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Variations in the Levels of Major Free Cytokinins and Free Abscisic Acid During Tuber Development of Sweet Potato

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Abstract. Changes in two plant growth substances were examined throughout tuber development of sweet potato (*Ipomoea batatas* Lam. cv. Minamiyutaka). Major free cytokinins [*t*-zeatin riboside, N^6-(Δ^2-isopentenyl)adenosine and 6-(3-methyl-2-butenyl amino)purine glucoside] and free abscisic acid in tubers were determined by HPLC and GC-ECD. An increase in *t*-ZR almost coincided with the onset of rapid tuberization 30–90 days after planting the vine cuttings. The levels of i^6Ado and ABA were much lower than that of *t*-ZR throughout tuber development. The maximum level of i^6Ado preceded the maximum of *t*-ZR. The level of iPG was higher than that of *t*-ZR, and the pattern of changes in the level was more complex. The maximum level of iPG reached about 270 $\mu g\ kg^{-1}$ fresh weight. Varied changes in the low levels of ABA appeared not to be related to tuber development. Longitudinal distribution of the cytokinins and ABA in the developing tubers showed that levels of *t*-ZR were higher in parts of the proximal side of the stem than in other, lower parts of the tubers.

The formation and development of tubers are among the most characteristic phenomena in the life cycle of sweet potato (*Ipomoea batatas* Lam.). However, there are only few data concerning the molecular mechanism of tuberization. Plant growth substances have been suggested to play an important role(s) in the formation and development of the tubers. Studying such mechanisms is expected to lead to methods of increasing tuber productivity.

Several cytokinins have already been identified in the extracts of sweet potato (Hashizume et al. 1981, 1982a,b, Matsuo et al. 1983). Sugiyama et al. (1983) used a mass spectrometric method using deuterium-labeled standards to precisely determine cytokinins in sweet potato plants. The relationship be-

tween cytokinin levels and the physiological development of this plant has not yet been examined in detail.

Okazawa and Chapman (1962) suggested that the tuberization of Irish potato (*Solanum tuberosum* L.) could be regulated by changes in the balance of promotive and inhibitory hormones. Abscisic acid (ABA) could play a role in cell and organ differentiation of tuberous roots by inhibiting root apical meristem activity and cell elongation, as noted by Melis and Van Staden (1985) in a recent review.

Our previous paper showed that the cytokinin content (mainly *t*-zeatin riboside; *t*-ZR) is higher in the larger sweet potato tubers among tubers harvested at the same time (Matsuo et al. 1983). Since several tubers and roots of various sizes are attached to each vine at different developmental stages, tuber sampling during development needs to be standardized.

The present paper describes variations in the levels of major free cytokinins, *t*-ZR, N^6-(Δ^2-isopentenyl)adenosine (i^6Ado), 6-(3-methyl-2-butenyl amino)purine glucoside (iPG), and ABA in the largest tubers among the tubers harvested from each vine at a given time during tuber development. The physiological significance of these hormones during tuber development is discussed.

Materials and Methods

Plant Materials and Chemicals

Vine cuttings of the sweet potato (*Ipomoea batatas* Lam., cv. Minamiyutaka), with seven leaves and about 45 cm in length, were planted in an experimental field at the Kagoshima University on May 1, 1984. Developing tubers were sampled eight times between May 22 and October 1. The cultivar "Minamiyutaka" is reported to be late developing, and in Japan the tuber can continue to develop and enlarge until mid-November. At each sampling date, 6–30 vines were harvested depending on tuber or root size. Only the largest tuber attached to the vine was collected at a given time. Average tuber diameter ranged from 1.5 to 68.2 mm, and the average fresh weight ranged from 1.3 to 340.2 g. The tubers were washed with ice-cold water, cut into pieces, and immersed in cold methanol (−20°C) immediately after collection.

Authentic i^6Ade, i^6Ado, *cis-trans*-zeatin, *cis-trans*-zeatin riboside, and ABA were purchased from Sigma, and *t*-zeatin and *t*-zeatin riboside from Calbiochem.

Extraction and Purification of Cytokinin and ABA

Pieces of tubers (about 100 g) were thoroughly homogenized in a porcelain mortar with a pestle in a given amount of cold methanol and then filtered. The residue was again extracted with cold methanol in the same manner. The combined filtrate (about 1 L) was concentrated to remove methanol, resulting in about 200 ml of an aqueous solution. The solution was adjusted to pH 3.5 with dilute acetic acid and partitioned three times against one-third volumes of dis-

tilled ethyl ether containing 1 mg/ml BHT. The ethyl ether phase was washed with 50 ml acidified water (pH 3.5, with dilute acetic acid). The organic solvent phase was used for ABA determination. The combined acidic aqueous solution was applied to a PVP column (volume = 100 ml; PVP purchased from Tokyo Kasei Kogyo). The column was washed with 300 ml acidified water (pH 3.5). The eluates were combined and then adsorbed on a column of Whatman P-1 cellulose (H^+ form, 100 ml volume). The column was washed with equal column volumes of 0.05 N acetic acid and 150 ml water. Cytokinins were eluted with 5 volumes of 1 N NH_4OH. The alkaline eluate was evaporated *in vacuo* to remove ammonia first in a cold water bath and then at 35°C to a volume of about 50 ml. The pH was adjusted to 8.0 with phosphate buffer (0.5 M) and extracted with three 25-ml volumes of *n*-butanol. After evaporating to remove *n*-butanol, the aqueous layer was further purified using a Sep-Pak cartridge (Waters Associates), as described previously (Matsuo et al. 1983). Up to the stage of Sep-Pak purification, the recoveries of authentic *t*-ZR and i^6Ado were shown to be 96% and 76%, respectively (Takagi et al. 1985).

The ethyl ether-soluble fraction was used to determine free ABA. To check the reproducibility of the assay, synthetic ABA was added to every individual sample at this stage at the rate of 500 ng/g fresh weight. The ether phase (about 240 ml) was extracted four times with a half volume of saturated $NaHCO_3$ solution. The aqueous phase was adjusted to pH 2.5 with 4 N HCl and then extracted with about 250 ml of ethyl acetate containing 1 mg/ml of BHT, and the combined organic phase was dried over Na_2SO_4 overnight. After filtration, the organic phase was evaporated *in vacuo* at 35°C. The gumlike substance obtained was dissolved in a small amount of methanol and applied to a PVP column (1.0 × 27 cm, column volume 85 ml). The column was washed with 0.8 column volumes of phosphate buffer (0.1 M, pH 8.0) and eluted with 2.7 column volumes of the same buffer. The eluate (from 0.8 to 3.5 column volumes) was adjusted to pH 3.0 with 4 N HCl and extracted four times with a half volume of ethyl acetate. After drying over Na_2SO_4 the organic phase was evaporated *in vacuo* at 35°C. The residual materials were dissolved in 50 μl of MeOH. The ABA in the extracts was purified by HPLC using Radial-Pak A (0.8 × 10 cm, Waters Associates). The elution was carried out with 60% MeOH containing 10 mM acetic acid at 1.0 ml/min. ABA was monitored in the eluate by measuring the OD at 260 nm. The ABA fraction was reduced to dryness and methylated with diazomethane.

Quantitative Determination of ABA with GLC

The content of methyl abscisate was determined by using a GC-ECD, Shimadzu GC-7APrFE after dilution of the sample solution. Samples were chromatographed at 215°C on a glass column (3 mm × 1.6 m, 3% SE-30 on Gaschrom Q). Flow rate of nitrogen gas as a carrier was 60 ml/min. Under these conditions authentic ABA methyl ester showed two peaks with retention times of 3.50 and 4.43 min. Determination of ABA methyl ester was calculated from the values of each peak area which was measured by Shimadzu Chromatopack B II. The standard curve was linear in the range of 10–50 pg of the ester. For

routine assays a single extraction of each tuber sample was carried out, and the resulting extract was assayed three times. The triple assays usually gave values within ±5% of the mean when a clean ECD was used. Overall recovery of the synthetic ABA was between 50% and 70%. All data in this paper have been corrected for recovery losses.

High-Performance Liquid Chromatographic Determination of Cytokinins

Details of high-performance liquid chromatography (HPLC) analyses for cytokinin determination are described in a previous paper (Matsuo et al. 1983). Amounts of iPG were estimated roughly by the areas of peaks of authentic i^6Ado on the chromatograms because of failure to synthesize iPG. This cytokinin showed a similar chromatographic behavior to it of *t*-ZR on purification and HPLC analysis. As well as ABA determination, for routine assays a single extraction of each tuber sample was carried out, and the resulting extract was assayed three times, which gave values within ±5% of the mean.

Gas Chromatography–Mass Spectrometry (GC-MS)

The GC-MS analyses were performed as described by Matsuo et al. (1983), with the following modifications. The silylating reagent was composed of bis-(trimethylsilyl)acetoamide-acetonitrile-trimethylchlorosilane (3:6:1, v/v/v). The reaction was carried out at 70°C for 15 min. The column temperature was 270°C for analysis of iPG TMS derivatives.

Identification of ABA methyl ester was also carried out by GC-MS analysis. In this case the silanized glass column (3 mm × 1.6 m) was packed with 3% OV-17 on Chromosorb AW-DMCS (80–100 mesh). The column temperature was 230°C, and the ionizing voltage was 25 eV.

Distribution of the Plant Growth Substances in the Developing Tubers of Sweet Potato

Longitudinal distribution of each hormone during tuber development was examined. Several sweet potato tubers were harvested 120 days after planting the vine cuttings. Their fresh weight averaged 345.3 g, and their average diameter was 74.1 cm.

Each tuber was washed with ice-cold water just after harvest and divided into five parts while at 4°C, as shown schematically in Fig. 4. The tubers were first cut 2 cm above and below the center line at their maximum diameters. This center part was called No. 4, and the lower part No. 5. After removing the enlarged stem (No. 1), the resulting upper part was divided equally to form part Nos. 2 and 3. Then each part was further cut into pieces and immersed in cold methanol (−20°C). Extraction, purification, and determination of cytokinins and ABA were performed in the same manner as previously described.

Results

Changes in Size and Weight of Tubers During Tuber Development

The changes in levels of free major cytokinins and free ABA were followed only in the largest tubers over the period May 22 to October 1—that is, during the 150 days after planting vine cuttings. During this period the average diameter of the largest roots or tubers increased from 1.5 mm to 68.2 mm, and their average fresh weights increased from 1.3 g to 340.2 g. A significant increase in the tuber diameter was found even at 30 days after planting vine cuttings. The diameter thereafter increased almost linearly until 120 days after planting (Fig. 1).

Changes in the Levels of Free Major Cytokinin During Tuber Development

Accompanying the greatest rate of change in the size and weight of tubers was a rapid change in *t*-ZR level in the developing sweet potato tubers (Fig. 2). In the newly developing tubers collected 20 days after planting, the level of *t*-ZR was 4.5 μg kg^{-1} of fresh weight. The level increased 20-fold during the following 30 days and attained a maximum of 84.6 μg kg^{-1} in the tubers collected 50 days after planting. Thereafter, the level decreased to 11.8 μg kg^{-1} of fresh weight at 90 days after planting. The large tubers (340.2 g and 68.2 mm) collected 150 days after planting had a *t*-ZR content of 7.3 μg kg^{-1}. During the last 2 months there was less change in the level.

Figure 2 also shows the changes in the level of i^6Ado during tuber development. The levels were considerably lower and about one-eleventh of *t*-ZR at maximum. In the tubers collected 20 days after planting the level was 2.6 μg

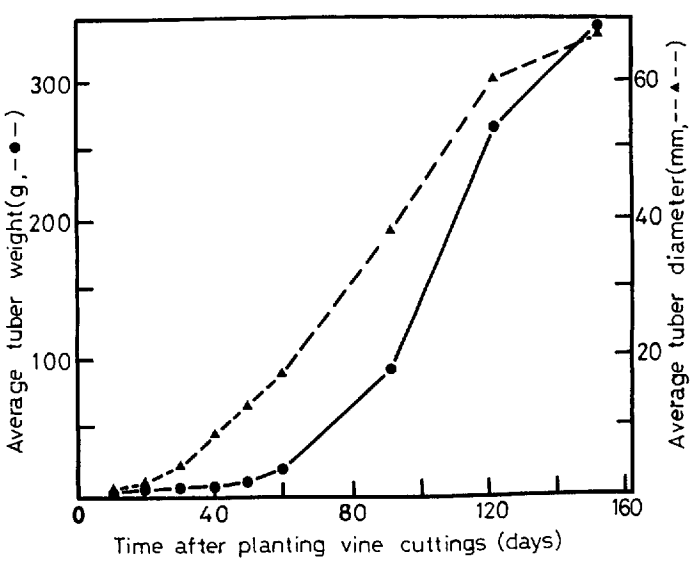

Fig. 1. Changes in size and weight of sweet potato tubers during development.

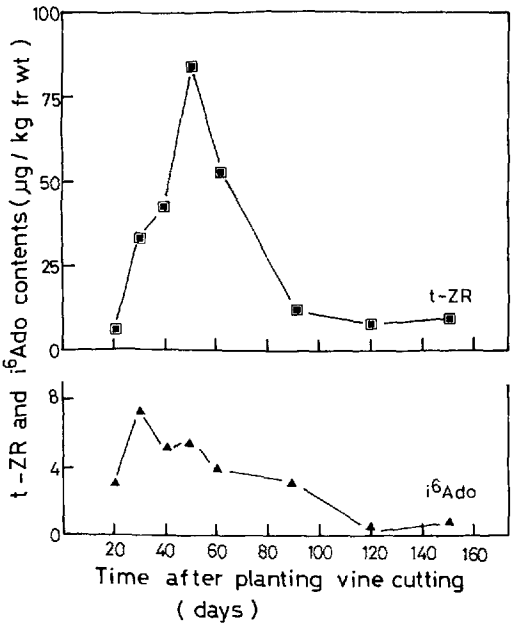

Fig. 2. Changes in the levels of i⁶Ado and *t*-ZR during tuber development.

kg^{-1} of fresh weight. The level of the compound reached a maximum in tubers 30 days after planting (7.5 μg kg^{-1}). Thereafter, the levels of i⁶Ado decreased gradually, to 0.5 μg kg^{-1} 120 days after planting.

The behavior of iPG was quite different from that of the other cytokinins. The content was higher throughout the developing period, and there were marked changes in level at an early stage of tuber development, as shown in Fig. 3. In tubers collected 20 days after planting, the level was 43.4 μg and 239 μg kg^{-1} of fresh weight at 30 days. The levels decreased by one-half during the following 10 days but again increased in the subsequent 20 days; they reached the maximum of 272.5 μg kg^{-1} 60 days after planting vine cuttings. The level again decreased between 90 days and 150 days, to 150 μg kg^{-1}.

Changes in Levels of Abscisic Acid During Tuber Development

The levels of ABA changed slightly during tuber development, although the levels were considerably lower than those of *t*-ZR and iPG (Fig. 3). The level of ABA was 4.0 μg kg^{-1} in the young tubers collected 20 days after planting. The peaks in ABA levels were observed at three sampling times—30, 60, and 150 days after planting. The three highest levels were 6.0, 12.5, and 11.0 μg kg^{-1} of fresh weight, respectively. The tubers collected 50 days after planting showed a minimum of 3.3 μg kg^{-1}, and then the levels increased remarkably, to 12.5 μg kg^{-1} (60 days after planting). After the maximum the level again decreased to 4.0 μg kg^{-1} in the large tubers collected 120 days after planting.

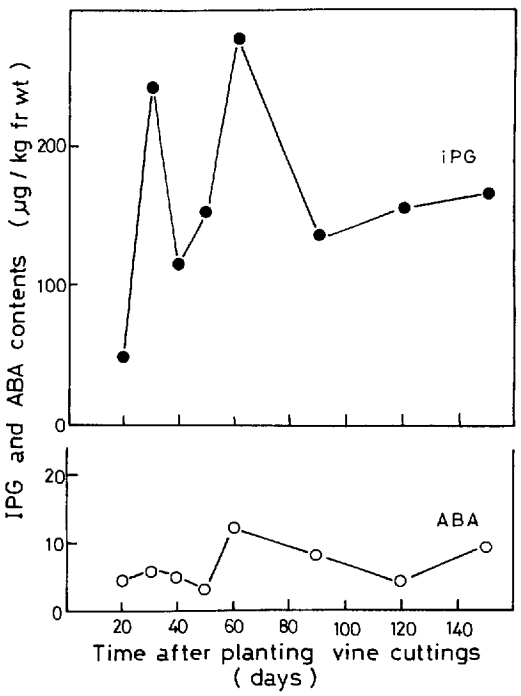

Fig. 3. Changes in the levels of iPG and ABA in the tubers of sweet potato during development.

Distribution of the Plant Growth Substances in the Developing Tubers of Sweet Potato

It was evident that *t*-ZR was localized longitudinally in the developing tubers. The level was remarkably high in the proximal side for stem parts No. 1 and 2, as shown in Fig. 4. The level in part No. 5 was 6.5 μg kg^{-1} of fresh weight, about one-fourth the level observed in part No. 2. It was noteworthy that the tissue of part No. 1 had a very high *t*-ZR level but showed less enlargement; that is, the diameters of the parts No. 1 and 4 were about 0.9 and 74.1 cm, respectively.

On the other hand, iPG was high in all parts of the tubers, and there was only a small difference in the levels among these parts, except for part No. 1. The average level was 148.2 μg kg^{-1} of fresh weight in parts No. 2, 3, 4, and 5.

The distribution pattern of ABA in the tubers was similar to that of iPG except ABA level was nearly the same in all parts.

Discussion

The present data show that there is a pronounced increase in the level of *t*-ZR which is coincident with the greatest rate of tuberization, that is, 30–90 days after planting (Figs. 1, 2). Kokubu (1973) examined the anatomical changes during development and found differentiation of root to tuber to be completed

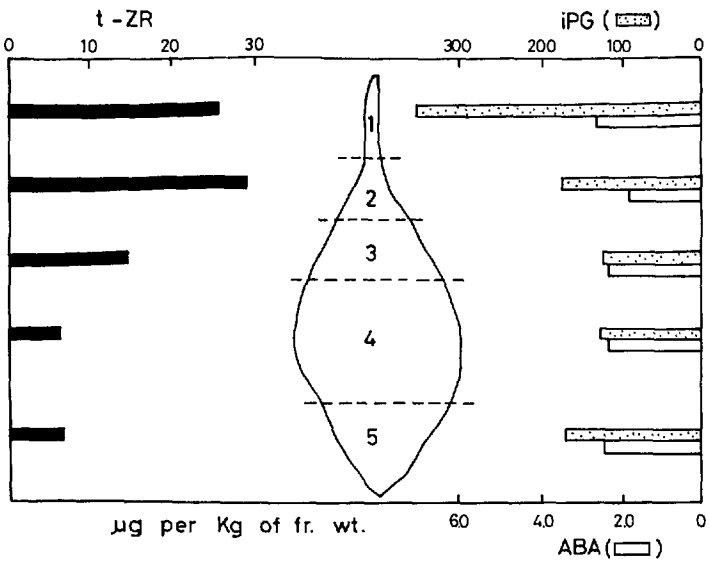

Fig. 4. Distribution of the cytokinins and ABA in developing sweet potato tubers.

about 20 days after planting vine cuttings with subsequent rapid cell division (about 30–50 days). The coincidence of this with a peak of *t*-ZR suggests that *t*-ZR may play a significant role in tuber development by influencing cell division and subsequent cell enlargement during early tuber development. There is much evidence to suggest that *t*-ZR and/or *t*-Z is present in high levels in tubers and other plant tissues or organs during active cell division as well as cell enlargement (Van Staden and Davey 1979, Beader 1980).

Stenlid (1982) reported that the elongation of wheat, flax, and cucumber seedlings roots was strongly inhibited by exogenous native cytokinins at relatively low concentrations (50% inhibition by zeatin at 2×10^{-7} M to 3×10^{-9} M). Melis and Van Staden (1984) discussed the function of this hormone in relation to the distribution and accumulation of carbohydrates in some tubers. From these observations and views, it is suggested that cytokinin may have several different roles depending on stage of the tuber development, such as (1) the inhibition of vertical growth of roots, (2) the induction and promotion of horizontal growth of roots including cell division and cell expansion, and (3) the promotion of transport and accumulation of carbohydrates accompanied by cell enlargement of tubers.

Kokubu observed less development of vascular bundles in the lower part of the tubers in contrast to the upper part (personal communication). The longitudinal distribution of *t*-ZR in the developing tubers (Fig. 4) shows high levels in the proximal side of the stem (parts No. 1 and 2). It is suggested that *t*-ZR localizes at vascular bundles because there are few parenchyma cells in the proximal side of the stem. Recently, Melis and Van Staden (1985) described the horizontal distribution of cytokinins in cassava tubers and found that most of the cytokinin activity was concentrated in the outer region of the xylem including the cambium. As suggested in their paper, the high levels of cytokinins could indicate that cytokinins are synthesized in this young tuber tissue and

control tuberous root growth. The view that hormones in a sink create a demand for carbohydrates by stimulating cell growth and differentiation has been discussed by Morris (1982). However, proof of its involvement at the reproduction site as well as the site of cytokinin function in sweet potato tubers needs further confirmation.

Hashizume et al. (1982b) reported that iPG showed only one-thousandth the activity as BA in the *Amaranthus* bioassay, which raises the question of its physiological role. The levels of iPG throughout tuber development were much higher than any of the other cytokinins, and changes in its level are rather complex (Fig. 3). Considering complex changes in the levels, it is suggested that this cytokinin might have some specific role that is different from that of *t*-ZR.

Considerable attention has been given to the possibility that tuberization is controlled by a balance between growth promoters and growth inhibitors. However, ABA content during tuber development is relatively low, and the change in its level appears not to be related to the increase in size and weight of the tuber throughout tuber development, as shown in Fig. 3. The level does not vary greatly among different parts of the tuber (Fig. 4). The results indicate that in the tuber of sweet potato the abrupt increase of *t*-ZR may have more important meanings that control tuber development, in contrast to ABA. Since much discussion has been carried out about a correlation between ABA levels and water stress in various plant tissues, further investigation is necessary to determine the role(s) of ABA in tuber development.

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