Effects of the Direction of Gravistimulation on Tuber Formation and Amyloplast Distribution in Tuber Tips of Chinese Yam

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Abstract: The effects of gravistimulation direction on tuber formation and amyloplast distribution in the tuber tips of Chinese yam were investigated. Tubers placed vertically for either 2 or 14 d elongated in the direction of gravity vector. The subapical parts of tubers that were positioned horizontally for 2 d curved slightly toward the gravity direction, and such tubers elongated downward after 14 d. In tubers that were inversed for 14 d, the tips bent and eventually elongated in the direction of the gravity vector. These results indicated that the subapical part of the tuber was the initial site of curvature in response to gravistimulation. Microscopy and measurement of amyloplast sedimentation ratios showed that many amyloplasts sedimented downward in each tip cell of tubers that were placed vertically, inversely or horizontally. The results indicated that amyloplast sedimentation direction corresponded with the eventual direction of tuber elongation under different gravistimulation orientations. Tubers rotated at 90° intervals every day for 14 d did not have an elongating shape and grew multiple tips at different angles. In the rotated tubers, amyloplasts dispersed within the tip cells. These results showed that proper sedimentation of amyloplasts would be essential for the normal elongation and formation of tubers. The results of this study supported the possibility that sedimentable amyloplasts in the tuber tip were involved in gravity sensing and tuber formation.

Key words: Amyloplast, Geotropism, Gravity, Nagaimo, Tuber, Yam.

Chinese yam (Dioscorea opposita Thunb.), called ‘nagaimo’ in Japanese, is an important root and tuber crop. The tuber is mainly used as human food and in oriental medicine in East Asia. In yams, the underground tuber can assume various shapes, such as elongate, spherical, or flattened. Elongate tubers grow in the direction of gravity vector (Kawasaki et al., 2008).

In higher plants, the columnella cells in the root caps and the endodermis cells in stems have been reported to be the primary gravity-sensing sites (Sack, 1991; Blancanclor et al., 1998; Fukaki et al., 1998; Chen et al., 1999; Tsugeki and Fedoroff, 1999; Kiss, 2000; Strohm et al., 2012a, 2012b; Hashiguchi et al., 2013). These cells contain amyloplasts that sediment to the lower side upon gravistimulation (Sack et al., 1985; Kiss et al., 1989; Saito et al., 2005; Leitz et al., 2009); such amyloplasts are also called statoliths. Many previous investigations have supported the theory that the sedimentable amyloplasts are involved in gravity sensing (Sack et al., 1985; Caspar and Pickard, 1989; Kuznetsov and Hasenstein, 1996; Blancanclor et al., 1998; Chen et al., 1999; Weise and Kiss, 1999; Weise et al., 2000; Kondrachuk and Hasenstein, 2001; Leitz et al., 2009; Strohm et al., 2012a, 2012b; Hashiguchi et al., 2013).

The formation of independent tissue, like root caps, at the tips of yam tubers has not been described to our knowledge. However, in Chinese yam, many amyloplasts formed locally and sedimented in downward direction in individual cells beneath the stele of the tuber tip (Kawasaki et al., 2008). When the tuber was grown on the surface of an inclined plate, the amyloplasts in the tuber tips relocated and sedimented at the new bottom of each cell. The tubers elongated vertically as soon as they passed through the barrier of the plate. These results suggested that the sedimentable amyloplasts in the tuber tips function as statoliths to sense gravity and participate in tuber formation (Kawasaki et al., 2008). However, which part of the tuber responded to the change in orientation by gravistimulation remains unknown and the relationship between amyloplast sedimentation and tuber formation requires further validation, because the tuber placed on
the inclined plate was investigated in our previous study (Kawasaki et al., 2008).

Tuber shape and its homogeneity are important factors determining the agricultural quality and productivity of yams. Therefore, elucidation of the relationships between amyloplast sedimentation, tuber gravity sensing and tuber formation is important to comprehensively understand how tuber shape is established underground. In this study, to clarify these relationships, we investigated the effects of gravistimulation by altering the direction of the gravity vector on tuber formation and amyloplast distribution in tuber tips of Chinese yam.

Materials and Methods

1. Plant material and gravistimulation

Chinese yam (Dioscorea opposita Thunb.) line Enshikei 6 was used in this experiment. The tuber of this line has an elongate shape that forms during the growth period. Aerial tubers were planted in plastic pots filled with culture soil containing 130 mg L⁻¹ of N, 700 mg L⁻¹ of P₂O₅, and 160 mg L⁻¹ of K₂O in May, 2013, and the plants were sprouted under natural temperature, light and humidity conditions in the greenhouse at Hirosaki University (40°59′ N, 140°47′ E, 53 m above sea level), Hirosaki, Japan.

After about 1 mo, when the plants were about 5 cm tall, they were transferred to plastic pipes (50.5 cm in length, 10.5 cm in inner diameter) filled with the culture soil containing 330 mg L⁻¹ of N, 280 mg L⁻¹ of P₂O₅, and 340 mg L⁻¹ of K₂O in the greenhouse. When they were about 1.5 m tall, after about 2 mo, they were treated as described below.

The pipes containing growing tubers were placed vertically (control), inversely (180° reorientation) or horizontally (90° reorientation) with respect to the gravity vector for 2 or 14 d in the greenhouse (Fig. 1a, b, c). The other pipes were rotated at 90° intervals daily (d, rotation). G, direction of gravity vector.

**Fig. 1.** Schematic diagrams of the gravistimulation treatments. Pipes containing growing tubers were placed vertically (a, control), inversely (b, 180° reorientation) or horizontally (c, 90° reorientation) with respect to the gravity vector. Other pipes were rotated at 90° intervals daily (d, rotation). G, direction of gravity vector.
2. Observation of tuber formation and amyloplasts in tip parts of tubers

The external appearances of sampled tubers were photographed with a digital camera (Nikon, D40, Tokyo, Japan). Six tubers per treatment of 2-d and 14-d gravistimulation were used for this observation. For bright-field optical microscopy, segments of tuber tips were fixed in 0.05 M sodium phosphate buffer (pH 7.2) containing 2% glutaraldehyde and 1% paraformaldehyde at 20ºC under reduced pressure for 1 hr then under normal pressure for 3 d. After fixed segments were dehydrated in an ethanol series, they were immersed in t-butyl alcohol and then embedded in paraffin. Next, longitudinal sections (4 μm in thickness) were made using a microtome (R2-40, Yamato Kohki, Saitama, Japan), and they were stained with hematoxylin and eosin stain after deparaffinization using a xylene-ethanol series. The sections were observed with using a bright-field optical microscope (BX51, Olympus Tokyo, Japan).

Fig. 2 shows the schematic diagrams of compartments in each cell in longitudinal sections of the tuber tips for investigating intercellular distribution of amyloplasts. The intercellular of each cell in longitudinal sections of tuber tips was divided into half area of the distal side (A) and half area of the proximal side (B), or both half areas of lateral sides of the tuber tips (C, D). The ratio of the number of amyloplasts in the half area of the distal side, proximal side and each of lateral sides per total number of amyloplasts in each cell in longitudinal sections of tuber tips were investigated.

3. Analysis of amyloplast distribution

The distribution pattern of amyloplasts was investigated using optical microscopic images of the longitudinal sections, as follows. After gravistimulation, segments of tuber tips were fixed by immersion in formalin-acetic acid-alcohol solution at 20ºC under reduced pressure for 1 hr then under normal pressure for 3 d. After fixed segments were dehydrated in an ethanol series, they were immersed in t-butyl alcohol and then embedded in paraffin. Next, longitudinal sections (4 μm in thickness) were made using a microtome (R2-40, Yamato Kohki, Saitama, Japan), and they were stained with hematoxylin and eosin stain after deparaffinization using a xylene-ethanol series. The sections were observed with using a bright-field optical microscope (BX51, Olympus Tokyo, Japan).

Fig. 2 shows the schematic diagrams of compartments in each cell in longitudinal sections of the tuber tips for investigating intercellular distribution of amyloplasts. The intercellular of each cell in longitudinal sections of tuber tips was divided into half area of the distal side (Fig. 2A) and half area of the proximal side (Fig. 2B), or both half areas of lateral sides of the tuber tips (Fig. 2C, D). Ratios of the number of amyloplasts in the half area of the distal side (Fig. 2A), proximal side (Fig. 2B) and each of lateral sides (Fig. 2C, D) per total number of amyloplasts in each cell in longitudinal sections of tuber tips were investigated.

Additionally, amyloplast sedimentation ratio that was the percentage of number of amyloplasts located at a part very near the bottom of each cell in the tuber tips per total number of amyloplasts in each cell in the tuber tips was calculated. One tuber per plant, three tubers per treatment of gravistimulation, two longitudinal sections per tuber tip, and nine cells per section were investigated. Analysis of variance (ANOVA) followed by Tukey’s test was performed for the amyloplast sedimentation ratio per cell.

Results

1. Effects of the gravistimulation on tuber formation

The control (vertical) tubers elongated downward in both the 2-d and 14-d treatments (Fig. 3a, b). In the tubers treated for 2 d, the subapical parts of tubers that were reoriented by 90º curved slightly in the direction of gravity vector (Fig. 3e). However, no difference in shape was observed between the control and 180º treatments (Fig. 3a, c). After 14 d, the tuber shapes differed substantially among treatments. The tubers that experienced 180º and 90º reorientation bent downward, with the distal parts of the tubers elongating toward a gravity direction (Fig. 3d, f). In the rotation treatment, 2 to 5 tips (the average was 3) per tuber formed at different angles in five out of six tubers (Fig. 3g).

2. Effects of the gravistimulation on amyloplast distribution

In control tubers after both 2-d and 14-d treatments, many amyloplasts were located at the lower part in each
cell beneath the stele of the tuber tips (Fig. 4a, b, Fig. 5a, b). Amyloplast sedimentation was also observed in the tips of the tubers in the 2-d reorientation treatments (Fig. 4c, e, Fig. 5c, e), but the amyloplasts had relocated toward the proximal side of the tubers in the 180° treatment (Fig. 4c, Fig. 5c) and toward the lower lateral side of the tubers in the 90° treatments (Fig. 4e, Fig. 5e). In the tuber reoriented by 180° and 90° for 14 d, many amyloplasts had sedimented and relocated toward the distal sides of tubers as in the control (Fig. 4d, f, Fig. 5d, f). Amyloplasts dispersed in each cell in the tuber tips in the rotation treatment (Fig. 4g, Fig. 5g).

In the 2-d treatments, there were no significant differences in the ratio of the number of amyloplasts located very near the bottom of each cell to the total number of amyloplasts of each cell in the tuber tips among all treatments; all ratios exceeded 53% (Fig. 6). Similarly, in 14-d treatment, there were no significant differences in the sedimentation rates among the control, 180° reorientation and 90° reorientation treatments, and these rates exceeded 60% (Fig. 6). However, the ratio in the rotation treatment was significantly lower than in the other treatments.

**Discussion**

Kawasaki et al. (2008) reported that the tubers of Chinese yam placed on the surface of an inclined plate elongated vertically as soon as they had elongated and exceeded the barrier. In this study, without such a plate, the subapical parts of tubers that were placed horizontally for 2 d curved slightly in the direction of the gravity vector (Fig. 3e), and the distal parts of tubers that were placed horizontally for 14 d also elongated downward (Fig. 3f). In inverted tubers, the distal parts bent and eventually elongated in the direction of the gravity vector (Fig. 3d). These results indicated that the subapical part of the tuber responded to changes in orientation by gravistimulation and that the basal or intermediate parts of tubers did not. Similarly, in the roots of plants, the initial site of curvature in response to gravistimulation is the distal elongation zone near root caps (Ishikawa and Evans, 1993; Miller et al., 2007; Strohm et al., 2012a), not the mature zones.
Hasenstein, 2001). The application of such fields to Arabidopsis root tips promoted the movement of amyloplasts toward the sides furthest from the fields, prompting the roots to curve in the direction of amyloplast movement (Kuznetsov and Hasenstein, 1996). In Arabidopsis phosphoglucomutase mutants defective in the ability to synthesize starch, the amyloplasts were starchless, and the gravitropic responses of roots and stems were slower than in the wild type (Kiss et al., 1989, 1996; Weise and Kiss, 1999). The roots of the mutant did not curve in response to high-gradient magnetic fields (Kuznetsov and Hasenstein, 1996; Kondrachuk and Hasenstein, 2001). In this study, tubers in the rotational treatment did not assume an elongated shape and grew several tips at different angles (Fig. 3g). In the rotated tubers, amyloplasts were dispersed in each cell of the tip area (Fig. 4g, Fig. 5g, Fig. 6). These results showed that the proper sedimentation of the amyloplasts would be essential for the normal elongation.

In the columella of roots and the endodermis of stems, many amyloplasts are positioned at the downward sides of the cells. After the roots or stems were turned horizontally, the amyloplasts sedimented in the direction of gravity vector and relocated to the new downward side (Sack et al., 1985; Yoder et al., 2001; Saito et al., 2005; Leitz et al., 2009). Our microscopy and amyloplast sedimentation ratio data showed that many amyloplasts sedimented in the downward direction in each cell within tuber tips in the control, 180° and 90° treatments (Fig. 4a–f, Fig. 5a–f, Fig. 6). The results indicated that the directions of amyloplast sedimentation corresponded with the eventual direction of tuber elongation in Chinese yam under varying gravistimulation vectors.

Studies using high-gradient magnetic fields have indicated that amyloplast movement triggered the gravitropic response in roots and shoots (Kuznetsov and Hasenstein, 1996; Weise et al., 2000; Kondrachuk and Hasenstein, 2001). The application of such fields to Arabidopsis root tips promoted the movement of amyloplasts toward the sides furthest from the fields, prompting the roots to curve in the direction of amyloplast movement (Kuznetsov and Hasenstein, 1996). In Arabidopsis phosphoglucomutase mutants defective in the ability to synthesize starch, the amyloplasts were starchless, and the gravitropic responses of roots and stems were slower than in the wild type (Kiss et al., 1989, 1996; Weise and Kiss, 1999). The roots of the mutant did not curve in response to high-gradient magnetic fields (Kuznetsov and Hasenstein, 1996; Kondrachuk and Hasenstein, 2001). In this study, tubers in the rotational treatment did not assume an elongated shape and grew several tips at different angles (Fig. 3g). In the rotated tubers, amyloplasts were dispersed in each cell of the tip area (Fig. 4g, Fig. 5g, Fig. 6). These results showed that the proper sedimentation of the amyloplasts would be essential for the normal elongation.

Fig. 4. Longitudinal sections of the tuber tips after the gravistimulation treatments. Sections were stained with toluidine blue O. Sections of tubers reoriented by 0° for 2 d (a), 0° for 14 d (b), 180° for 2 d (c), 180° for 14 d (d), 90° for 2 d (e), and 90° for 14 d (f) and sections of tubers rotated at 90° intervals every day for 14 d (g) were investigated. Arrowheads indicate amyloplasts. G, direction of gravity vector. Bars = 20 μm.
and formation of tubers. Additionally, temporary translocation of amyloplasts by daily rotation may cause the formation of multiple tuber tips.

Our results supported the possibility that the sedimentable amyloplasts in the tuber tips function as statoliths and are involved in gravity sensing and tuber formation in Chinese yam. Recently, physiological and molecular biological studies have revealed important new details related to sensing, signal transduction, and growth response during gravitropism in plant roots and shoots (Strohm et al., 2012a, 2012b; Hashiguchi et al., 2013). Further investigations will be needed to comprehensively understand the mechanisms related to the gravitropism and formation of yam tubers.

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Fig. 6. Amyloplast sedimentation ratio in cell in tuber tips after the gravistimulation treatments. Amyloplast sedimentation ratio that was the percentage of the number of amyloplasts located at a part very near the bottom of each cell in the tuber tips to the number of total amyloplasts of each cell in the tuber tips was calculated. Different letters indicate a significant difference at the 0.1% level by Tukey’s test. Bars show means ± SE (n = 3). Three tubers per treatment of gravistimulation, two longitudinal sections per tuber tip, and nine cells per section were investigated in this analysis.