High Concentrations of Endogenous Phytohormone are Presented in Cucumber Fruit Apoplasts

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Summary

Apoplast (AP) and symplast (SY) solutions were collected by centrifugation from sections of cucumber fruit pulp and placenta. Indole-3-acetic acid (IAA), abscisic acid (ABA), trans-zeatin (tZ), isopentenyladenine (iP), jasmonic acid (JA), methyl jasmonic acid (MeJA), gibberellin$_1$ (GA_1), and gibberellin$_4$ (GA_4) in AP solution, SY solution, and pericarp tissue were simultaneously quantified by liquid chromatography-mass spectrometry (LC-MS). IAA concentrations were 4.0 times higher in AP solution than in SY solution in the placenta and 6.5 times higher in AP solution than in SY solution in the flesh. tZ concentrations were 2 times higher in AP solution than in SY solution in the placenta and 5.5 times higher in AP solution than in SY solution in the flesh. iP concentrations were 2 times higher in AP solution than in SY solution in the placenta and 5.5 times higher in AP solution than in SY solution in the flesh. The concentration of GA_1 was 8 times higher than that of SY solution in the placenta and 3.5 times higher than that of SY solution in the flesh. The concentration of ABA in the placenta was 1.5 times higher than that in SY solution, and that in the flesh was 2 times higher than that in SY solution. The concentrations of the major phytohormones quantified in cucumber fruit were higher in AP solution than in SY solution in both the flesh and placenta.

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Key words : ABA, cytokinin, GA, IAA, phytohormone

Introduction

The outer space of the cell membrane, comprising the cell wall and elements such as vessel cells, is known as the apoplast (AP) (Seto and Yamaguchi, 2015). The various substances that are synthesized in the cell are secreted into the AP through the cell membrane. The AP plays an important role in the transport of signaling molecules because the vessel fluid contains these low-molecular-weight substances and signaling molecules.

The mechanism by which plant hormones in AP solutions cause physiological phenomena has been studied by the phenomenon of stomatal closure by abscisic acid (ABA). Water stress lowers the pH of the chloroplasts and cytoplasm of mesophyll cells, increased the pH, and increased the ABA concentration of AP solution (Hartung and Slovik, 1991). Furthermore, under water stress, the ABA concentration of the AP solution increases first, followed by its increase in the in the whole leaf (Cornish and Zeevaart, 1985). Therefore, stomatal closure is postulated to occur because of the pH change in the intracellular compartment due to water stress, which increases the ABA concentration of the AP solution.

Furthermore, it has been reported that the phytohormone receptors have binding sites on the AP side outside the cell membrane. Hornberg and Weiler (1984) reported a specific binding site of ABA in the cell membrane of guard cells. It was observed that directly injecting ABA into the guard cells of *Commelina communis* did not cause stomatal closure; however, injecting ABA outside the guard cells caused stomatal closure (Anderson *et al*., 1994). In another study, extracellular ABA conjugated with BSA (ABA–BSA) induced gene expression (Schultz and Quatrano, 1997). On injecting ABA–BSA outside the cultured *Arabidopsis* cells, an induction of ABA-responsive genes, such as RAB18, was observed (Jeannette *et al*., 1999). These results indicate that the ABA receptors in the guard cells are presented with the ABA binding site facing the AP.

Venis *et al*. (1990) used indole-3-acetic acid (IAA) analogues, which cannot penetrate the cell membrane, and reported that their presence on the outer surface of the cell membrane, indicating that IAA functions without entering the cell. Tsurusaki *et al*. (1997) reported that the concentration of IAA in the AP solution was several times higher than that in the symplast (SY) solution of the hypocotyl of squash and correlated this with the elongation rate of the hypocotyl, which further supported the hypothesis that IAA exerted its physiological effects from outside the cell membrane (Löbler and Klämbt, 1985).

In general, gibberellins (GAs) are synthesized in the leaves (Wareing and Phillips, 1981) and also in roots (Davies, 2002) and move through vascular bundles. Hooley *et al*. (1992) prepared protoplasts from the aleurone layer of *Avena fatua* and GA_4 derivative attached to Sepharose beads, and the administration of this derivative confirmed the induction of α-amylase and revealed the presence of GA receptors on the cell membrane of the aleurone layer.

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It has been suggested that cytokinins (CKs) are not synthesized in seeds, and instead, are transported from the roots (Bohner and Bangerth, 1988). Furthermore, an analysis using mutants suggested that vessels cells transport the CKs synthesized in the roots aboveground, which then promote aboveground growth (Kiba *et al*., 2013).

The present study aimed to clarify the concentration of phytohormones in the AP solution of cucumber fruits. The cucumber fruits after collected were divided into pericarp, pulp, and placenta and their AP and SY solutions were collected The IAA, ABA, active CKs (tZ and iP), and active GAs (GA_1 and GA_4) were quantified using liquid chromatography–mass spectrometry (LC–MS) .

Materials and Methods

Plant material

The fruits used in this study were house-grown cucumbers (*Cucumis sativus*; rootstock, all-star Ikki; scion, high green 22) obtained from a cucumber farmer in Niigata City, Niigata Prefecture, Japan, in early October. As few insects are available for pollination during this period, cucumbers are mostly produced through parthenocarpy. Twenty-four 22 cm long fruits were selected, which were ice-packed and immediately brought to the laboratory. A 0.5 cm portion at both ends of each fruit was discarded, and the fruit was cut into 1 cm long sections. The pericarp was peeled off with a knife and separated into the placenta and flesh by a cork borer.

AP and SY solutions were collected by centrifugation from sections of placenta and flesh (Kojima *et al*., 2002). The sections were placed on a plastic mesh and centrifuged at 4500 g for 5 min at 4°C to obtain AP solution.

Extraction, separation and purification of IAA and ABA

Hormone analysis was performed according to the procedure of Kojima *et al*. (2020) Briefly, the pericarp was ground, filtered, and made into a stock solution of about 80% ethanol. The equivalent of 1 g fresh weight was weighed and $^{13}C_6$-IAA and d_6-ABA were added as internal standards. Stock solutions of AP and SY solutions from pulp and placenta were also weighed to the equivalent of 1 g fresh weight, and also added as internal standards. The concentrated aqueous solutions were adjusted to pH 2.8, filtered, partitioned and extracted with diethyl ether, and concentrated.

HPLC for IAA and ABA

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) was isocratically eluted with a solution of 40% ethanol. Eluates corresponding to the retention times of IAA and ABA were collected separately and dried under reduced pressure.

Second HPLC: After fractionation by "First HPLC", all fractions were further purified with the same HPLC system. 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.

Extraction, separation and purification of GAs and CKs

Extraction and partition: We added 200 pmol of d_5-tZ, d_6-iP, d_2-GA_1, and d_2-GA_4 as internal standards to a stock solution (9 g FW equivalent). The sample solution was concentrated to approximately 20 mL under reduced pressure. 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).

The ethyl acetate layer: Previously reported extraction rates within the ethyl acetate layer for each partition were near 85% for GA_1, 98% for GA_4 (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 (GA_1, GA_4, 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 022 μm syringe filter.

The aqueous layer: pH of the aqueous layer (tZ and iP) was adjusted to 7.0 using a 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 tZ 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

Extracts from the ethyl acetate and butanol layers were fractionated using the same HPLC system used for "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 GA_1, GA_4, and iP were collected. Extracts from the butanol layer were injected into the HPLC system, and eluates corresponding to the retention times of tZ 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.

After fractionation by "First HPLC for GAs and CKs," the extracts were further fractionated using the same method for "Second HPLC for IAA, ABA, and JAs". The fractions of GA_1, GA_4, tZ, and iP were injected separately, and eluates corresponding to the retention times of GA_1, GA_4, tZ, and iP were collected. The collected fractions were dried under reduced pressure.

Analysis by LC-MS

A liquid chromatography–mass spectroscopy system (LCMS 2010EV, Shimadzu) to identify the hormones was used: column, Cadenza CD-C18 (3 μm, 250 × 2 mm, Imtakt Corp., Kyoto); flow rate, 0.1 mL/min; and eluent, 70% ethanol + 30% ultrapure water + 0.1% acetic acid. Analysis mode was selected for ion monitoring (SIM method), and phytohormone concentrations were calculated from the ratio of the peak areas of natural and labeled ions as an internal standard.

Results

The ratio of the weight of the AP solution to the weight of the solution collected by centrifugation was 11.3% in the placenta and 8.2% in the pulp (Fig. 1.). The hormone

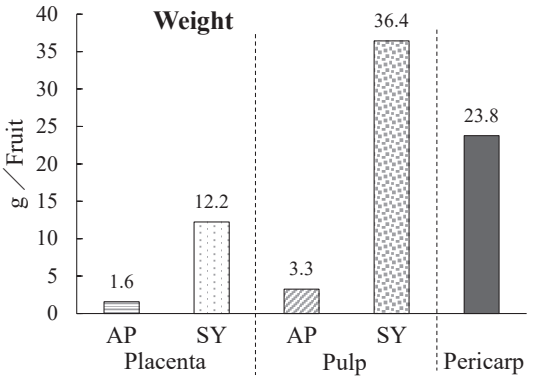

Fig. 1. AP solution and SY solution of placenta and flesh of cucumber fruits and fresh weight of pericarp. The AP solution and SY solution of 24 individuals were mixed and measured.

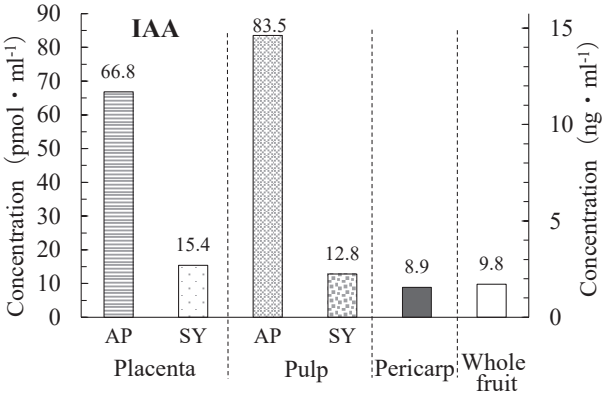

Fig. 2. Endogenous IAA concentrations in AP and SY solutions from the placenta and flesh and pericarp of cucumber fruits. The solutions of 24 individuals were mixed into one and analyzed. An internal standard was added, the mixture was extracted with diethyl ether, fractionated, purified by HPLC, and quantified by LC-MS.

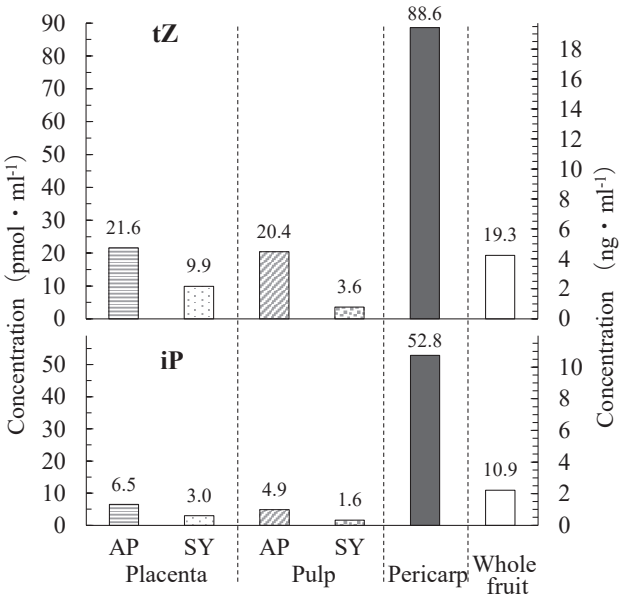

Fig. 3. Endogenous tZ (A) and iP (B) concentrations in AP and SY solutions from the placenta and flesh and pericarp of cucumber fruits. The solutions of 24 individuals were mixed and analyzed. An internal standard was added, extracted with butanol, fractionated, purified by HPLC, and quantified by LC-MS.

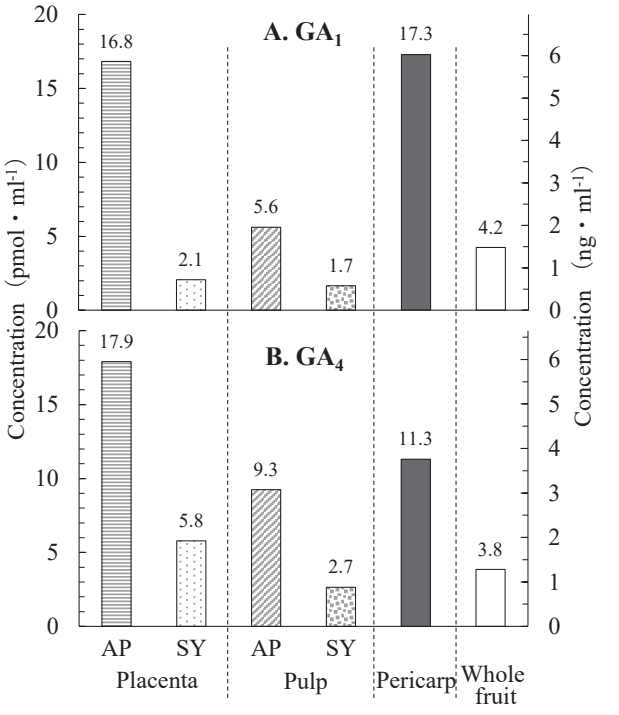

Fig. 4. Endogenous GA_1 (A) and GA_4 (B) concentrations in AP and SY solutions from the placenta and flesh and pericarp of cucumber fruits. The solutions of 24 individuals were mixed and analyzed. An internal standard was added, the mixture was extracted with ethyl acetate, fractionated, purified by HPLC, and quantified by LC-MS.

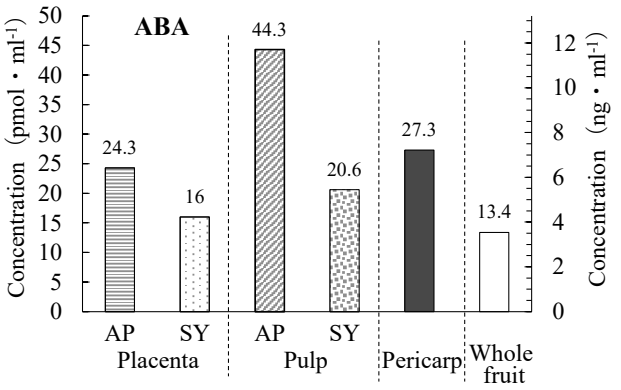

Fig. 5. Endogenous ABA concentrations in AP and SY solutions from the placenta and flesh and pericarp of cucumber fruits. The solutions of 24 individuals were mixed and analyzed. An internal standard was added, the mixture was extracted with diethyl ether, fractionated, purified by HPLC, and quantified by LC-MS.

concentrations of the whole fruit were calculated by summing up the amount of hormones in the AP solution, SY solution, and pericarp per fruit and dividing the total amount by the fresh weight, including residue per fruit. The IAA concentration was 4.0 times that of the placenta SY solution and 6.5 times that of the flesh SY solution (Fig. 2.). When comparing the concentrations of the active CKs, tZ and iP, the concentrations of tZ were found to be higher than that of iP at any given site (Fig. 3.). The tZ concentration was twice that of the placenta SY solution and 5.5 times that of the flesh SY solution. The iP concentration was twice that of the placenta SY solution and three times that of the flesh SY solution. The GA_1 concentration was eight times that of the placenta SY solution and 3.5 times that of the flesh SY solution (Fig. 4A.). The GA_4 concentration was three times that of the placenta SY solution and 3.5 times that of the flesh SY solution (Fig. 4B.). The ABA concentration was 1.5 times that of the placenta SY solution and twice that of the flesh SY solution (Fig. 5.).

Discussion

Using LC–MS and centrifugation (Tsurusaki *et al*., 1997), the major endogenous phytohormones in the AP solution of the cucumber fruits were quantified and their presence was reported. Because the AP solution per individual fruit was miniscule and below the analytical limit of the employed laboratory analysis system, analysis could not be repeated, and the solutions from the 24 cucumber fruits were analyzed as one.

As shown by Kojima *et al*. (2003), there is a possibility of the mixing of the AP and SY solutions during centrifugation. However, had the mixing occurred, the difference between the higher and lower concentrations would have been lesser, and the difference in the phytohormone concentrations between the AP and SY solutions would have been greater.

In the cucumber fruits, the IAA concentration of the placenta and flesh AP solutions was 84 and 67 pmol/gFW, respectively. In the upper part of the lower hypocotyl of squash, the IAA concentration of the AP solution was 226 pmol/ml (Tsurusaki *et al*., 1997). In the upper and lower stem of a three-month-old tomato, the IAA concentration of the AP solution was 150 and 300 pmol/gFW, respectively (Kojima *et al*., 2002). In other plants, whose AP solutions were quantified, similar concentrations of IAA were reported.

So far, the quantification of CK in the AP solution of fruits has reported a slightly higher concentration of tZ in the flesh AP solution than in the SY solution in both 6 and 8 cm diameter tomato fruits with a slow growth rate (Kojima *et al*., 2003). In the cucumber fruits, the concentrations of tZ and iP were higher in the pericarp than in the placenta and flesh (Fig. 3.). Previous studies analyzed the tZ and iP concentrations separately for the pericarp and flesh of grape (Kojima *et al*., 2020), watermelon (Kojima *et al*., 2021a), and melon (Kojima *et al*., 2021b), all of which showed lower concentrations of the CKs in the pericarp than in the flesh. The main physiological effect of CK in fruits is to promote cell division (Naylor, 1984); however, cell division is not particularly active in the cucumber pericarp. Therefore, the high CK concentrations of the pericarp may be related to the rapid growth physiology of cucumber fruit; further research is expected to provide more clarification.

In general, GAs are synthesized in the leaves (Wareing and Phillips, 1981) and roots (Davies, 2002). In the cucumber fruit, GA_1 and GA_4 were highly concentrated in the placenta AP solution, and GA_1 was also highly concentrated in the pericarp. These results suggest that GA_1 and GA_4 may be synthesized in the placenta AP and GA_1 in the pericarp as well.

The cucumber fruits were considered to be in a state of water stress because the AP solution was collected several hours after harvesting. Due to the pH change of the intracellular compartment due to this water stress, the amount of ABA in the AP solution increased, which is hypothesized to stimulate stomatal closure (Hartung and Slovik, 1991; Cornish and Zeevaart, 1985).

Tsurusaki *et al*. (1997) reported that the concentration of IAA in the AP solution was several times higher than that of the SY solution in the lower hypocotyl of squash and correlated this with its elongation rate. Venis *et al*. (1990) reported that IAA receptors are located on the outer surface of the cell membrane, and hence, IAA can function without entering the cell. The concentrations of all the phytohormones quantified in the cucumber fruit were higher in the AP solution than in the SY solution in both the flesh and placenta. It was reported that ABA receptors are presented with their binding site facing the AP (Anderson *et al*., 1994), and a receptor for GA was also reported to be presented on the plasma membrane (Hooley *et al*., 1992). Therefore, it has been suggested that these phytohormones

are transported from the tissue of their synthesis to the tissue of their action via AP where they bind directly to these receptors without permeating the cell membrane and efficiently transmit signals and produce physiological responses in the cucumber fruit, such as rapid growth.

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References

Anderson, B. E., J. M. Ward and J. I. Schroeder. 1994. Evidence for an extracellular reception site for abscisic acid in Commelina guard cells. Plant Physiol., **104**: 1177-1183. https://doi.org/10.1104/pp.104.4.1177

Bohner, J. and F. Bangerth. 1988. Effects of fruit set sequence and defoliation on cell number, cell size and hormone levels of tomato fruits (*Lycopersicon esculentum* Mill.) within a truss. Plant Growth Regul., **7**: 141-155.

Cornish, K. and J. A. Zeevaart. 1985. Abscisic acid accumulation by roots of *Xanthium strumarium* L. and *Lycopersicon esculentum* Mill. in relation to water stress. Plant Physiol., **9**: 653-658.

Davies, P. J. 2002. Gibberellins: Regulators of Plant Height. p. 461-492. In: Taiz L. and E. Zeiger (eds). Plant Physiol., 3rd edn. Sinauer Associates, Sunderland.

Durley, R. C. and R. P. Pharis. 1972. Partition coefficients of 27 gibberellins. Phytochemistry **11**: 317-326.

Hartung, W. and S. Slovik. 1991. Physicochemical properties of plant growth regulators and plant tissues determine their distribution and redistribution: stomatal regulation by abscisic acid in leaves. New Phytol., **119**: 361-382. https://doi.org/10.1111/j.1469-8137.1991.tb00036.x

Hooley, R., M. H. Beale, S. J. Smith, R. P. Walker, P. J. Rushton, P. N. Whitford and C. M. Lazarus. 1992. Gibberellin perception and the *Avena fatua* aleurone: do our molecular keys fit the correct locks? Biochem. Soc., Trans. **20**: 85-89. https://doi.org/10.1042/bst0200085

Hornberg, C. and E. W. Weiler. 1984. High-affinity binding sites for abscisic acid on the plasmalemma of *Vicia faba* guard cells. Nature, **310**: 321-324.

Jeannette, E., J. P. Rona, F. Bardat, D. Cornel, B. Sotta and E. Miginiac. 1999. Induction of RAB18 gene expression and activation of K^+ outward rectifying channels depend on an extracellular perception of ABA in *Arabidopsis thaliana* suspension cells. Plant J., **18**: 13-22. https://doi.org/10.1046/j.1365-313X.1999.00423.x

Kiba, T., K. Takei, M. Kojima and H. Sakakibara. 2013. Side-chain modification of cytokinins controls shoot growth in *Arabidopsis*. Dev. Cell, **27**: 452-461. https://doi.org/10.1016/j.devcel.2013.10.004

Kojima, K., E. Ohtake and Z. Yu. 2002. Distribution and transport of IAA in tomato plants. Plant Growth Regul., **37**: 249-254.

Kojima, K., Y. Tamura, M. Nakano, D. S. Han and Y. Niimi. 2003. Distribution of indole-acetic acid, gibberellin and cytokinins in apoplast and symplast of parthenocarpic tomato fruits. Plant Growth Regul., **41**: 99-104.

Kojima, K., H. Ikarashi, D. Andou and T. Matsumoto. 2020. Endogenous plant hormones profile in growing grape berries of Campbell Early. Hort. J., **89**: 509-515.

Kojima, K., D. Andou and M. Ito. 2021a. Plant hormone changes in growing small watermelon fruit. Hort. J., UTD-209. https://doi.org/10.2503/hortj.UTD-209

Kojima, K., H. Nomura and D. Andou. 2021b. Endogenous plant hormone profiles in growing melon fruit. Environ. Control Biol., **59**: 141-146. https://doi: 10.2525/ecb.59.141

Letham, D. S. 1974. Regulators of cell division in plant tissues ＸＸＩ. Distribution coefficients for cytokinins. Physiol. Plant., **118**: 361-364.

Löbler, M. and D. Klämbt. 1985. Auxin-binding protein from coleoptile membranes of corn (*Zea mays* L.). I. Purification by immunological methods and characterization. J. Biol. Chem., **260**: 9848-9853.

Naylor, A. W. 1984. Functions of hormones at the organ level of organization. p. 172-218. In: Scott, T. K. (ed.). Hormonal regulation of development Ⅱ. Springer, Berlin, Heidelberg.

Schultz, T. F. and R. S. Quatrano. 1997. Evidence for surface perception of abscisic acid by rice suspension cells as assayed by Em gene expression. Plant Sci., **130**: 63-71. https://doi.org/10.1016/S0168-9452(97)00204-5

Seto, Y. and S. Yamaguchi. 2015. Movement and functions of signaling molecules in the apoplast. 2015. Regul. Plant Growth Dev., **50**: 70-75 (In Japanese). https://doi.org/10.18978/jscrp.50.1_70

Tsurusaki, K., Y. Masuda and N. Sakurai. 1997. Distribution of indole-3-acetic acid in the apoplast and symplast of squash (*Cucurbita maxima*) hypocotyls. Plant Cell Physiol., **38**: 352-356. https://doi.org/10.1093/oxfordjournals.pcp.a029173

Venis, M. A., E. W. Thomas and H. Barbier-Brygoo, G. Ephritikhine and J. Guern. 1990. Impermeant auxin analogues have auxin activity. Planta., **182**: 232-235.

Wareing, P. F. and I. D. J. Phillips. 1981. Growth and Differentiation in Plants. Pergamon Press, Oxford.

キュウリ果実のアポプラストには高濃度の内生植物ホルモンが存在する

児島清秀

（2022年12月25日受付）

要　約

キュウリ果実の果肉と胎座の切片から遠心法でアポプラスト（AP）溶液とシンプラスト（SY）溶液が採取された。AP 溶液、SY 溶液および果皮組織のインドール -3- 酢酸（IAA）、アブシジン酸（ABA）、トランスゼアチン（tZ）、イソペンテニルアデニン（iP）、ジャスモン酸（JA）、ジャスモン酸メチル（MeJA）、ジベレリン1（GA_1）およびジベレリン4（GA_4）が同時に液体クロマトグラフィー質量分析（LC-MS）で定量された。

IAA 濃度は胎座において AP 溶液は SY 溶液の4.0倍で、果肉においても AP 溶液は SY 溶液の6.5倍であった。tZ 濃度は胎座において AP 溶液は SY 溶液の２倍で、果肉においても AP 溶液は SY 溶液の5.5倍であった。iP 濃度は胎座において AP 溶液は SY 溶液の２倍で、果肉においても AP 溶液は SY 溶液の３倍であった。GA_1濃度は胎座において AP 溶液は SY 溶液の８倍で、果肉においても AP 溶液は SY 溶液の3.5倍であった。GA_4濃度は胎座において AP 溶液は SY 溶液の３倍で、果肉においても AP 溶液は SY 溶液の3.5倍であった。ABA 濃度は胎座において AP 溶液は SY 溶液の1.5倍で、果肉においても AP 溶液は SY 溶液の２倍であった。

キュウリ果実で定量を行った主要な植物ホルモンにおいて、果肉部と胎座部とも、AP 溶液の方が SY 溶液よりも高い濃度であった。

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キーワード：ABA、サイトカイニン、GA、IAA、植物ホルモン

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