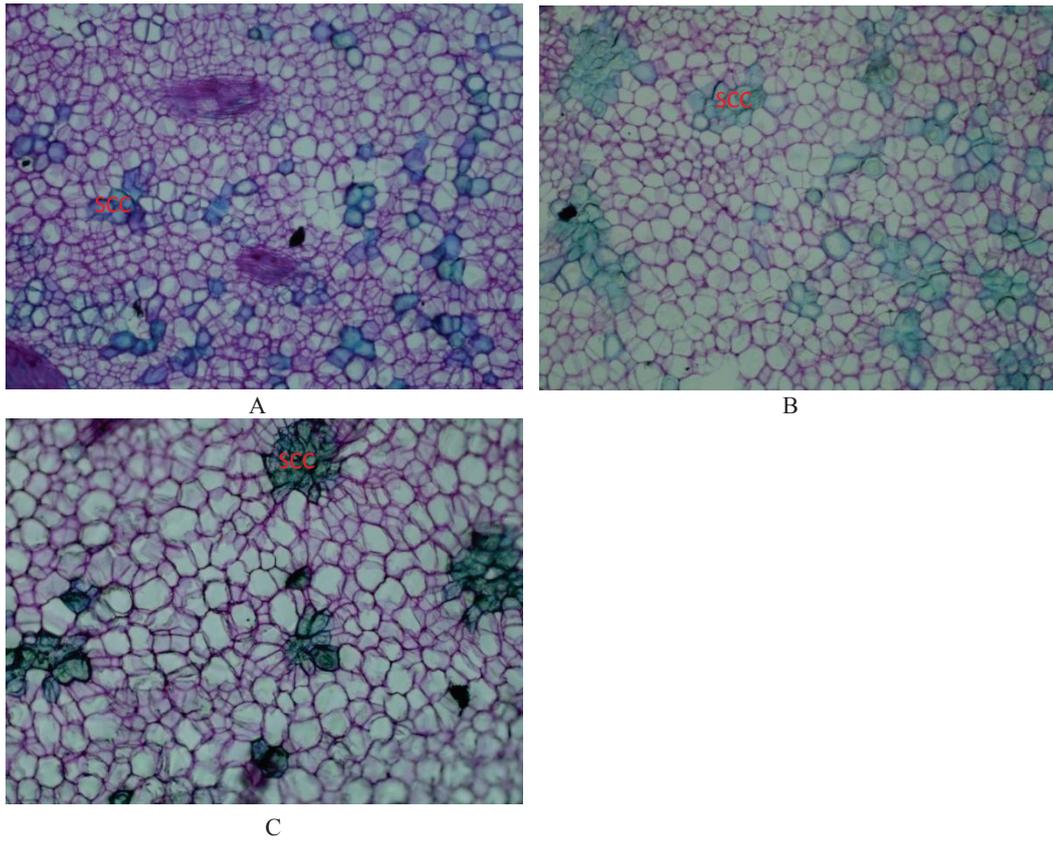


Fig. 4-2 Changes in flesh tissue observed by biological microscopy (100×) at 30 DAFB (A), 45 DAFB (B), and 60 DAFB (C) in the 'Hosui' pear cultivar. (DAFB: days after full bloom. SCC: stone cell clusters.)

Early fruit development is usually divided into three phases: development of the ovary, cell division, and subsequent cell expansion (Fu et al., 2008). Studies have shown that the formation of stone cells and the clustering of these cells occur between 30 and 60 DAFB (Choi and Lee, 2013; Martin-Cabrejas et al., 1994).

In the present study, our findings suggested that the development of stone cell cluster in Japanese pears were coincident, although the stone cell cluster numbers and areas were differed based on cultivars. Take the rough cultivar 'Zuisyu' and the smooth cultivar 'Hosui' (Fig. 4-1 and Fig. 4-2) as examples, the majority of stone cells were gathered in small clusters with a few independent cells at 30 DAFB (Fig. 4-1A and Fig. 4-2A). The clusters were spread randomly throughout the flesh tissue at 45 DAFB (Fig. 4-1B and Fig. 4-2B), and at 60 DAFB, the stone cells continued to develop as clusters scattered irregularly throughout the fruit flesh but appeared to be more concentrated in some areas than in others (Fig. 4-1C and Fig. 4-2C). We will focus on the differences of stone cell cluster numbers and areas between cultivars follows.

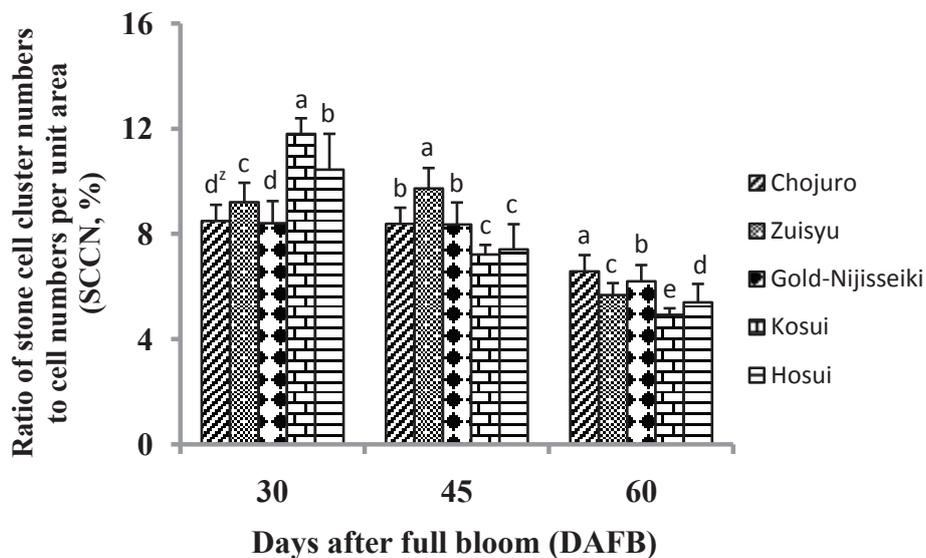


Fig.4-3 Changes in SCCN in Japanese pear during the early growth period.

(^z: Different letters indicate significant difference, $P < 0.05$, Tukey's test; $n = 5$; Vertical bar indicates standard error.

SCCN: ratio of stone cell cluster numbers to cell numbers per unit area.)

Ratio of stone cell cluster numbers to cell numbers per unit area (SCCN) increased from 30 to 45 DAFB, and then decreased to 60 DAFB in the rough 'Zuisyu' cultivar, while SCCN continuously decreased in 'Kosui', 'Gold-Nijisseiki', 'Chojuro', and 'Hosui' cultivars from 30 to 60 DAFB (Fig. 4-3). At 30 DAFB, SCCN was higher in the smooth cultivars 'Kosui' and 'Hosui' than in the rough cultivars 'Zuisyu', 'Chojuro', and 'Gold-Nijisseiki'. At 45 and 60 DAFB, SCCN was higher in the rough cultivars 'Zuisyu', 'Chojuro', and 'Gold-Nijisseiki' than in the smooth cultivars 'Hosui' and 'Kosui'. The studies have shown that high levels of cell division take place in the first few weeks after full bloom (Joubes et al., 1999), and capability for cell division slows down as development proceeds (Bertin et al., 2003). In our study, cell division in 'Kosui' and 'Hosui' cultivars finished or became slowly from 30 to 60 DAFB, however, these changes in 'Chojuro',

'Zuisyu', and 'Gold-Nijisseiki' cultivars took place from 45 to 60 DAFB (nearly all the tested cultivars reached the full-bloom stage in mid to late April, but the harvest stages were earlier in 'Kosui' and 'Hosui' than in the other rough cultivars). We inferred that the stages of cell division influenced the development of SCCN which higher in rough cultivars than in smooth ones at 45 and 60 DAFB.

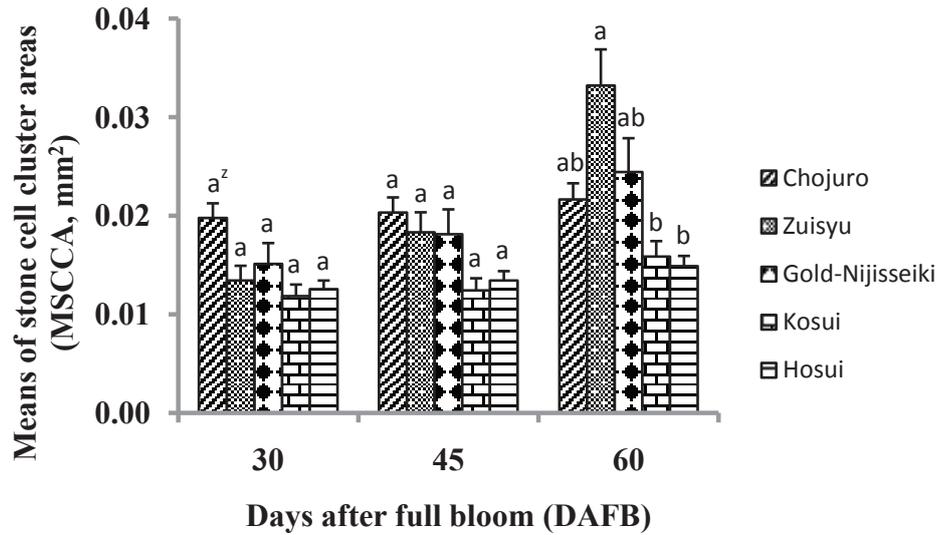


Fig. 4-4 Changes in MSCCA in Japanese pear during the early growth period.

(^z: Different letters indicate significant difference, $P < 0.05$, Tukey's test; $n = 5$; Vertical bar indicates standard error.

MSCCA: means of stone cell cluster areas.)

Means of stone cell cluster areas (MSCCA) increased from 30 to 60 DAFB in all the tested cultivars (Fig. 4-4). MSCCA was higher in the rough cultivar 'Zuisyu' than in the smooth cultivars 'Kosui' and 'Hosui' at 60 DAFB. The studies have shown that the cell expansion increases cell volume (Czerednik et al., 2015) provided the foundation for the increasing of MSCCA in our study, and we thought these stages kept longer in 'Zuisyu' than in 'Kosui' and 'Hosui' cultivars. In addition, there were studies have shown that stone cell formation capability (Lu et al., 2011) is differed based on cultivars. We inferred that the cell expansion and stone cell formation capability influenced the development of MSCCA between cultivars.

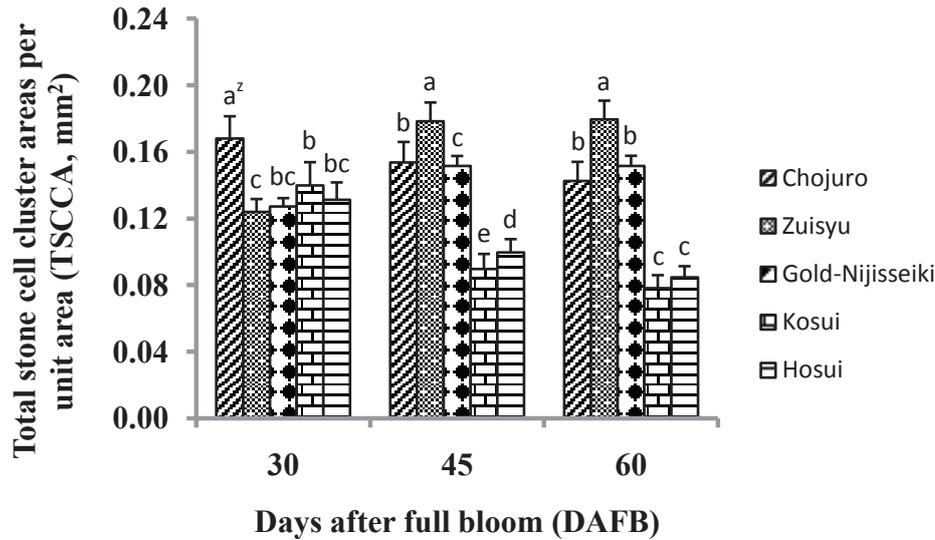


Fig. 4-5 Changes in TSCCA in Japanese pear during the early growth period.

(^z: Different letters indicate significant difference, $P < 0.05$, Tukey's test; $n = 5$; Vertical bar indicates standard error.

TSCCA: total stone cell cluster areas per unit area.)

Total stone cell cluster areas per unit area (TSCCA) decreased from 30 to 60 DAFB in the smooth cultivars 'Kosui', 'Hosui', and rough 'Chojuro', and increased from 30 to 45 DAFB in the rough cultivars 'Gold-Nijisseiki' and 'Zuisyu', there were no obvious changes from 45 DAFB to 60 DAFB in these two cultivars (Fig. 4-5). At 30 DAFB, TSCCA was higher in the 'Chojuro' cultivar than in the 'Kosui', 'Hosui', 'Gold-Nijisseiki', and 'Zuisyu' cultivars; at 45 and 60 DAFB, TSCCA was higher in the rough cultivars 'Zuisyu', 'Chojuro', and 'Gold-Nijisseiki' than in the smooth cultivars 'Kosui' and 'Hosui'. The changes and differences of TSCCA among cultivars were based on the combination of SCCN and MSCCA which the cell division finished or became slowly earlier in smooth 'Kosui' and 'Hosui' cultivars than in the other rough cultivars, and the cell expansion kept longer in rough 'Zuisyu' than in the smooth cultivars. In a word, we inferred that the stages of cell development influenced the development of TSCCA which higher in rough cultivars than in smooth ones at 45 and 60 DAFB.

4.4 Summary

In the present study, we found that the SCCN and TSCCA at 45 and 60 DAFB were higher in the rough cultivars than in the smooth cultivars. These differences were consistent with our previous study described in Chapter 3, which showed that EIS content was higher in the rough cultivars ‘Chojuro’, ‘Zuisyu’, and ‘Gold-Nijisseiki’ than in the smooth cultivars ‘Kosui’ and ‘Hosui’. We inferred that the SCCN and TSCCA at 45 and 60 DAFB were higher in rough cultivars than in smooth ones and played important roles in influencing EIS content in Japanese pear fruits.

Chapter 5

Studies on Enzymatic Analysis

5.1 Introduction

In Chapter 4, we noted that the SCCN and TSCCA at 45 and 60 DAFB differed according to cultivar and influenced EIS content. Although the mature stone cells only contain 20%-30% lignin (Lu et al., 2011), their formation is considered a lignification process. Studies have shown that the development of stone cells is closely related to the synthesis, transfer, and deposition of lignin (Holtman et al., 2006; Li et al., 2007). In addition, there is evidence that lignin plays an important role not only in cell wall thickening (Capeleti et al., 2005; Kaczkowski, 2003; Kuc, 1997; Solecka, 1997), but also in stone cell formation (Crist and Batjer, 1931; Ranadive and Haard, 1973; Tao et al., 2009). In Chapter 2, we reported that lignin content during late May to early June influenced the EIS content at these stages. It is necessary to clarify the effect of lignin formation on stone cell clusters and EIS content in order to confirm our earlier results.

Lignin is a complex, noncrystalline polymer with a three-dimensional network structure. The distribution, content, and structure of lignin in cell walls vary in different plants and in different parts of the same plant (Wang et al., 2012). Cai *et al* (2006) showed that increased fruit firmness was a result of fruit lignification and was closely related to the activity of the enzymes PAL, cinnamyl alcohol dehydrogenase (CAD), and POD.

The lignin biosynthetic pathway involves at least 10 enzymes (Li et al., 2008), most of which

have been isolated and characterized (Zhong et al., 2007). PAL catalyzes the first step of the phenylpropanoid pathway by deaminating L-phenylalanine to cinnamic acid (Boerjan et al., 2003; Boudet, 2000; Korth et al., 2001). PAL is indirectly associated with the synthesis of phenol polymers including lignin and suberin (Heldt, 2005; Parr and Bolwell, 2000). Antisense suppression of PAL activity leads to reductions in lignin content, as well as alterations of lignin subunit composition (Bate et al., 1994; Korth et al., 2001; Sewalt et al., 1997).

POD, the last major enzyme in lignin synthesis (Cassab, 1998; Ingham et al., 1998), is involved in the oxidative polymerization of lignin precursors (Boerjan et al., 2003) to form lignin (Sitbon et al., 1999). Studies have shown that POD activity was positively correlated with lignin content during the development of pear fruits (Tao et al., 2004). Plants with a lower POD activity showed less lignin deposition in the cell wall (Sitbon et al., 1999), and POD may exert some effect on stone cell formation (Lee et al., 2007). PAL and POD can catalyze the biosynthesis of secondary metabolites and lignification in plant tissue; changes in the activities of PAL and POD could affect the edible quality of the fruits (Cao et al., 2006).

In the present study, we used the rough cultivars ‘Chojuro’, ‘Zuisyu’, and ‘Gold-Nijisseiki’, and the smooth cultivars ‘Hosui’ and ‘Kosui’. We measured the levels of PAL and POD activity at 30, 45, and 60 DAFB.

5.2 Materials and Methods

5.2.1 Fruit Materials

The rough cultivars ‘Chojuro’, ‘Zuisyu’, ‘Gold-Nijisseiki’, smooth cultivars ‘Kosui’, and ‘Hosui’, flesh hardness is 26.3 N, 23.5 N, 22.8 N, 14.3 N, and 15.0 N, respectively, in which the EIS content during the early growth period influences the EIS content at harvest periods and determines flesh hardness (Chapter 3), were employed in this study. Ten fruits were collected from three trees of each cultivar at development stages of 30 DAFB, 45 DAFB, and 60 DAFB. All fruit were obtained from the orchard in Tottori University, Japan (35.5°N, 134.2°E). After selecting for uniformity of size and freedom from defects, the harvested fruits were transported to the laboratory in plastic boxes packed with ice.

5.2.2 Treatment for Determination of Enzyme Activity

Small cubes of tissue cut from the flesh of Japanese pears were immediately frozen in liquid nitrogen and then stored at -80°C (Sanyo, Tokyo, Japan) until the enzymatic activity assay took place. All extraction procedures were conducted at 4°C. Samples of 100 mg from five fruits were taken and ground with 3 mg polyvinyl polypyrrolidone (PVPP) with different buffers to assay different enzymes: 0.25 mL of 0.05 M sodium borate buffer (pH 8.8, containing 5 mM β -mercaptoethanol) for PAL, and 2 mL of 0.1 M phosphate buffer [pH 6.1, containing 0.5% (v/v) Triton X-100] for POD. The samples were ground by mortar and pestle, and then centrifuged at 15,

000 ×g (CF16RX, Hitachi, Japan) at 4°C for 1 h (Tian et al., 2006). The supernatants were used as the crude enzyme source to assay enzymatic activities.

PAL activity was assayed according to the method described by Assis *et al* (2001), with slight modifications. Briefly, 1 mL of enzyme extract was incubated with 2 mL of borate buffer (50 mM, pH 8.8) and 1 mL of L-phenylalanine (20 mM) for 1 h at 37°C (Tokyo Rikakikai Co. LTD, Tokyo, Japan). The reaction was stopped with 1 mL of HCl (1 M). PAL activity was determined by the production of cinnamate, whose absorbance was measured at a wavelength of 290 nm (U-1800, Hitachi, Japan). Specific enzyme activity was defined as nanomoles of cinnamic acid produced per hour per milligram of protein.

POD activity was assayed according to the method described by Wang *et al* (1991), with slight modifications. Briefly, 0.4 mL of enzyme extract was incubated with 2 mL of phosphate buffer [pH 6.1, containing 0.5% (v/v) Triton X-100], 1.08 µL of guaiacol, and 120 µL of 60 mM H₂O₂ at 25°C for 5 min. The activity was determined by measuring the increase in absorbance at 420 nm (U-1800, Hitachi, Japan), and was expressed as the change in absorbance per minute per milligram of protein.

Protein concentrations were determined using the Bradford method (1976), employing bovine serum albumin (BSA) as a standard. Briefly, dye reagent was prepared by diluting 1 part Bio-Rad Protein Assay Dye Reagent Concentrate with four parts distilled, deionized water. Next, 5 dilutions were prepared of a protein standard, representative of the protein solution to be tested. BSA solution (0 to 1.48 mg/ml) was also prepared. 10 µl of each standard and sample solution were pipetted into a clean, dry test tube, and then added 0.5 ml of diluted dye reagent was added to each tube and

vortex. The mixed solution was incubated 5 min to 1 h at room temperature, and then the absorbance was measured at a wavelength of 595 nm (U-1800, Hitachi, Japan).

5.2.3 Statistical Analysis

Statistical analysis of the data was carried out by one-way analysis of variance, and the means were separated by Tukey's test using the SPSS software package for Windows version 17.0. A probability of $p > 0.05$ was considered non-significant.

5.3 Results and Discussion

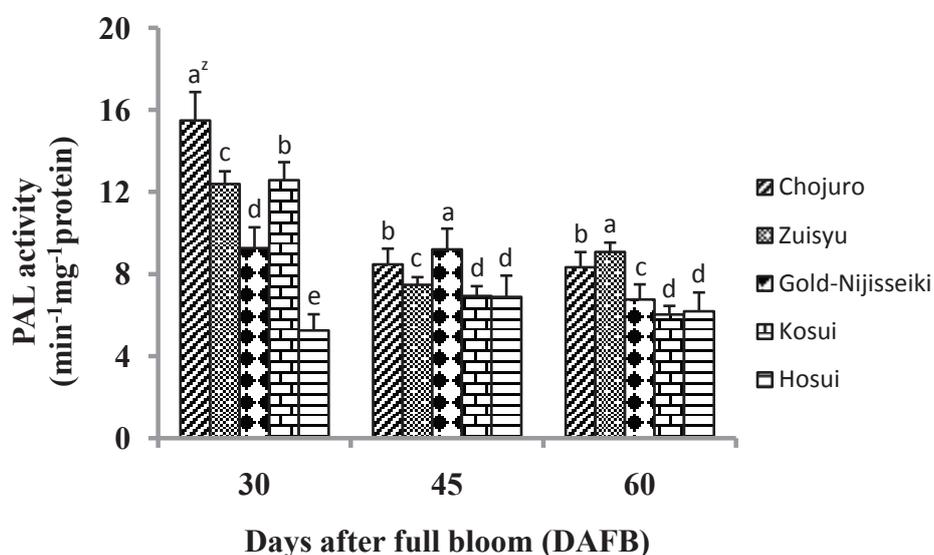


Fig. 5-1 Changes in PAL activity in Japanese pear during early growth periods.

(^z: Different letters indicate significant difference, $P < 0.05$, Tukey's test; $n = 5$; Vertical bar indicates standard error).

Our results showed that PAL activity decreased from 30 to 60 DAFB in 'Kosui', 'Gold-Nijisseiki', and 'Chojuro' cultivars, whereas it increased from 30 to 45 DAFB and then decreased to 60 DAFB in 'Hosui', and decreased from 30 to 45 DAFB and then increased to 60 DAFB in 'Zuisyu' (Fig. 5-1). At 30 DAFB, PAL activity was higher in 'Chojuro', 'Kosui', and 'Zuisyu' cultivars than in 'Gold-Nijisseiki' and 'Hosui' cultivars. At 45 and 60 DAFB it was higher in the rough cultivars 'Chojuro', 'Gold-Nijisseiki', and 'Zuisyu' than in the smooth cultivars 'Hosui' and 'Kosui'. PAL activity was higher in rough fruit than in control fruit from 30 to 50 DAFB (Lu et al., 2015) provided the foundation for our results. Studies have shown that PAL activity is highest at 20 DAFB, and then rapidly decreases until fruit maturity in pears (Yang et al., 2015). Other studies have shown that PAL activity in pears decreases during fruit growth (Steyn et al., 2004). These

studies have provided the foundation for our study results. In addition, there were studies have shown that PAL activity plays an important role in the formation of stone cell clusters (Cai et al., 2006). We inferred that PAL activity was higher in rough cultivars than in smooth ones and influenced the differences of SCCN and TSCCA at 45 and 60 DAFB.

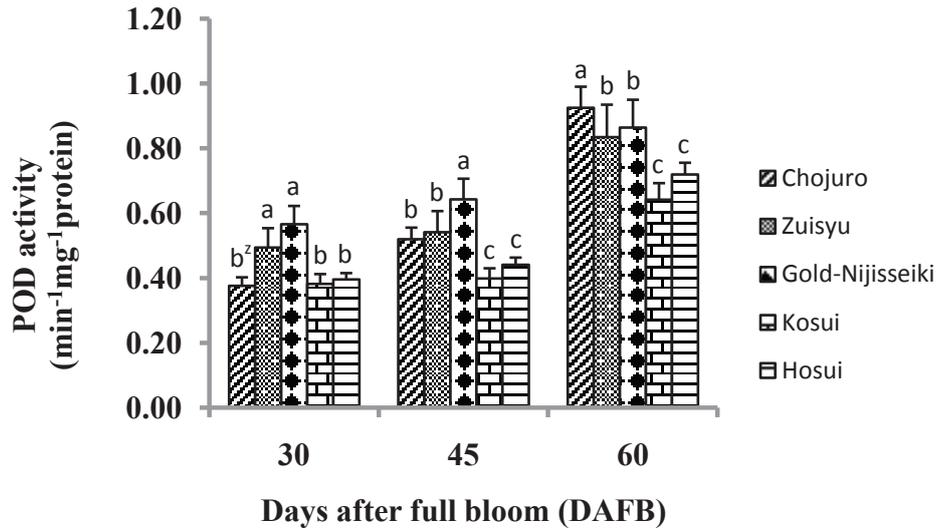


Fig. 5-2 Changes in POD activity in Japanese pear during early growth periods.

(^z: Different letters indicate significant difference, $P < 0.05$, Tukey's test; $n = 5$; Vertical bar indicates standard error).

POD activity increased from 30 to 60 DAFB in all tested cultivars (Fig. 5-2). At 30 DAFB, POD activity was higher in the 'Gold-Nijisseiki' and 'Zuisyu' cultivars than in the 'Hosui', 'Kosui', and 'Chojuro' cultivars. At 45 and 60 DAFB, POD activity was higher in the rough cultivars 'Gold-Nijisseiki', 'Zuisyu', and 'Chojuro' than in the smooth cultivars 'Hosui' and 'Kosui'. Studies have shown that POD activity spikes abruptly at 60 DAFB in pear fruits (Lee et al., 2007). Other studies have also shown that POD plays an important role in the stiffening of cell walls (Sanchez et al., 1995) and in increasing their thickness (Asselbergh et al., 2007). In addition, the positive relationship between POD activity and stone cell content in pear fruit has been established in previous studies (Zhang et al., 2006). These studies provided a foundation for our research into the differences in POD activity between cultivars. At 45 and 60 DAFB, these differences influenced the formation of stone cell clusters and played important role in determining the development of SCCN

and TSCCA.

5.4 Summary

Our data showed that PAL activity and POD activity differed according to Japanese pear cultivar and the differences in activity were consistent with the differences in SCCN and TSCCA at 45 and 60 DAFB noted in Chapter 4. We inferred that PAL and POD activity influence the formation of stone cell clusters and play important roles in the development of SCCN and TSCCA, and then indirectly influencing the flesh hardness in the tested cultivars.

Chapter 6

General Discussion

Selecting Japanese pear cultivars with excellent texture is the major focus of recent cultivation and breeding research. Researchers are also exploring the use of existing cultivars to breed new Japanese pear cultivars to meet market demand. Studies have shown that the characteristics of Japanese pear cultivars closely match those observed in both parents (Abe et al., 1993a, 1993b, 1995). Therefore, we inferred that if we breed Japanese pear cultivars with excellent texture, the new cultivars will also have excellent texture. It is necessary to determine the texture differences and the factors influence texture in different Japanese pear cultivars.

In the present study, we used major pear cultivars in Japan. We determined the amount of EIS, main component of cell walls, and the amount of cell wall polysaccharides during fruit growth (Chapter 2). We found that the EIS content influenced the flesh hardness at harvest; the amount of EIS was higher in Japanese pear cultivars with harder flesh. We also found that the EIS content during late May to early June was correlated with the amount at harvest, and the amounts of cellulose and lignin during these stages played an important role in influencing the EIS content. However, there were no differences in the amounts of soluble pectin or 24% KOH hemicellulose polysaccharides among Japanese pear cultivars. In addition, previous studies have shown that the structure of cell wall polysaccharides (i.e., diameter, length, and branching of polysaccharides may be important in fruit texture) (Chen et al., 2009; Cybulska et al., 2013; Liu et al., 2009; Yang et al., 2005; Zhang et al., 2010). Therefore, it is necessary to determine the structure of polysaccharides to

further clarify the effect of cell wall on the texture of Japanese pear cultivars.

Studies have shown that the EIS is the main component of dry matter (Prinzivalli et al., 2006; Rose et al., 2003). In addition, approximately 40% of a plant's dry matter consists of carbon, fixed during photosynthesis (Feng et al., 2015). The accumulation of dry matter in fruit is a result of the integrated processes of photosynthetic carbon assimilation and subsequent partitioning and utilization of photo-assimilates. It is necessary to determine the effect of the accumulation of photosynthetic products on EIS. We found that ^{13}C content was higher in the residue of 'Chojuro' than in 'Kosui' (see Chapter 3). These results proved that the accumulation of photosynthetic products was higher in the rough cultivar 'Chojuro' than in the soft cultivar 'Kosui' and these products strongly influenced the EIS content during the early growth stages of Japanese pear cultivars. However, studies in cereal crops showed that at harvest, 40% to 60% of carbon initially fixed by plants at different stages of development was lost through respiration (Austin et al., 1977; Yamagata et al., 1987). Therefore, it is necessary to increase test more stages of development and more cultivars to further confirm the effect of the accumulation of photosynthetic products on EIS content.

We found that the lignin content during late May to early June influenced the EIS content and was higher in the rough cultivars than in the smooth ones (see Chapter 2). Although the mature stone cells only contain 20%-30% lignin (Lu et al., 2011), their formation is considered a process of lignification. Studies looking at whether or how stone cell clusters affect EIS content during the early stages of growth are essential to further understand texture differences of Japanese pear

cultivars. We compared the development of stone cell clusters between cultivars and found that the SCCN and TSCCA during late May to early June was higher in the rough cultivars than in the smooth ones (see Chapter 4). These differences were consistent with the differences in EIS content described in Chapter 2. A previous study that showed pear cultivars are characterized by the amounts of stone cells in their flesh (Dibuz, 1998) provided the foundation for categorizing cultivars as rough and smooth in texture. We inferred that the SCCN and TSCCA during late May to early June played important roles in influencing EIS content in Japanese pear fruits.

PAL activity is known to play an important role in the formation of stone cell clusters (Cai et al., 2006). In addition, stone cell formation is influenced by POD activity (Lee et al., 2007). Next, it is essential to understand how PAL and POD activity influence stone cell clusters in the early growth stages, in order to further understand texture differences in Japanese pear cultivars. We measured PAL and POD activity (see Chapter 5) and determined that both were higher in the rough cultivars than in the smooth ones during late May to early June. These differences were consistent with the differences in SCCN and TSCCA noted in Chapter 4. We inferred that PAL and POD activity during late May to early June strongly influenced the formation of stone cell clusters, determined the development of SCCN and TSCCA, and thus indirectly influenced EIS content. We also found that the lignin content during these stages influenced the EIS content (see Chapter 2). It is necessary to determine the effect of PAL and POD activity on lignin content during these stages to further clarify how PAL and POD activity affect the texture of Japanese pear cultivars. Molecular marker studies and analysis of the gene expression of PAL and POD can inform efforts to breed

superior new Japanese pear cultivars.

In conclusion, PAL and POD activity influence the development stone cell clusters during the early growth stages in Japanese pear cultivars; higher PAL and POD activity are associated with higher SCCN and TSCCA. In addition, the Japanese pear cultivars with higher SCCN and TSCCA are rough in texture, associated with higher EIS content during late May to early June and harder flesh.

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Abstract

The Japanese pear fruits are crisp, and their sugar content, organic acid content, and texture differ among cultivars. Studies have shown that the content and quality of cell wall materials influences the texture differences between cultivars, and that the size and density of stone cells in Japanese pear help determine the texture. However, such studies are incomplete. In order to clarify which factors influence texture differences between Japanese pear cultivars, we compared Japanese pear cultivars with different textures. We measured the cell wall component in Japanese pear flesh, conducted a morphological observation of stone cells, and recorded the activity of enzymes. Our primary results are presented below.

1. Cell Wall Composition During Japanese Pear Fruit Growth

In order to clarify the relationship between cell wall composition and fruit texture, we compared and measured the amounts of ethanol-insoluble solid (EIS), cellulose, and lignin during Japanese pear fruit growth. Flesh hardness at harvest differed according to cultivar and was higher in the rough cultivars ‘Chojuro’ and ‘Gold-Nijisseiki’ than in the soft cultivars ‘Kosui’ and ‘Hosui’. The EIS content increased from late May to early June and then decreased to harvest in all the tested cultivars. The EIS content in late May and early June was strongly correlated with the content at harvest and influenced flesh hardness; it was higher in the rough cultivars ‘Chojuro’ and ‘Gold-Nijisseiki’ which have harder flesh, and was lower in the soft cultivars ‘Hosui’ and ‘Kosui’.

which have softer flesh. Cellulose content, lignin content, and 4% KOH hemicellulose content decreased during the early-middle growth stages. We inferred that the EIS content during late May to early June played an important role in the EIS content at harvest and also influenced the flesh hardness, the growth of cells, and the softening of Japanese pear cultivars.

2. Accumulation of Photosynthetic Products in the Japanese Pear

The primary component of cell walls is carbon, and studies have shown that the fruit texture is influenced by cell wall composition. In order to determine the relationship between the accumulation of photosynthetic products and texture differences in the early growth stages of Japanese pear cultivars, we compared the representative rough cultivar ‘Chojuro’ with the soft cultivar ‘Kosui’. We determined the accumulation of photosynthetic products by using ¹³Carbon (¹³C) labeling. The EIS content was higher in the rough cultivar ‘Chojuro’ than in the soft cultivar ‘Kosui’. The ¹³C content in the core and the residue, after the starch has been removed from EIS were also higher in ‘Chojuro’ cultivar than in ‘Kosui’. We inferred that the accumulation of photosynthetic products during the early growth stages was one of the factors that influenced the differences in EIS content and texture in Japanese pear cultivars.

3. Development of Stone Cells in Japanese Pear

The quantity and density of stone cells are important factors that determine the quality of Japanese pear. In order to clarify the effect of the development of stone cells on EIS content, we

compared the Japanese pear cultivars which have different textures, and determined the ratio of stone cell cluster numbers to cell numbers per unit area (SCCN), means of stone cell cluster areas (MSCCA), and total stone cell cluster areas per unit area (TSCCA) at 30, 45, and 60 days after full bloom (DAFB). The SCCN decreased from 45 to 60 DAFB in all the tested cultivars; however, the MSCCA increased during these stages. The SCCN and TSCCA were higher in the rough cultivars ‘Chojuro’, ‘Zuisyu’, and ‘Gold-Nijisseiki’ than in the smooth cultivars ‘Kosui’ and ‘Hosui’ at 45 and 60 DAFB. These differences were consistent with the differences in EIS content in these stages. In addition, we determined that the flesh cells around the stone cells presented a chrysanthemum appearance during fruit development in the rough ‘Zuisyu’ cultivar. We inferred that that the quantity and the density of stone cells played an important role in the texture differences during the growth of Japanese pear fruit.

4. Enzymatic Analysis in Japanese Pear

In order to clarify the effect of biosynthesis of lignin on the development of stone cells, we compared the Japanese pear cultivars which have different textures, and determined phenylalanine ammonia lyase (PAL) and peroxidase (POD) activity at 30, 45, and 60 DAFB. PAL is involved in catalysis of the first step of phenylpropanoid pathway by deaminating L-phenylalanine to cinnamic acid, and its activity decreased from 30 to 60 DAFB. POD is the last major enzyme in lignin synthesis, and its activity increased from 45 to 60 DAFB. In addition, PAL and POD activity were higher in the rough cultivars than in the smooth ones at 45 and 60 DAFB. We inferred that the PAL

and POD activity were factors that influenced the differences in SCCN and TSCCA in Japanese pear cultivars.

学位論文要旨

ニホンナシの果実にはクリスピー感という独特の食感があり，酸味と甘みのバランスおよび肉質は品種によって大きく異なっている．この肉質の差異の原因は細胞壁成分の量的あるいは質的な差異，ならびに石細胞の大小と粗密性が関与するといわれているが，その詳細は不明な点が多い．本研究では，ニホンナシ果実の肉質の品種間差異の原因を明らかにするため，肉質に特徴のある品種を用いて，果肉細胞壁成分の分析，果肉細胞の形態観察ならびに酵素活性の測定を行った．

1. 果実の発育期における細胞壁成分の経時的変化

細胞壁成分が肉質に及ぼす影響を明らかにするため，ニホンナシ果実の生育期におけるエタノール不溶性固定物（EIS）含量，ならびにセルロース含量とリグニン含量の経時的な変化を調査した．成熟期におけるニホンナシ果実の硬度は品種によって異なっており，肉質の粗い‘長十郎’および‘ゴールド二十世紀’の果肉硬度は，肉質の柔らかい‘幸水’および‘豊水’に比べて高かった．果実内における EIS 含量は，5 月下旬～6 月上旬にかけて増加し，その後の成熟期にかけて低下した．また，細胞壁の主要成分である EIS 含量は肉質の粗い品種の方が柔らかい品種より高く，5 月下旬と 6 月上旬の含量が成熟期の含量に影響を及ぼし，成熟期の硬度を決定する要因であると考えられた．また，

生果重あたりのセルロース含量とリグニン含量，ならびに 4%KOH ヘミセルロース含量は生育初期から中期にかけて，いずれの品種においても減少した．以上の結果より，ニホンナシの果肉硬度には5月下旬から6月上旬にかけての EIS 含量が深く関与し，細胞生長およびそれに続く果実の軟化に関与していることが示唆された．

2. 光合成産物の蓄積

細胞壁成分の多くは炭素を主要な骨格としているため，光合成により合成される炭水化物の蓄積が果実の肉質にも影響していると考えられる．ニホンナシ果実の生育初期における光合成産物の分配と果実の肉質との関係を明らかにするため，¹³C でラベルした安定同位体を用いて‘長十郎’と‘幸水’における光合成産物の蓄積様式を比較した．5月下旬に採取した‘長十郎’の果実の EIS 含量は，‘幸水’に比べて有意に高かった．さらに，EIS からデンプンを除いた残渣，ならびに果芯における ¹³C の含量も‘幸水’に比べて‘長十郎’で有意に高かったため，生育初期の光合成産物の分配が EIS 含量ならびに細胞壁成分の品種間差異を引き起こす要因であると考えられた．

3. 石細胞の発達

ナシの果肉内に存在する石細胞の数と密度は，果実品質を決定する重要な要因である．ニホンナシの石細胞の発達が EIS 含量に及ぼす影響を明らかにする

ため、果実の肉質が異なる品種を用いて開花後 30 日、45 日および 60 日に単位面積あたりの石細胞群数と細胞数の比率 (SCCN)、石細胞群面積の平均値 (MSCCA)、単位面積あたりの石細胞群面積 (TSCCA) を測定した。いずれの品種においても、SCCN の値は開花後 45 日と 60 日にかけて減少し、同時期において MSCCA の値は増加した。肉質の粗い‘長十郎’、‘瑞秋’および‘ゴールド二十世紀’においては、開花後 45 日と 60 日の SCCN および TSCCA の値が、肉質の密な‘幸水’や‘豊水’に比べて高く、その差は同時期の EIS 含量の差と一致した。さらに‘瑞秋’においては、成熟に伴い石細胞群を中心に果肉細胞が菊花状に変化するという形態的特徴が観察され、果実生育期における石細胞数の数と石細胞の密度が果実の粗密に影響を及ぼすと推察された。

4. 酵素活性の分析

ニホンナシ果実の石細胞の発達に及ぼすリグニン合成の影響を明らかにするため、肉質の異なる品種におけるフェニルアラニンアンモニアリアーゼ (PAL) とペルオキシダーゼ (POD) の活性測定を行った。L-フェニルアラニンから桂皮酸への生合性に関与する PAL の活性は開花後 30 日～60 日にかけて低下し、リグニン合成経路における最終段階の酵素である POD の活性は開花後 45 日～60 日にかけて増加した。また、肉質の粗い品種においては、開花後 45 日と 60 日における PAL および POD の活性が柔らかい品種に比べて高く、これらの活性は SCCN と TSCCA の品種間差異に及ぼす要因のひとつであると考えられた。

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List of Related Publication

1. Mingfeng Jiang, Yoshihiro Takemura, Katsuou Kuroki and Fumio Tamura. 2016. Changes in cell wall composition in Japanese pear during fruit growth. *Journal of Japanese Society of Agricultural Technology Management*. 23(2): 41-48. (The corresponding content is in Chapter 2 and Chapter 3)
2. Mingfeng Jiang, Yoshihiro Takemura, Katsuou Kuroki and Fumio Tamura. 2016. Relationship between the development of stone cell clusters and ethanol-insoluble solid in Japanese pear. *Journal of Japanese Society of Agricultural Technology Management*. 24(1): in press. (The corresponding content is in Chapter 4 and Chapter 5)