

# Site-specific changes in concentrations of endogenous phytohormones in growing 'Le Lectier' fruit

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## Summary

To elucidate the site-specific concentration changes of plant hormones associated with various stages of fruit growth in pears (*Pyrus communis* cv. 'Le Lectier'), the fruits were harvested 36-177 days after flowering (DAFB), and divided into pericarp, pulp, core, and seed. Indole-3-acetic acid (IAA), trans-zeatin (tZ), isopentenyl adenine (iP), gibberellin<sub>1</sub> (GA<sub>1</sub>), gibberellin<sub>4</sub> (GA<sub>4</sub>) and abscisic acid (ABA) from each tissue were quantified simultaneously by liquid chromatography mass spectrometry. The IAA concentration in seeds remained higher than that in other tissues during all harvesting periods, and peaked at 123 DAFB. In the pericarp and pulp, the IAA concentrations were almost constant and low and the entire period. In seeds, tZ maintained a higher concentration than other tissues during all harvesting periods, and peaked at 65 and 150 DAFB. The GA<sub>1</sub> concentration was higher than that of GA<sub>4</sub> in all tissues during all periods. The GA<sub>1</sub> concentration in seeds was at 36 DAFB at the highest concentration (406 pmol·g<sup>-1</sup>FW) and decreased sharply to 65 DAFB. The GA<sub>4</sub> concentration was low and almost constant up to 95 DAFB in seeds but increased after 123 DAFB. The concentrations of core and pulp were low. The fruit core peaked at 123 DAFB and had the highest concentration in the fruit (7610 pmol·g<sup>-1</sup>FW), with similar changes in the pulp. This study elucidates the concentration changes of the plant hormones at various stages during the growth of 'Le Lectier' fruits.

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**Key words** : abscisic acid, auxin, cytokinin, gibberellin, trans-zeatin.

## INTRODUCTION

Pear fruit growth exhibits two distinct stages (Lindo-García *et al.*, 2020). The first stage is associated with ovary development and rapid cell division, while the second stage of growth is associated with cell expansion due to increased cell volume. A comprehensive analysis of the major endogenous plant hormones in tissues is crucial for understanding the role of plant hormones in controlling physiological processes in the two stages of fruit growth. However, few studies report on the role of hormones in the transition from cell division to the stage of cell expansion and the key event of cell expansion (McAtee *et al.*, 2013).

The function of seeds, which play a central role in fruit growth, was studied with strawberries (Nitsch, 1950). In strawberries, all seeds are on the surface, and removing these seeds reduced fruit growth. Additionally, Nitsch conducted an experiment that replaced seeds by auxin application for growth, and demonstrated that seeds promote fruit growth by auxin. Analysis of strawberry endogenous plant hormones also supported the role of seeds (Kojima *et al.*, 2021a). Many other reports also demonstrated that seeds control many aspects of fruit growth (Leopold and Kriedemann, 1964). Particularly, there is a high correlation

between seed number and fruit size, which is consistent with the notion that seeds are the source of fruit growth-promoting signals. Even in pears, the uneven distribution of seeds significantly changed the shape of the fruit, and when seeds were present on only one side, only that side of the fruit grew, resulting in a distorted asymmetric fruit (Luckwill, 1959).

Pear (*Pyrus communis* L.) cv. 'Le Lectier' is a late-maturing variety that is mainly cultivated in Niigata Prefecture, Japan and produces a relatively large fruit weighing 350-400 g, thus is suitable for physiology research. The biological assay of auxin, gibberellin (GA) and abscisic acid (ABA) like substance in growing pear have been reported (Gil *et al.*, 1972). Furthermore, analysis of GA, indole-3-acetic acid (IAA) and ABA by Enzyme-Linked Immunosorbent Assay (ELISA) immediately after fruit set has been reported (Liu *et al.*, 2018). Additionally, there are reports of simultaneous analysis of major phytohormones throughout the growth of pear fruit by current reliable mass detectors (Niu *et al.*, 2014; Oikawa *et al.*, 2015; Quinet *et al.*, 2019; Lindo-García *et al.*, 2020). However, there are no reports of simultaneous analysis of major plant hormones in the tissue of growing pear fruits. Therefore, in this study, eight

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major endogenous plant hormones in the tissue in the growth process from the young fruit of the 'Le Lectier' fruit to the fruit at the optimum harvest time, changes in IAA, transzeatin (tZ), isopentenyladenine (iP), gibberellin<sub>1</sub> (GA<sub>1</sub>), gibberellin<sub>4</sub> (GA<sub>4</sub>) and ABA were quantified by instrumental analysis simultaneously. The purpose of this study is to elucidate the concentration change of the plant hormones in the tissue at various stages during the growth of 'Le Lectier' fruits.

## MATERIALS AND METHODS

### Plant material

The fruits (*Pyrus communis* cv. 'Le Lectier') were collected six times in 2009 at the research orchard of Niigata Prefectural Agricultural Research Institute: 36 DAFB (days after full bloom) (June 8), 65 DAFB (July 7), 95 DAFB (August 6), 123 DAFB (September 3), 150 DAFB (September 30), and 177 DAFB (October 27). At each sampling, 18 fruits were collected from three 15-year-old trees. The fruits were cut on a pericarp, pulp, core and seed with a knife. However, at only 36 DAFB, the fruits were divided into seeds and the remaining tissues. Together with each tissue from the 18 fruits, after measuring the weight, it was immersed in 80% ethanol and stored at  $-80^{\circ}\text{C}$  until extraction.

### Hormone analysis

Hormone analysis was performed according to the procedure of Kojima *et al.* (2020).

#### 1. Preparation of IAA and ABA sample for LC-MS

Extraction, separation and purification: Hormone analysis was performed according to the procedure of Kojima *et al.* (2020). Briefly, achenes and receptacles were homogenize and the three tissues were filtered into a stock solution of about 80% ethanol. We added <sup>13</sup>C<sub>6</sub>-IAA and d<sub>6</sub>-ABA as internal standards, concentrated aqueous solution, adjusted pH to 2.8 and filtered. Partition extraction was performed with diethyl ether, which was concentrated and filtered.

*High-performance liquid chromatography (HPLC):* The extracts were fractionated using HPLC (LC-20AD; Shimadzu Corporation, Japan) system I equipped with an ultraviolet detector (Kojima *et al.*, 2002). The HPLC column (Inertsil ODS-3, 3 $\mu\text{m}$ , 10 $\times$ 250 mm; GL Sciences Inc., Japan) was isocratically eluted with a solution of 40% ethanol. Eluates corresponding to the retention times of IAA and ABA were collected separately. IAA and ABA fractions were dried under reduced pressure. After fractionation using HPLC system I, all fractions were further purified with the same type HPLC system II. HPLC column (C-30-S-Select) was isocratically eluted with a solution of 40% ethanol. Eluates corresponding to each retention time of IAA and ABA were collected and concentrated.

#### 2. Preparation of GAs and CKs sample for LC-MS

*Extraction, separation, and purification.* We added d<sub>5</sub>-tZ, d<sub>6</sub>-iP, d<sub>2</sub>-GA<sub>1</sub>, and d<sub>2</sub>-GA<sub>4</sub> as internal standards to a stock solution of approximately 80% ethanol (equivalent to 9 g fresh

weight). The concentrated solution was adjusted to pH 3.5 and filtered. Partition extraction was performed using ethyl acetate (Kojima *et al.*, 2003). *The ethyl acetate layer:* Anhydrous sodium sulfate was added to the ethyl acetate layer for dehydration and allowed to stand overnight. The ethyl acetate layer was decanted, concentrated and filtered. *The aqueous layer:* pH of the aqueous layer was adjusted to 7.0, partitioned, and extracted with butanol. The butanol layer was concentrated, dissolved in 50% ethanol, and filtered.

Extracts from the ethyl acetate and butanol layers were fractionated using HPLC system I. From the ethyl acetate layer, eluates corresponding to GA<sub>1</sub>, GA<sub>4</sub>, and iP were collected separately. From the butanol layer, eluates corresponding to tZ and iP were collected separately. The collected fractions were dried and dissolved in 80% ethanol. After fractionation using HPLC system I the extracts were further fractionated using the same method for HPLC system II. The HPLC column (C-30-S-Select, 5 $\mu\text{m}$ , 4.6 $\times$ 250 mm; GL Sciences) was isocratically eluted with a solution of 40% ethanol + 60% ultrapure water containing 0.1% acetic acid. The fractions of GA<sub>1</sub>, GA<sub>4</sub>, tZ, and iP were injected separately, and eluates corresponding to GA<sub>1</sub>, GA<sub>4</sub>, tZ, and iP were collected and dried.

#### 3. Analysis of plant hormones by LC-MS

We used an LC-MS (LCMS 2010EV; Shimadzu) to identify the hormones: column, Cadenza CD-C18 (3 $\mu\text{m}$ , 250 $\times$ 2 mm; Imtakt Corporation, Japan); flow rate, 0.1 mL $\cdot$ min<sup>-1</sup>; and eluent, 70% ethanol + 30% ultrapure water + 0.1% acetic acid. The selected ion monitoring (SIM) method was selected, and ion monitoring and mode were according to Kojima *et al.* (2020). Plant hormone concentrations were calculated from the ratio of the peak areas of natural and labeled ions as an internal standard.

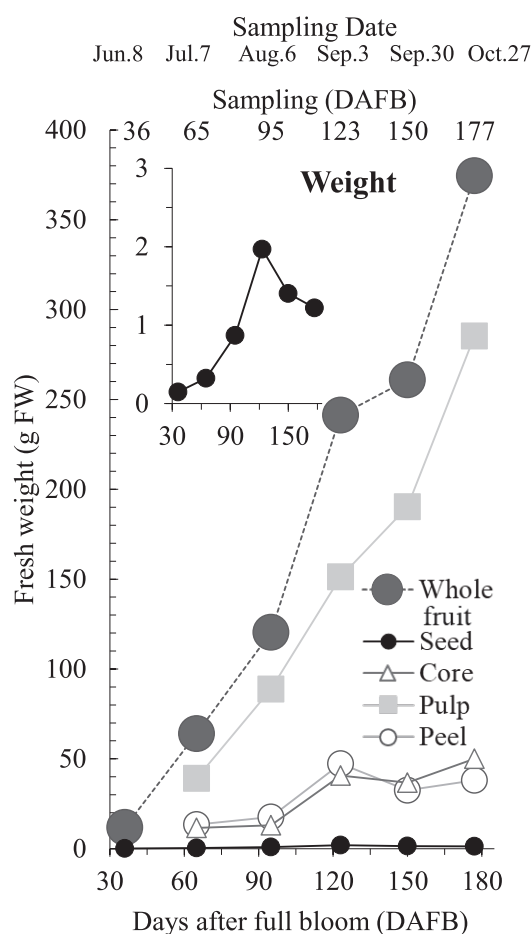
## RESULTS

The weight of the seeds of 'Le Lectier' continued to increase from 36 to 95 DAFB and then decreased (Fig. 1). This decrease may be due to seed drying. The pulp continued to increase from 65 to 177 DAFB. The weight of the pericarp and the core (which does not contain seeds) increased to 123 DAFB and then was almost constant.

The hormone concentration of the whole fruit was calculated by summing the hormone quantities of the pericarp, pulp and seed per fruit and dividing this total value by the total of each raw weight per fruit. Since the plant hormones were analyzed by combining the tissues from the 18 fruits, the analysis data of the hormones are the average values of the 18 fruits.

The IAA concentration in seeds remained higher than that in other tissues during all harvesting periods, and peaked at 123 DAFB (Fig. 2). In the pericarp and pulp, the concentrations were low and almost constant and low over the entire period. The core also had a low concentration that was almost constant except for the increase at 177 DAFB.

In seeds, tZ maintained a higher concentration than



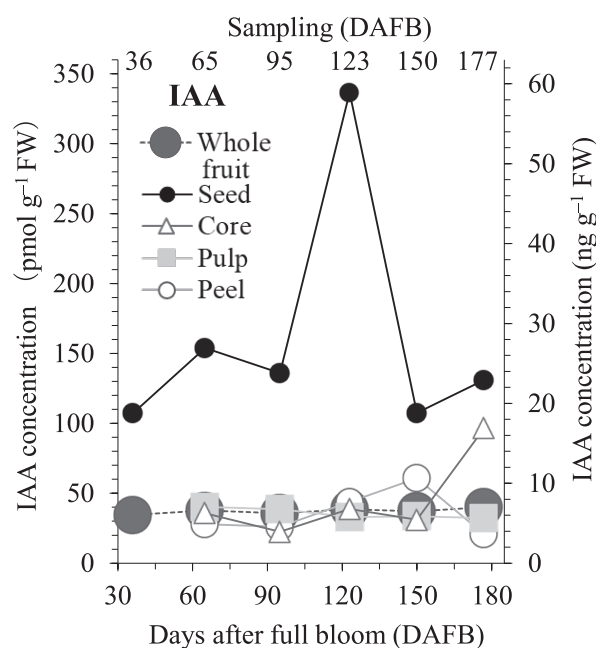
**Fig. 1** Changes in weight in 'Le Lectier' fruits from 36 to 177 days after full bloom (DAFB). Eighteen fruits were collected at each sampling, and all the fruits were divided into pericarp, pulp, core and seeds with a knife, and each tissue at each sampling was mixed.

other tissues during all harvesting periods, and peaked at 65 and 150 DAFB (Fig. 3A). Concentrations in the core, pulp and pericarp were lower than  $180 \text{ pmol}\cdot\text{g}^{-1}\text{FW}$  over all periods.

iP was lower than tZ in all tissues over all periods (Fig. 3B). Seeds tended to decline with growth. Concentrations in the core and pulp were lower than  $44 \text{ pmol}\cdot\text{g}^{-1}\text{FW}$  over all periods. In the pericarp, iP increased at 120 DAFB.

The  $\text{GA}_1$  concentration was higher than that of  $\text{GA}_4$  in all tissues during all periods (Fig. 4). The  $\text{GA}_1$  concentration in the seeds was at the highest concentration at 36 DAFB ( $406 \text{ pmol}\cdot\text{g}^{-1}\text{FW}$ ), decreased sharply to 65 DAFB, increased at 95 and 123 DAFB, and decreased after 150 DAFB (Fig. 4A). The core tended to decrease with growth. The pulp increased to 123 DAFB and decreased after 150 DAFB. The pericarp had high concentrations at 65 and 95 DAFB, but dropped sharply to 123 DAFB and peaked at 150 DAFB.

The  $\text{GA}_4$  concentration was low and almost constant low



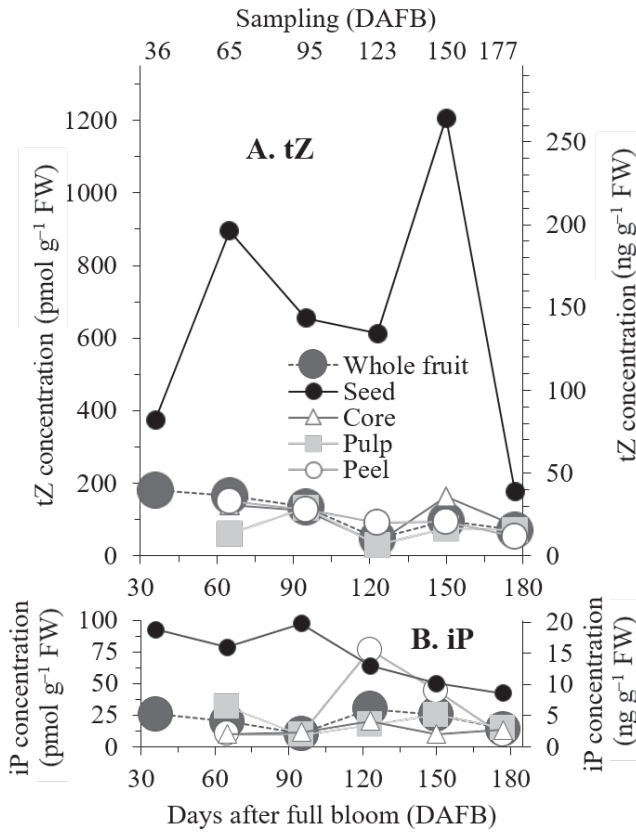
**Fig. 2** Changes in endogenous concentration of IAA of whole fruits and tissues in developing 'Le Lectier' fruits from 36 to 177 DAFB. Each of pericarp, pulp, core and seeds of 18 fruits at each sampling was mixed. An internal standard was added, fractionated by extraction with diethyl ether, purified by HPLC, and quantified by LC-MS.

up to 95 DAFB in seeds, but increased after 123 DAFB. The concentrations of core and pulp were low, but increased at 177 DAFB. The pericarp rose slightly at 95 DAFB.

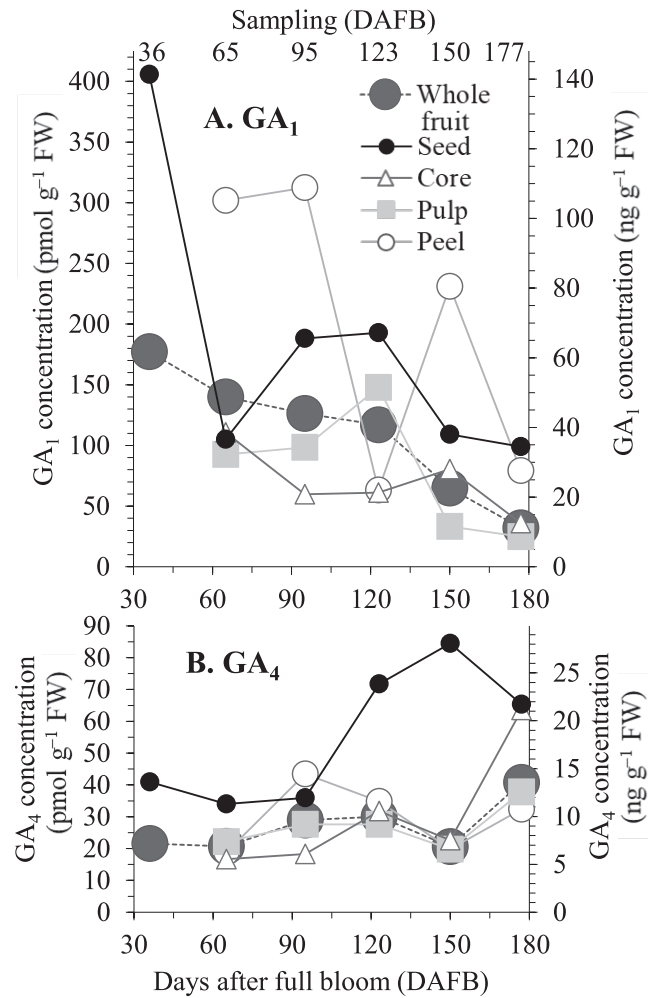
In seeds, the ABA concentration increased at 65 DAFB, decreased to 95 DAFB, and then increased (Fig. 5). The fruit core peaked at 123 DAFB and had the highest concentration in the fruit ( $7610 \text{ pmol}\cdot\text{g}^{-1}\text{FW}$ ), with similar changes in the pulp. The pericarp concentration rose at 95 and 150 DAFB.

## DISCUSSION

Pear growth is divided into two stages: cell division stage, namely, pulp cell division, up to approximately 54 days after full bloom (DAFB); cell expansion stage, pulp cell expansion, after approximately 54 DAFB (Bain, 1961). Therefore, 36 DAFB of 'Le Lectier' in this study corresponds to the cell division stage, and after 65 DAFB, it corresponds to the cell expansion stage. Although fruit expansion is an crucial event, little literature studies the role of plant hormones in the transition from the cell division to cell expansion stage and in the continuation of cell expansion (McAtee *et al.*, 2013). Comparing the levels of endogenous phytohormones of whole fruit by molar concentration ( $\text{pmol}\cdot\text{g}^{-1}\text{FW}$ ), they can be divided into three groups: a) IAA (34–40), iP (10–30), and  $\text{GA}_1$  (21–41); b) tZ (45–180) and  $\text{GA}_1$



**Fig. 3** Changes in endogenous concentration of tZ (A) and iP (B) of whole fruits and tissues in developing 'Le Lectier' fruits from 36 to 177 DAFB. Each of pericarp, pulp, core and seeds of 18 fruits at each sampling was mixed. Internal standards were added, fractionated by extraction with butanol, purified by HPLC, and quantified by LC-MS.



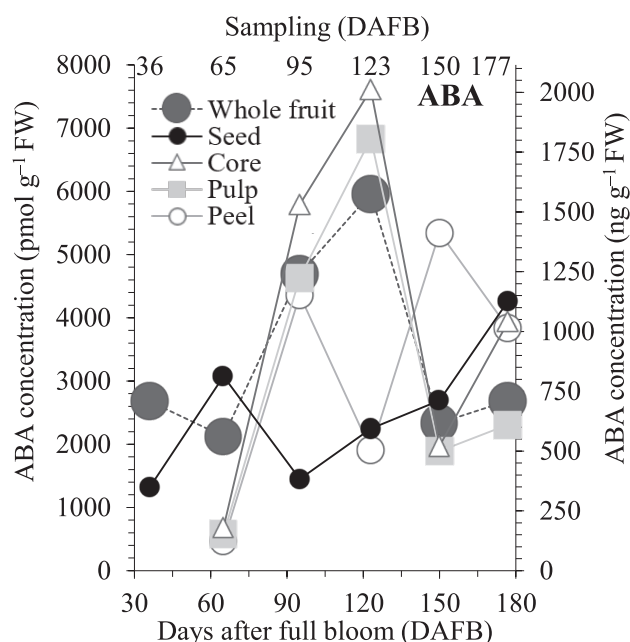
**Fig. 4** Changes in endogenous concentration of GA<sub>1</sub> (A) and GA<sub>4</sub> (B) of whole fruits and tissues in developing 'Le Lectier' fruits from 36 to 177 DAFB. Each of pericarp, pulp, core and seeds of 18 fruits at each sampling was mixed. Internal standards were added, fractionated by extraction with ethyl acetate, purified by HPLC, and quantified by LC-MS.

(32-180); c) ABA (2100-6000). iP and GA<sub>4</sub> in a group are hormones that are not considered major bioactive types in each hormone group. Conversely, tZ and GA<sub>1</sub>, which are considered to be the major bioactive types, are included in b group. The molar concentration of ABA in c group is approximately 100 times higher. Further studies on the analysis of endogenous hormones are required to understand whether such a tendency is a phenomenon common to pears.

The auxin-like activity was measured by bioassay using the whole fruit of the pear 'Bartlett' (Gil *et al.*, 1972). The activity value of all seeded fruits of 'Bartlett' was almost constant 30-90 DAFB, consistent with 'Le Lectier' in this report.

The IAA concentration of 'Le Lectier' seeds peaked at 123 DAFB, and strawberry seeds (achenes) also peaked in the middle of growth (Archbold and Dennis, 1984). The peak IAA concentration of strawberries was 520 ng · g<sup>-1</sup>FW, but the peak of seeds of 'Le Lectier' was as low at approximately 1/10 (59 ng · g<sup>-1</sup>FW).

The seeds of 'Le Lectier' maintained a higher tZ concentration than other tissues during all harvesting periods (Fig. 3A). It has also been reported that tomatoes also have high cytokinin activity in growing seeds and low in surrounding tissues (Gillaspy *et al.*, 1993). The tZ concentration of grape seeds was the highest at 14 DAFB immediately after cell division arrest and remained higher than that of other tissues (Kojima *et al.*, 2020). In strawberry fruits, the tZ concentration in seeds (achenes) was higher than that in receptacles (Kojima *et al.*, 2021a). Furthermore, in watermelon, the tZ concentration in seeds remained higher than that in the flesh and skin at a substantially constant concentration during growth (Kojima *et al.*, 2021b). Melon



**Fig. 5** Changes in endogenous concentration of ABA of whole fruits and tissues in developing 'Le Lectier' fruits from 36 to 177 DAFB. Each of pericarp, pulp, core and seeds of 18 fruits at each sampling was mixed. An internal standard was added, fractionated by extraction with diethyl ether, purified by HPLC, and quantified by LC-MS.

fruits also had high concentrations in the placenta containing seeds (Kojima *et al.*, 2021c). In these fruits, tZ is considered to be synthesized in seeds because it has the highest concentration in seeds, but CK is not synthesized in seeds and may be transported from other tissues such as the roots (Bohner and Bangerth, 1988). Gillaspay *et al.* (1993) suggested that CK from growing seeds may rapidly degrade in surrounding tissues after promoting cell division.

The concentration of GA<sub>1</sub> in the major bioactive form was highest in seeds, at 36 DAFB, which is considered to be in the cell division stage, and then decreased sharply (Fig. 4A). In watermelon seeds, the GA<sub>1</sub> concentration was the highest at 7 DAFB immediately after cell division, and then decreased sharply to a low concentration (Kojima *et al.*, 2021a). Higher concentrations of GA<sub>1</sub> in seeds during this period are thought to regulate fruit cell division. (Gillaspay *et al.*, 1993; Mesejo *et al.*, 2016). Since 54 DAFB and later correspond to the cell expansion stage of the pulp, it is suggested that the peak-like increase in the GA<sub>1</sub> concentration of 123 DAFB in the pulp promotes the cell expansion. In tomato fruits also GA has also been reported to promote cell expansion (Serrani *et al.*, 2008).

ABA concentration tended to increase slightly with the progression of DAFB in seeds (Fig. 5), and this tendency was also the same in strawberry seeds (Kojima *et al.*, 2021a).

However, the ABA concentration of strawberry seeds was 130–160 pmol·g<sup>-1</sup>FW, while that of 'Le Lectier' was 3–7 times higher (349–1129 pmol·g<sup>-1</sup>FW).

In the core and pulp, the ABA concentration peaked at 123 DAFB, which is the period of cell expansion, and then decreased (Fig. 5). This tendency was also seen in grapes (Kojima *et al.*, 2020). However, compared to the peak ABA concentration of 290 pmol·g<sup>-1</sup>FW in grape pulp, the concentration in 'Le Lectier' was approximately 24 times higher (6835 pmol·g<sup>-1</sup>FW).

Mutants deficient in ABA in tomatoes have reduced fruit size (Nitsch *et al.*, 2012), and ABA is thought to be associated with the promotion of fruit enlargement (Gillaspay *et al.*, 1993). High levels of ABA were associated with high sink activity in soybean (Schussler *et al.*, 1984). The application of ABA to the seed coat of the common bean increased the unloading of the assimilation product (Clifford *et al.*, 1986). From these results, Brenner *et al.* (1989) claimed that ABA stimulates phloem unloading and promotes sink activity in pericarp and locule tissues during high growth rate stages of tomato fruit. Therefore, the increase in ABA concentration in the core and pulp of 'Le Lectier' (Fig. 5) may be related to the uptake of assimilation product required for cell expansion.

The whole fruit of two pear varieties was used as a material, and the internal standard of stable isotope labeling was added and quantified by a mass detector (Lindo-Garcia *et al.*, 2020). From 30 DAFB to the harvest period, the concentration of the whole fruit of 'Le Lectier' in this study was compared as follows. For the IAA concentration, 'Blanquilla' was almost constant in the range of 20–36 ng·g<sup>-1</sup>FW, and 'Conference' tended to decrease in the range of 42.5 ng·g<sup>-1</sup>FW, which was the same level as 'Le Lectier' in this report. (Fig. 2). The GA<sub>1</sub> concentration of 'Blanquilla' tended to decrease (13–3 ng·g<sup>-1</sup>FW) and that of 'Conference' tended to increase (5–18 ng·g<sup>-1</sup>FW), which is the same level as that of 'Le Lectier' in this report. (Fig. 4A). The ABA concentration of 'Blanquilla' tended to decrease (5000–800 ng·g<sup>-1</sup>FW), and that of 'Conference' also tended to decrease (6500–100 ng·g<sup>-1</sup>FW), which is the same level as that of 'Le Lectier' in this report. (Fig. 5). Although the varieties and cultivation conditions were different, the IAA, GA<sub>1</sub> and ABA concentrations of the whole fruit were similar in these three pear varieties.

The whole fruit of the pear 'La France' was used as a material and quantified by a mass detector with an internal standard added (Oikawa *et al.*, 2015). From 30 DAFB to the harvest season, the concentration of the whole fruit of 'Le Lectier' in this study was compared as follows. For the IAA concentration, 'La France' was almost constant and was about several ng·g<sup>-1</sup>FW, which was the same level as 'Le Lectier' in this report (Fig. 2). The tZ concentration was almost constant in 'La France' and was 0.1 ng·g<sup>-1</sup>FW or less. However, 'Le Lectier' tended to decrease, and the concentration range was 40–10 ng·g<sup>-1</sup>FW, which was about 100 times higher (Fig. 3A). The iP concentration was almost constant in 'La France' and was 0.01 ng·g<sup>-1</sup>FW or less. 'Le Lectier' was also almost

constant, but the concentration range was 2.1-6.1 ng ·g<sup>-1</sup>FW, which was about 100 times higher (Fig. 3B). The GA<sub>4</sub> concentration tended to be almost constant at about 0.02 ng ·g<sup>-1</sup>FW in 'La France'. 'Le Lectier' tended to rise slightly, but the concentration range was 7-14 ng ·g<sup>-1</sup>FW, which was about 100 times higher (Fig. 4B). The ABA concentration was almost constant at about 100 ng ·g<sup>-1</sup>FW in 'La France' and increased to about 400 ng ·g<sup>-1</sup>FW during the harvest season. 'Le Lectier' peaked at 123 DAFB, but the concentration range was 561-1576 ng ·g<sup>-1</sup>FW, which was about 3-5 times higher (Fig. 5). In 'Le Lectier', the concentrations of tZ, iP, GA<sub>1</sub> and GA<sub>4</sub> were about 100 times higher than those reported in 'La France'.

The whole fruit of the pear 'Conference' was used as a material and quantified by a mass detector with an internal standard added (Quinet *et al.*, 2019). The fruits of 30 DAFB were compared with the overall concentration of the fruits of 'Le Lectier' in this study as follows. In the IAA concentration, the 'Conference' was almost constant at about 5000-6000 pmol·g<sup>-1</sup>FW, which is 170 times the level of the 'Le Lectier' level in this report. However, the ABA concentration was about 6000-9000 pmol·g<sup>-1</sup>FW in 'Conference', which is about 2-3 times that of 'Le Lectier' (Fig. 2).

Differences in the concentration of pear fruits were observed several to 100 times, and this may be due to differences in varieties, cultivation conditions, weather, and other factors. Further analysis of endogenous plant hormones in the future will clarify the cause.

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## 生長中の「ル レクチエ」果実の内生植物ホルモンの部位別の濃度変化

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### 要 約

「ル レクチエ」果実の生長のさまざまな段階に関連する植物ホルモンの部位別の濃度変化を解明するために、開花後36~177日 (DAFBB) に「ル レクチエ」果実を採取し、果皮、果肉、中心部、種子に分けた。各組織からのインドール-3-酢酸 (IAA)、トランスゼアチン (tZ)、イソペンテニルアデニン (iP)、ジベレリン<sub>1</sub> (GA<sub>1</sub>)、ジベレリン<sub>4</sub> (GA<sub>4</sub>) およびアブシジン酸 (ABA) を同時に液体クロマトグラフィー質量分析 (LC-MS) で定量した。種子中の IAA 濃度は、すべての収穫期間中、他の組織よりも高いままであり、123DAFB でピークに達した。果皮と果肉では、IAA 濃度はほぼ一定で低い値であった。種子では、tZ はすべての収穫期間中、他の組織よりも高い濃度を維持し、65および150DAFB でピークに達した。GA<sub>1</sub>濃度は、すべての期間ですべての組織で GA<sub>4</sub>の濃度よりも高かった。種子中の GA<sub>1</sub>濃度は最高濃度で36DAFB であり、65DAFB まで急激に減少した。GA<sub>4</sub>濃度は低く、種子中の95DAFB まではほぼ一定であったが、123DAFB 以降は増加した。果芯と果肉の濃度は低かった。果実の芯は123DAFB でピークに達し、果実の濃度が最も高く、果肉にも同様の変化が見られた。

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