Endogenous Plant Hormone Profiles in Growing Campbell Early Grape Berries

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To elucidate the profiles of plant hormones involved in growth and ripening of grape fruit at various stages, grape bunches of Campbell Early grape bunches (*Vitis labrusca* ‘Bailey’) which were harvested from 14 to 63 days after full bloom (DAFB) were divided into skin, pulp and seeds. Endogenous plant hormones, which included indole-3-acetic acid (IAA), abscisic acid (ABA), trans-zeatin (Z), isopentenyl adenine (iP), jasmonic acid (JA), methyl jasmonate (MeJA), gibberellin₁ (GA₁), and gibberellin₄ (GA₄), were quantified by liquid chromatography-mass spectrometry (LC-MS). Throughout the sampling period, higher IAA concentrations were maintained in the seeds than those in the skin and pulp. In the seeds, the Z concentration was highest at 14 DAFB and then decreased rapidly. However, in the skin and pulp, the iP concentration increased over the course of sampling. The ABA concentration in the pulp was high around veraison. The JA concentration in the skin reached 4,100 pmol·g⁻¹FW at 27 DAFB. The MeJA concentration in the skin also increased rapidly. These results suggest that increases in iP concentration in the skin and pulp are involved in maturation.

Key Words: ABA, cytokinin, GA, IAA, jasmonic acid.

Introduction

Fruit growth may require interactions among various plant hormones, because the normal fruit growth is induced by the external application of a combination of plant hormones (Vivian-Smith and Koltunow, 1999). Various plant hormones, which are used for uniform ripening and fruit quality improvement, have been studied in non-climacteric fruits such as grapes (Fortes et al., 2015) and strawberries (Symons et al., 2012). In general, abscisic acid (ABA) plays a role in promoting ripening of fruits, whereas auxin, cytokinin (CK), and gibberellin (GA) are considered to play a role in inhibiting ripening (Leopold and Kriedemann, 1964). Grapes show a slight increase in ethylene synthesis and no typical respiration peak (Coombe and Hale, 1973).

Plant hormones synthesized in seeds mainly may promote fruit growth (Varga and Bruinsma, 1976; Naylor, 1984). Especially, the auxin concentration in the seeds of most fruits is higher than that in surround-

ing tissues (Rayle and Cleland, 1992), which is a major factor in cell expansion of fruit tissues. GA triggers the fruit growth and can induce normal fruit growth even without fertilization (Gillaspy et al., 1993).

In grape fruit, there is a period called veraison between the second and third stage of fruit growth, and during veraison chlorophyll degradation, anthocyanin biosynthesis, sugar accumulation, fruit softening and acid reduction all occur (Castellarin et al., 2011).

Bioassays for auxin, GA, CK, and ABA in pulp and seeds of grapes have been reported (Coombe and Hale, 1973; Inaba et al., 1976). However, the reliability of bioassay analyses has been proven to be problematic. Even so, advances in Mass Spectrometry (MS) detectors have led to them being much more reliable than bioassays (Hedden, 1993). Simultaneous analysis of major plant hormones is important, because fruit growth is regulated by various plant hormones (Gillaspy et al., 1993). However, there is no report of simultaneous analysis of major plant hormones of each various tissue of growing grape berries using this method. Therefore, in this study, we analyzed eight endogenous plant hormones involved in the growing process of grapes using LC-MS: indole-3-acetic acid

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(IAA), trans-zeatin (Z), isopentenyl adenine (iP), gibberellin₁ (GA₁), gibberellin₄ (GA₄), ABA, JA, and methyl jasmonate (MeJA). The purpose of this study was to elucidate the phytohormone profiles at various stages of growth and ripening of grape fruit.

Materials and Methods

Plant materials

The materials were harvested five times from the 30-year-old grapes Campbell Early grapes (*Vitis labrusca* 'Bailey') in the research orchard of the Niigata Agricultural Research Institute: 14 (July 4), 27 (July 17), 41 (July 31), 50 (August 9), and 63 DAFB (days after full bloom) (August 22) (Fig. 1). Five clusters were collected at each sampling and all the clusters were divided into skin, pulp and seeds with a knife. The skins and seeds of the five clusters were weighed together, and then immersed in 80% ethanol and stored at -40°C until extraction. The pulps of the five fruit bunches were weighed together and then ground, and the soluble solids were measured with a digital refractometer using about 1 g of the ground liquid (RP-101, Atago Co., Ltd., Japan). The remaining grinding liquid was immersed in 80% ethanol and stored at -40°C until extraction.

Extraction, separation and purification of IAA, ABA and JAs

The skin and seeds were homogenized. The three tissues were filtered into a stock solution of about 80% ethanol. We added 200 pmol of $^{13}\text{C}_6$ -IAA (Cambridge Isotope Laboratories Inc., USA), d_6 -ABA (OIChemIm s.r.o., Czech Republic), d_2 -JA, and d_2 -MeJA (Tokyo Chemical Industry Co., Ltd., Japan) as internal standards to a stock solution of about 80% ethanol (equivalent to 1 g fresh weight). The aqueous sample solution was concentrated using a rotary evaporator. We adjusted the pH of the concentrated aqueous solution to 2.8 with dilute hydrochloric acid and filtered it through a membrane filter (0.22 μm). Partition extraction was

performed with the same amount of diethyl ether, and the diethyl ether layer and aqueous layer were recovered (CKs were present in the diethyl layer and GAs were present in the aqueous layer). The diethyl ether layer was concentrated under reduced pressure to approximately 1 mL and filtered through a 0.22 μm membrane filter.

HPLC for IAA, ABA, and JAs

First HPLC: The extracts were fractionated with a high-performance liquid chromatography (HPLC) system 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 + 60% ultrapure water containing 0.1% acetic acid. 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.

Second HPLC: After fractionation by the "First HPLC", all fractions were further purified with the same HPLC system. The HPLC column (C-30-S-Select, 5 μm , 4.6×250 mm, GL Sciences Inc.) was isocratically eluted with a solution of 40% ethanol + 60% ultrapure water containing 0.1% acetic acid. Fractions of IAA, ABA, JA, and MeJA were injected separately, and eluates corresponding to the retention times of IAA, ABA, JA, and MeJA were collected. IAA and ABA fractions were dried under reduced pressure, and JA and MeJA fractions were concentrated to approximately 1 mL under reduced pressure.

Extraction, separation and purification of GAs and CKs

Extraction and partition: We added 200 pmol of d_5 -Z, d_6 -iP, d_2 -GA₁, and d_2 -GA₄ (OIChemIm s.r.o., Czech Republic) as internal standards to a stock solution of about 80% ethanol (equivalent to 9 g fresh weight). The sample solution was concentrated to approximately 20 mL under reduced pressure. The pH of the aqueous layer was adjusted to 3.5 with dilute hydrochloric acid and filtered with a 0.22 μm membrane filter. Partition extraction was performed using the same amount of ethyl acetate, and the ethyl acetate and the aqueous layers were recovered (Kojima et al., 2003).

Ethyl acetate layer: Previously reported extraction rates within the ethyl acetate layer for each partition were near 85% for GA₁, 98% for GA₄ (Durley and Pharis, 1972), and 60% for iP (Letham, 1974). Anhydrous sodium sulfate (approximately 1 g/10 mL) was added to the ethyl acetate layer (GA₁, GA₄, and iP) for dehydration and allowed to stand overnight. The ethyl acetate layer was decanted, concentrated under reduced pressure, dissolved in approximately 1 mL of 50% ethanol, and filtered through a 0.22 μm syringe filter.

The aqueous layer: the pH of the aqueous layer (Z

Fig. 1. Photos of typical grape clusters of Campbell Early from 14 to 63 days after full bloom (DAFB).

and iP) was adjusted to 7.0 using an NaOH aqueous solution, partitioned, and extracted with the same amount of butanol. Previously reported extraction rates within the butanol layer for each partition were about 85% for Z and 39% for iP (Letham, 1974). The butanol layer was concentrated under reduced pressure, dissolved in approximately 1 mL of 50% ethanol, and filtered through a syringe filter (0.22 μm).

HPLC for GAs and CKs

First HPLC: Extracts from the ethyl acetate and butanol layers were fractionated using the same HPLC system used for the “First HPLC for IAA, ABA, and JAs”. Extracts from the ethyl acetate layer were injected into the HPLC system, and eluates corresponding to the retention times of GA₁, GA₄, and iP were collected. Extracts from the butanol layer were injected into the HPLC system, and eluates corresponding to the retention times of Z and iP were collected separately. The iP fractions from the ethyl acetate and butanol layers were mixed. The collected fractions were dried under reduced pressure and the residue was dissolved in 0.3 mL of 80% ethanol.

Second HPLC: After fractionation by the “First HPLC” the extracts were further fractionated using the same method for the “Second HPLC for IAA, ABA, and JAs”. The fractions of GA₁, GA₄, Z, and iP were injected separately, and eluates corresponding to the retention times of GA₁, GA₄, Z, and iP were collected. The collected fractions were dried under reduced pressure.

Analysis by LC-MS

We used an LC-MS (LCMS 2010EV, Shimadzu Corporation, Japan) to identify the hormones: column, Cadenza CD-C18 (3 μm , 250 $\times$ 2 mm; Imtakt Corp., Kyoto); flow rate, 0.1 mL·min⁻¹; and eluent, 70% ethanol + 30% ultrapure water + 0.1% acetic acid. The selected ion monitoring (SIM) method was selected as the analysis mode, and the mode for monitoring ions is shown in Table 1. Plant hormone concentrations were calculated from the ratio of the peak areas of natural and labeled ions as an internal standard (Table 1).

Table 1. Monitored pairs of ions of LC-MS for calculation of endogenous plant hormones.

			Cold/Label (m/z)	Mode
IAA	130/136	176/182	IAA/ ¹³ C ₆ IAA	Positive
Z		220/225	Z/d ₅ -Z	Positive
iP	136/137	204/210	iP/d ₆ -iP	Positive
GA ₁		347/349	GA ₁ /d ₂ -GA ₁	Negative
GA ₄		331/333	GA ₄ /d ₂ -GA ₄	Negative
ABA		263/269	ABA/d ₆ -ABA	Negative
JA		209/211	JA/d ₂ -JA	Positive
MeJA		225/227	MeJA/d ₂ -MeJA	Positive

Results

Changes in skin color, fresh weight and soluble solids

The growth of grape berries was divided into three stages (Fig. 2): Stage I, rapid growth (ca. 0–28 DAFB); Stage II, deceleration of growth (ca. 28–42 DAFB); and Stage III, rapid resumption of growth (ca. 42–71 DAFB) (Inaba et al., 1976). The rapid growth in Stage III was primarily due to cell enlargement of the pulp (Coombe and Hale, 1973). At approximately 42 DAFB, during the veraison period, maturation begins, the skin changes from green to black, sugar accumulates, the fruit softens, and acid decrease begins (Castellarin et al., 2011). In grape berries, cell division in the pulp continues until approximately 10 DAFB, after which the fruit grows with increasing cell volume (Nakagawa and Nanjo, 1965). Cell division in the pericarp continues until about 33 DAFB. The FW of the skin and pulp of grapes of Campbell Early grapes continued to increase during the sampling, but the FW of seeds varied a little (Fig. 2a). The soluble solids of the pulp increased gradually until 27 DAFB, accelerated until 51 DAFB, and increased gradually until 63 DAFB (Fig. 2b). The skin was green at 14 and 27 DAFB, partly red at 41 DAFB around veraison, and black at 50 and 63 DAFB (Fig. 1).

Fig. 2. Changes in weight and soluble solids in the developing Campbell Early grape berries from 14 to 63 DAFB. Five clusters were collected at each sampling, and all the clusters were divided into skin, pulp and seeds with a knife, all tissues were mixed.

Changes in IAA concentration

The hormone concentration of whole berries was calculated by summing the amount of seed, pulp, and skin hormones per cluster and dividing this value by the FW per fruit cluster. In the seeds, the concentration of IAA was $158 \text{ pmol} \cdot \text{g}^{-1} \text{FW}$ at 14 DAFB, but this decreased gradually to $73 \text{ pmol} \cdot \text{g}^{-1} \text{FW}$ at 51 DAFB and then increased rapidly to $775 \text{ pmol} \cdot \text{g}^{-1} \text{FW}$ (Fig. 3). Throughout the sampling period, the seeds maintained a higher IAA concentration than that in the pulp or skin. At 14 DAFB, the IAA concentration in the skin was $21 \text{ pmol} \cdot \text{g}^{-1} \text{FW}$ and that in the pulp was $5 \text{ pmol} \cdot \text{g}^{-1} \text{FW}$, however, at 27 DAFB, IAA concentrations in both tissues decreased to $2.4 \text{ pmol} \cdot \text{g}^{-1} \text{FW}$ or less (Fig. 3 inset).

Changes in Z and iP concentrations

The Z concentration in the seeds was $200 \text{ pmol} \cdot \text{g}^{-1} \text{FW}$ at 14 DAFB, but decreased to $9.8 \text{ pmol} \cdot \text{g}^{-1} \text{FW}$ at 63 DAFB (Fig. 4a). However, throughout the sampling period, the concentration of Z in the seeds remained higher than that in the pulp and skin. In the pulp, the Z concentration increased from 0.61 to $2.9 \text{ pmol} \cdot \text{g}^{-1} \text{FW}$ (14–63 DAFB) (Fig. 4a inset). In the skin, the Z concentration was as low as 0.25 – $0.71 \text{ pmol} \cdot \text{g}^{-1} \text{FW}$ throughout the sampling period. The iP concentration in the seeds was almost the same level from 14 to 63 DAFB (Fig. 4b). The change in iP concentration was in contrast to that in Z, and the iP concentration in the skin and pulp increased over the course of sampling.

Fig. 3. Changes in endogenous concentration of indole-3-acetic acid (IAA) of whole berries and tissues in developing Campbell Early grape berries from 14 to 63 DAFB. Skin, pulp, and seeds of five clusters at each sampling were mixed. An internal standard was added, then fractionated by extraction with diethyl ether, purified by high performance liquid chromatography (HPLC), and quantified by liquid chromatography-mass spectrometry (LC-MS).

Changes of GA_1 and GA_4 concentrations

The GA_1 concentration in the seeds increased slowly from 14 to 42 DAFB, increased rapidly until 51 DAFB, and then decreased rapidly (Fig. 5a). Throughout the sampling period, the GA concentration in the pulp ranged from 0.36 to $0.96 \text{ pmol} \cdot \text{g}^{-1} \text{FW}$. In the skin, the GA_1 concentration peaked at 27 and 51 DAFB. In the seeds, the highest concentration of GA_4 ($145 \text{ pmol} \cdot \text{g}^{-1} \text{FW}$) was observed at 14 DAFB. This dropped rapidly until 41 DAFB, and then peaked at 51 DAFB (Fig. 5b). In the pulp, the GA_4 concentration ranged from 0.55 to $3.0 \text{ pmol} \cdot \text{g}^{-1} \text{FW}$ throughout the sampling period and that in the skin peaked at 27 and 51 DAFB (Fig. 5b inset).

Changes in ABA, JA and MeJA concentrations

The ABA concentration in the seeds was the highest at 14 DAFB, but this decreased until 27 DAFB and then

Fig. 4. Changes in endogenous concentration of Trans-zeatin (Z) (a) and isopentenyl adenine (iP) (b) of whole berries and tissues in developing Campbell Early grape berries from 14 to 63 DAFB. Skin, pulp and seeds of five clusters at each sampling were mixed. Internal standards were added, then fractionated by extraction with butanol, purified by HPLC, and quantified by LC-MS.

Fig. 5. Changes in endogenous concentration of gibberellins₁ (GA₁) (a) and gibberellins₄ (GA₄) (b) of whole berries and tissues in developing Campbell Early grape berries from 14 to 63 DAFB. Skin, pulp and seeds of five clusters at each sampling were mixed. Internal standards were added, then fractionated by extraction with ethyl acetate, purified by HPLC, and quantified by LC-MS.

remained stable (260–300 pmol·g⁻¹FW) (Fig. 6). In the pulp, the ABA concentration rose until 27 DAFB, remained stable at 260–295 pmol·g⁻¹FW until 51 DAFB, and then decreased. In the skin, the ABA concentration increased from 27 to 63 DAFB.

The JA concentration in the seeds increased until 27 DAFB and continued to decrease thereafter (Fig. 7a inset). In the pulp, the JA concentration ranged from 6 to 96 pmol·g⁻¹FW during the sampling period. In the skin, the JA concentration peaked at 27 DAFB (4,100 pmol·g⁻¹FW) and increased again at 63 DAFB (Fig. 7a). The MeJA concentration in the seeds remained at a low level (Fig. 7b). In the pulp, the MeJA concentration ranged from 21 to 74 pmol·g⁻¹FW during the sampling period, and after 27 DAFB it was lower than that in other tissues. In the skin, the MeJA

Fig. 6. Changes in endogenous concentration of abscisic acid (ABA) of whole berries and tissues in developing Campbell Early grape berries from 14 to 63 DAFB. Skin, pulp, and seeds of five clusters at each sampling were mixed. An internal standard was added, then fractionated by extraction with diethyl ether, purified by HPLC, and quantified by LC-MS.

concentration rapidly increased until 27 DAFB (3,400 pmol·g⁻¹FW), decreased until 41 DAFB, and stayed at almost the same level thereafter (Fig. 7b).

Discussion

The roles of plant hormones during berry development

Seeds are a rich source of growth substances that diffuse to surrounding tissues (Mullins et al., 1992). It has been suggested that plant hormones (primarily auxins) synthesized in seeds promote fruit growth (Nitsch, 1971; Varga and Bruinsma, 1976). IAA synthesized in seeds is transported to surrounding tissues to promote cell division and cell expansion, affecting fruit growth (Mullins et al., 1992; Tiwari et al., 2013). In seeds of grapes, the IAA concentration also remained higher than that in the pulp and skin throughout the whole sampling period (Fig. 3). Therefore, it appears that IAA synthesized in grape seeds moves from the pulp to the skin and promotes fruit growth. However, one study has reported that grape seeds do not play a major role in the growth patterns (Peynaud and Ribéreau-Gayon, 1971); the correlation between seed number and berry size was low among seeded varieties. In addition, the seedless variety showed the same double-sigmoid growth pattern as the seeded variety. Further research is expected to elucidate the role of IAA in the seeds.

At 14 DAFB immediately after cessation of cell division, seed Z had the highest concentration of 200 pmol·g⁻¹FW (Fig. 4a). Gillaspay et al. (1993) reported that in tomato CK activity is high in growing seeds and low in surrounding tissues. It has also been reported

Fig. 7. Changes in the endogenous concentration of Jasmonic acid (JA) (a) and Methyl jasmonate (MeJA) (b) of whole berries and tissues in developing Campbell Early grape berries from 14 to 63 DAFB. Skin, pulp, and seeds of five clusters at each sampling were mixed. Internal standards were added, then fractionated by extraction with diethyl ether, purified by HPLC, and quantified by LC-MS.

that CK is not synthesized in seeds but may be transported from other tissues (Bohner and Bangerth, 1988). Moreover, Gillaspay et al. (1993) suggested that CK from developing seeds may promote cell division and that in surrounding tissues degrades rapidly. Therefore, the reason for the low Z concentration of pulp at 14 DAFB is unknown, but high Z in the seeds may contribute to the promotion of cell division of berries after flowering (Fig. 4a).

In grape berries, GA_4 was generally at a higher concentration than GA_1 (Fig. 5b), suggesting that GA_4 is the major GA. In seeds, GA_4 at 14 DAFB of Stage I had the highest concentration (Fig. 5b). GA levels in tomato fruits also tended to reach the highest concentration around Stage I (Gillaspay et al., 1993). As a result, the GA_4 of the seeds in grape berry seeds in Stage I is suggested to promote rapid berry growth.

The roles of plant hormones during fruit ripening

In the skin and pulp, iP concentrations increased over the course of sampling (Fig. 4b). Böttcher et al. (2015) reported that in grapes, iP levels increased during ripening, suggesting that increased iP is involved in the maturation process. Therefore, the increase in iP concentration in this study also suggests that iP is involved in the maturation process.

Application of GA during fruit growth reportedly delayed ripening in many fruits (McDonald et al., 1997; Zilkah et al., 1997). However, in grapes, GA_1 and GA_4 peaked at 51 DAFB in the Stage III (coloring stage after veraison) (Fig. 5a, b). Further research is needed to resolve the contradiction between the study of external application and GA increase in this study in the coloring phase.

Auxin has been reported to suppress anthocyanin synthesis. External application of auxin slows down the synthesis of anthocyanins, and removing berries (a source of auxin) from strawberry fruits during coloring induces anthocyanins synthesis (Manning, 1993). However, although there were low levels of IAA in all tissues up to 51 DAFB in grapes (Fig. 3), the skin at 51 DAFB was colored (Fig. 1). Therefore no inhibitory effect of IAA on coloring was observed in grapes. The cause and physiological significance of the high concentration of IAA in the seeds at 63 DAFB are unknown (Fig. 3), but there are two possibilities: (1) little transfer of IAA to the pulp due to the enhanced impermeability of substances by the seed coat and (2) rapid metabolism after diffusion to the surrounding pulp.

In grape berries, the ethylene peak occurs before the ABA peak (Sun et al., 2010) and just before veraison in grape berries (Chervin et al., 2004). Sun et al. (2010) suggested that some ABA effects may be mediated by ethylene. Therefore, conducting a study that combines the determination of major endogenous hormones and the measurement of ethylene production will help to elucidate the physiology of grape ripening.

In conclusion, our results revealed an endogenous plant hormone profile in grape fruit growth. The following points were further suggested: 1) The high concentration of Z in seeds at 14 DAFB immediately after cessation of cell division promotes cell division of berries after flowering. 2) Since GA_4 is generally at a higher concentration than GA_1 , GA_4 is the main GA. 3) The increase in the iP level of the skin and flesh during the ripening is involved in the maturation process.

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