Plant Hormone Changes in Growing Small Watermelon Fruit

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To elucidate the phytohormone profiles associated with various stages of growing and ripening of small watermelon fruits, small watermelon fruits were collected 7 to 35 days after flowering (DAF) and divided into rind, pulp, and seeds. Indole-3-acetic acid (IAA), abscisic acid (ABA), trans-zeatin (tZ), isopentenyl adenine (iP), jasmonic acid (JA), methyl jasmonate (MeJA), gibberellin1 (GA1), and gibberellin4 (GA4) from each tissue were simultaneously quantified by liquid chromatography mass spectrometry (LC-MS). From 7 to 28 DAF, pulp weight increased rapidly due to cellular expansion. The IAA concentration increased up to 21 DAF in seeds, but then decreased up to 35 DAF. Similar changes occurred in the pulp approximately 7 days after the pattern of rising and falling IAA levels in the seeds. The GA concentration was higher in the seeds, and peaked prior to the seed growth peak. The ABA level in the pulp peaked at the time of fruit enlargement (21 DAF). The concentrations of iP, GA1, and GA4 tended to decrease with growth in all tissues. The results revealed the changes in endogenous plant hormones involved in the growth of small watermelon fruit.

Key Words: abscisic acid, auxin, cytokinin, gibberellin, jasmonic acid.

Introduction

Cucurbitaceous vegetables are grown in all over the world, and watermelon is an important crop plant with the highest yield (FAO, 1994). Watermelons produce large fruits, which can weigh over 30 kilograms (Sinnott, 1960). Therefore, the concentrations of plant hormones controlling growth and developmental changes are remarkable, and these can contribute valuable data to elucidate the growth physiology of fruits. As watermelons are a relatively inexpensive crop and do not show distinct physiological changes during maturation and distribution, little physiological research has been conducted (Mizuno and Pratt, 1973).

Fruit growth was reported to be regulated by various plant hormones (Gillaspy et al., 1993). Seeds, a rich source of plant hormones, are involved in promoting the growth of surrounding tissues and determining fruit size (Crane, 1969; Ozga and Reinecke, 2003). GA is present in the highest concentrations in the most actively growing seeds (Garcia-Martinez and Hedden, 1997).

Additionally, it was reported that synthetic auxin and synthetic cytokinin could induce seedless watermelon fruit (Hayata et al., 1995; Huitrón et al., 2007).

Endogenous plant hormones were reported to be involved in watermelon fruit growth and development. Bioassays of immature watermelon seeds showed a peak of cytokinin activity, followed by rapid seed growth (Prakash and Maheshwari, 1970). However, studies on cytokinin activity were carried out before the development of reliable techniques to quantify plant hormones. On the other hand, watermelon fruits were harvested at various developmental stages and studied in detail with regards to respiratory rate and ethylene production (Mizuno and Pratt, 1973), showing that until 35 DAF there is no climacteric respiration increase or ethylene synthesis.

Because harvested fruits are relatively easy to manipulate, much research has been done on fruit maturation. However, the growth physiology often fluctuates during the long and complex growth process from young fruit to the largest fruit at cultivation and researchers tend to avoid research on pre-harvest fruits (Coombe, 1976; Gillaspy et al., 1993). This report covers all stages of watermelon fruit maturity from early growth to harvest.

Since fruit growth is regulated by various plant hormones (Gillaspy et al., 1993), a simultaneous analysis...
of key plant hormones is important. However, there is no previous report on the simultaneous analysis of major plant hormones in the tissues of growing watermelon fruit using currently available, reliable mass detectors. Therefore, in this study, during the growth process of watermelon fruit from fruit to mature fruit, eight major endogenous plant hormones, indole-3-acetic acid (IAA), abscisic acid (ABA), and trans-zeatin (tZ), isopentenyladenine (iP), jasmonic acid (JA), methyl jasmonate (MeJA), and gibberellins (GA$_1$, GA$_3$) were simultaneously determined by instrumental analysis. The aim of this study was to elucidate the profile of plant hormones in tissues at various stages during watermelon fruit growth and ripening.

Materials and Methods

**Plant material**

Due to the ease of cultivation and the limited size of the field, small watermelons were used instead of large watermelons. We purchased small watermelon seedlings (*Citrullus lanatus* Thunb.) that were produced by grafting the seedling “Benikodama” to the rootstock “Sintosakabotha”. Beds (1.5 m wide) in a field of the Faculty of Agriculture at Niigata University were covered with black polyethylene mulch, and 60 seedlings were planted on 6 May 2013 and cultivated at 80 cm separation. Plants were artificially fertilized with male pollen on the day of flowering. The fruits were collected five times, at 7, 14, 21, 28, and 35 days after pollination (DAF), and five fruits were collected on each collection day. After harvesting, the fruits were divided with a knife into three tissues: rind, pulp, and seed. The tissues of the five fruits were combined, weighed, immersed in 80% ethanol, and stored at −40°C until extraction. Thus, there were no biological replicates for each sample.

**Hormone analysis**

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

1. **Preparation of IAA, ABA, and JAs sample for LC-MS**

   Briefly, the rind and seeds were homogenized and the three tissues were filtered into a stock solution of approximately 80% ethanol. We added $^{13}$C$_6$-IAA, d$_6$-ABA, d$_6$-JA, and d$_2$-MeJA as internal standards to the stock solution of approximately 80% ethanol (equivalent to 1 g fresh weight). We then concentrated an aqueous solution, adjusted pH to 2.8 and filtered. Partition extraction was performed with concentrated and filtered diethyl ether.

   **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 μm, 10 × 250 mm; GL Sciences Inc., Japan) was isocratically eluted with a solution of 40% ethanol. Eluates corresponding to the retention times of IAA, ABA, JA, and MeJA were collected separately. IAA and ABA fractions were dried under reduced pressure, and JA and MeJA fractions were concentrated under reduced pressure to approximately 1 mL. 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, ABA, JA, and MeJA were collected and concentrated.

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

   **Extraction, separation, and purification**

   We added d$_4$-tZ, d$_4$-iP, d$_3$-GA$_1$, and d$_2$-GA$_4$ 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$_1$, GA$_3$, 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 μm, 4.6 × 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$_1$, GA$_3$, tZ, and iP were injected separately, and 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) was isocratically eluted with a solution of 40% ethanol + 60% ultrapure water + 0.1% acetic acid. The selected ion monitoring (SIM) method was selected, and ion monitoring 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. Figure 1 shows a representative chromatogram (selective ion monitoring). Contaminants were removed by two HPLC purifications,
leaving only peaks derived from watermelon and internal standard.

Results and Discussion

Changes in pulp color and fresh weights of fruit parts

Figure 2 shows a typical color change of small watermelon pulp over 7–35 days after flowering (DAF): 14 DAF pink around seeds; 21 DAF uniformly pink; 35 DAF deep red. The weight of the rind and pulp of the watermelon fruit increased rapidly from 7 to 28 DAF (Fig. 3, inset). In many varieties of herbaceous fruits, cell division continues for about one week after flowering, after which the cells enlarge due to cell expansion (Gillaspy et al., 1993). In watermelon, cell division stops at 6 DAF, and the fruit enlarges due to cell expansion thereafter (Kano, 1993). Subsequently, the cells of the watermelon grow large enough to be recognized by the naked eye (Nitsch, 1970). From 7 to 28 DAF in this study, it was considered that the weight of the pulp increased rapidly due to the expansion of the watermelon cells. The weight of the whole fruit was slightly reduced at 35 DAF, probably due to bias from an insufficient sample size.

The role of plant hormones in growing fruits

The hormone concentration in the whole fruit was calculated by summing the amount of hormones in the rind, pulp and seed per fruit, and dividing this total value by fresh weight per fruit.

1. IAA profiles

It has been suggested that IAA synthesized in seeds is transported to surrounding tissues, promotes cell expansion, and affects fruit growth (Nitsch, 1970; Varga and Bruinsma, 1974; Mullins et al., 1992; Tiwari et al., 2013; Kojima et al., 2020). In watermelon, IAA concentration increased to 21 DAF in seeds, but then decreased up to 35 DAF (Fig. 4). In the pulp, it rapidly
increased from 14 DAF to 28 DAF, but decreased slightly up to 35 DAF. A similar change occurred in the pulp 7 days after the change in seeds. This is probably because IAA in the seed diffused into the pulp, causing a similar increase in the pulp. In the rind, the low concentration of 0.25–0.71 pmol·g⁻¹ FW during all sampling periods suggests that high concentrations of IAA in the seeds and pulp did not affect the rind tissue outside the pulp.

2. CK profiles

Because all tissues contained a higher concentration of iP than tZ, iP is considered to be the major CK in watermelon (Fig. 5). The tZ concentration was low at 1.6 pmol·g⁻¹ FW or lower in all tissues and was almost constant during growth. iP concentration tended to decrease with growth in all tissues. CK is generally thought to play an important role in promoting cell division during fruit growth (Srivastava and Handa, 2005; Mariotti et al., 2011). A reasonable correlation between cell division and endogenous CK concentration was reported in avocado (Gazit and Blumenfeld, 1970) and peas (Burrows and Carr, 1970). In this study, plant hormones were not quantified during the cell division period (before 6 DAF), but the high concentration in the pulp immediately after the cell division period (Fig. 5) may be due to the high concentration of CK in the cell division period (7 DAF), which suggests a role for CK in promoting cell division.

During growth of watermelon fruit, iP was higher in the pulp than in the seeds. However, in tomato, CK activity was reported to be higher in seeds than in the pulp during growth (Gillaspy et al., 1993). It was reported that CK is not synthesized in seeds and may be transported from other tissues (Bohner and Bangerth, 1988). Thus, the cause of lower iP concentration in seeds could not be determined, but the higher iP concentration in pulp is thought to contribute to the promotion of fruit growth.

3. GA profiles

The role of GA during fruit growth is poorly understood, but was postulated as necessary for promoting cell division and maintaining cell expansion (Gillaspy et al., 1993). GA₄ is generally higher than GA₃ in watermelon fruit (Fig. 6), suggesting that GA₄ is the major GA. The GA₄ concentration in the seeds was at the highest concentration (37.9 pmol·g⁻¹ FW) at 7 DAF, sharply reduced at 14 DAF, and lowered to 1.5 pmol·g⁻¹ FW after 21 DAF (Fig. 5a). GA₄ concentration decreased while oscillating in the seeds.

GA activity measured by bioassay showed it was approximately 100 times higher in Phaseolus seeds than in vegetative organs (Radley, 1958). The correlation between seed growth rate and GA activity was reported in various plants, where the GA activity peak precedes the seed growth rate peak (Goodwin, 1978). The weight of watermelon seeds increased up to 14 DAF and remained almost constant thereafter (Fig. 3, inset). There-
Therefore, consistent with previous reports, this report shows that GA concentration is high in seeds, and the peak of GA concentration comes ahead of the peak of seed growth, suggesting that seed growth is promoted by GA.

GA$_1$ levels in the pulp ranged at low concentrations from 3.3 to 3.6 pmol·g$^{-1}$FW during all growth periods (Fig. 6). The rind also ranged at low concentrations from 0.7–1.5 pmol·g$^{-1}$FW. The levels of GA$_1$ in the pulp was highest at 7 DAF (51.2 pmol·g$^{-1}$FW) and dropped sharply to below 17.3 pmol·g$^{-1}$FW at 14 DAF. The rind had a low concentration of 11.4 pmol·g$^{-1}$FW or less for all growth periods.

4. ABA profiles

The ABA level was at the highest concentration (418 pmol·g$^{-1}$FW) in seeds at 14 DAF, but continued to decrease up to 35 DAF (Fig. 7). The pulp increased in ABA levels up to 21 DAF and continued to decrease up to 35 DAF. The rind had a low concentration of less than 41.8 pmol·g$^{-1}$FW in all growth periods.

ABA was implicated in controlling assimilation partitioning in both vegetative and reproductive organs (Brenner et al., 1989). In the reproductive organs, the correlation between ABA levels and growth of the reproductive organ was reported in grapes (Coombe and Hale, 1973), apples (Berüter, 1983), and soybeans (Hein et al., 1984). Nitsch et al. (2012) reported that an ABA-deficient double mutant was produced in tomato, and the fruit became smaller in the ABA-deficient state. They suggested that in wild-type tomatoes, ABA promotes ethylene synthesis, cell expansion and sink capacity/dry matter accumulation, and leads to growth of normal-sized fruits. As for the growth physiology of watermelon fruit, the future simultaneous measurement of major plant hormones and ethylene will lead to a resolution of this point. The ABA level in the pulp of watermelon peaked at 21 DAF (Fig. 7), which is the stage of cell expansion, suggesting that ABA promotes fruit growth.

Endogenous levels of plant hormones during watermelon growth were quantified by an enzyme linked immune sorbent assay (ELISA) (Dou et al., 2017). Compared to the data in this study, the quantitative values of IAA and ABA by ELISA were about 1/100th. Despite differences in the varieties and cultivation conditions of the watermelons used, further studies are necessary to test the reliability of the ELISA method, which was not an internal standard method.

5. Jasmonates (JA and MeJA) profiles

JA concentration in seeds decreased from 7 to 21 DAF and peaked at 28 DAF (Fig. 8). Similar changes were observed in the rind, pulp and whole fruit. The MeJA concentration was the highest in seeds at 7 DAF (238 pmol·g$^{-1}$FW), but decreased up to 14 DAF, and gradually increased thereafter. The rind and pulp also peaked at 7 DAF, but declined thereafter.

Jasmonate is present extensively in plant organs, but is found abundantly in fruit tissues (Meyer et al., 1984). In apples of the same climacteric fruit, the JA concentration in seeds was high in the early growth stage (Kondo et al., 2000), and the watermelon in this study showed the same tendency.

Role of plant hormones in ripening fruits

1. Growth and maturation progress

Watermelon is a climacteric fruit (Thompson, 2015), and the onset of ripening in climacteric fruit is characterized by changes in respiration rate, ethylene synthe-
sis, rind color, and texture (Abeles et al., 1992). Mizuno and Pratt (1973) reported the relationship between DAF and characteristic changes during watermelon fruit growth: pink around the seeds (20 DAF); uniformly pink (30 DAF); and deep red for best quality (45 DAF). In this study, the color of the pulp changed around 21 DAF. Thus, it was suggested that ripening started at this time, causing an increase in respiration and ethylene.

2. Physiological effects of endogenous plant hormones

High levels of endogenous CK delay fruit ripening and CK level decreases as ripening progresses (Ludford, 1995). These changes were reported in cherry tomatoes (Isenberg et al., 1987) and tomatoes (Varga and Bruinsma, 1974; Desai and Chism, 1978), suggesting an inhibitory effect on CK maturation (Srivastava and Handa, 2005). In this report, iP, which is thought to be a major CK, also tended to decrease with ripening in all tissues, suggesting an inhibitory effect on CK maturation (Fig. 5).

GA was reported to delay ripening in many climacteric fruits (Martinez-Romero et al., 2000; Singh et al., 2007; Sudha et al., 2007). Our results support the idea that GA results in the suppression of maturation in the climacteric fruit watermelon: 1) The GA\(_1\) level in seeds was the highest concentration at 7 DAF, and at 21 DAF it was a lower level (Fig. 6); 2) The GA\(_3\) concentration tended to decrease in seeds; 3) The GA\(_4\) was the highest concentration in the pulp at 7 DAF and a lower concentration after 14 DAF.

External ABA administration increased anthocyanin levels in strawberry fruits (Jiang and Joyce, 2003). Increased endogenous ABA levels in strawberry fruits were consistent with the onset of red coloring of the pulp (Jia et al., 2011; Symons et al., 2012). In the watermelon fruits in this study, an increase in ABA coincided with the onset of red coloring of the pulp, suggesting that ABA promotes red coloring.

Comparison of phytohormonal change patterns with fruits of other horticultural crops

There are some common characteristics when comparing the change patterns of grape phytohormones (Kojima et al., 2020) with the watermelon in this study: 1) The IAA concentration in whole fruits increased during late growth. 2) Seeds had high tZ and GA\(_4\) concentration. 3) The ABA concentration increased in the pulp during the middle growth stage. 4) The JA concentration was higher than other tissues in the skin (rind).

The results of hormonal changes in grape and watermelon fruits suggested the following (Kojima et al., 2020): 1) IAA synthesized in seeds migrated from the pulp to the pericarp and promoted fruit growth. 2) After CK in growing seeds promoted cell division, CK was rapidly degraded in surrounding tissues (Gillaspy et al., 1993), although it was reported that CK is not synthesized in seeds and may be transported from other tissues (Bohner and Bangert, 1988). 3) Increased ABA levels in the mid-stage were found in the rind (skin) and pulp (flesh), which contributed to phenomena such as changes in color and sugar content.

There are some common characteristics when comparing the change patterns of pear plant hormones (Okawa et al., 2015) and the watermelon in this study with the whole fruit: GA\(_1\), GA\(_3\), and iP were high at the early stage of growth, then decreased sharply and continued to be low. The accumulation of changes in endogenous hormone levels in many fruits shown in publications is important for inductively understanding the growth and maturation physiology of fruits.

In future studies, it is necessary to investigate the number and enlargement of cells in pulp, the enlargement of seeds and the production of ethylene and biological replicates for each sample.

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