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Dormancy-related Change in Endogenous ABA, Batatasin, and Sugar in Stored Tuber and Bulbil of Chinese Yam

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ABSTRACT: Endogenous ABA and batatasins were first quantified in the stored tubers and bulbils of the Chinese yam, *Dioscorea opposita* Thunb. cv. 'Tsukune' by GC-MS with comparison of internal standard, and sugar content was also analyzed by HPLC with comparison of external standard. Endogenous ABA content in stored tubers and bulbils was rapidly decreased as storage period prolonged. ABA content of the bulbils was always higher than that of tuber during storage periods. All batatasins of stored tubers and bulbils at 30 days after storage decreased steadily compared to 120 days after storage. On the contrary, batatasin-III of the stored tuber and bulbils was only decreased at 60 days after storage. In *Dioscorea opposita* Thunb. cv. 'Tsukune' like *D. japonica*, *D. alata*, and *D. bulbifera*, may be controlled by endogenous batatasins and ABA, In these compounds, batatasins rather than ABA might be closely related to dormancy-inducing compound during dormancy of the Chinese yam, *Dioscorea opposita* Thunb. cv. Tsukune.

Keywords : Chinese yam, *Dioscorea opposita*, tuber, bulbil, dormancy, ABA, batatasins, sugars

Naturally occurring growth inhibitors, termed batatasins, discovered in *Dioscorea oposita*, have been found to increase during bulbil development and to be associated with capacity for dormancy (Hasegawa and Hashimoto, 1973). Moreover, exogenously applied batatasins have been shown to extend bulbil dormancy in an effect which was reversible by low temperature stratification (Hashimoto *et al.*, 1972).

In addition, exogenously applied gibberellic acid (GA_3) was found to extend bulbil dormancy and CCC (2-chloroethyltrimethyl-ammonium chloride) to promote sprouting in bulbils of temperate *Dioscorea* species, leading to the suggestion that endogenous GA_3 also played some physiological roles in bulbil dormancy and dormancy of yam is not broken by gibberellin (in particular, GA_3) and is reversely induced when stratified bulbils and tubers are treated with gibberellins. This action of gibberellin is inconsistent with the dormancy-breaking activity of this growth promoter well-known in seeds or buds of many plant species (Okagami and Nagao, 1971).

Apart from these compounds, ABA has been isolated from the bulbils of yam (*Dioscorea opposita*), but this ABA could not reinduce dormancy efficiently in non-dormant bulbils which had been fully stratified (Hasegawa and Hashimoto, 1974; Hasegawa and Hashimoto, 1975). Otherwise, On the other hand, the application of gibberellins (GA_1, GA_3, and GA_4) strengthens or deepens the dormancy of the tubers and bulbils in Chinese yams. Dormancy mechanism of *Dioscorea opposita* is still questioned in relation to post-harvest storage, although the mechanism of dormancy in tropical yam species is well documented (Wickham *et al.*, 1984). Until now, plant growth retardants like ABA and batatasins, and sugars have not been quantified in the Chinese yam.

Thus, This paper basically focuses on the quantitative changes of endogenous batatasins and ABA during dormancy periods in the Chinese yam.

MATERIALS AND METHODS

Plant material and growth conditions

Tubers of *Dioscorea opposita* Thunb. cv. Tsukune were cut into four pieces (about 70 g, fresh weight). Tuber pieces were pre-sprouted on a mixture of vermiculite and sand (1:1, v/v) in a plastic pots (0.5 × 0.3 × 0.15 m [high]; total volume 2.3 m^3), and then kept in a chamber at 30°C under dark conditions. At 30 days after incubation, the sprouted tuber pieces were planted to 30×30 cm with four replications under randomized block design and grown in the experimental field at the Institute of Bioresources at Kyongbuk Provincial Agricultural Technology Administration on June 15, 2001.

After harvesting on November 22, ripened tubers and matured bulbils were stored in a temperature-controlled room in the dark at 4 ± 0.5°C at an approximate relative humidity of 85 ± 3%. The contents of batatasins and endogenous abscisic

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acid (ABA) in the tuber and bulbil were determined periodically at every 30 day intervals. Tubers and bulbils were frozen in liquid nitrogen immediately, stored at -70°C until required for extraction and analysis of ABA, batatasins, and sugars.

Extraction and analysis of ABA

For analysis of ABA by GC-MS-SIM followed by some combined modifications (Browning and Wignall, 1987; Qui *et al.*, 1998; Kamboj *et al.*, 1999). the lyophilized tubers (1 g, DW) and the bulbils (1 g, DW) were extracted and filtered in 30 Ml of solution (isopropanol : acetic acid, 95:5, v/v), adding 100 ng of deuterated ABA [(±)3,5,5,7,7-d_6-ABA]. The filtrate was evaporated to dryness. The residue was dissolved in 4 ml of 1 N NaOH and washed three times with 3 ml of methylene chloride to remove lipophilic materials. The aqueous phase was adjusted to pH 3.5 with 6 N HCl and partitioned three times into EtOAc. The EtOAc extract was combined and then evaporated.

The dried residue was dissolved in phosphate buffer (pH 8.0) and then run through PVPP column. The phosphate buffer was adjusted to pH 3.5 with 6 N HCl and partitioned three times into EtOAc. The EtOAc extract was combined and evaporated. The residue was dissolved in methyle chloride and passed through a 'Sep-Pak' silica cartridge (Waters Associates Co.) which were prewashed with 10 ml of diethyl ether : methanol (3:2, v/v) and 10 ml of methylene chloride. ABA was recovered from cartridge by elution with 10 ml of diethyl ether : methanol (3:2, v/v).

The extract was reduced to dryness. ABA samples were methylated with diazomethane for GC-MS analysis. Finnigan GCQ equipped with a fused silica DB-1 column (30 m × 0.25 mm ID, film thickness 0.25 μm, J & Scientific) was used for the analysis of endogenous ABA in the samples. The column temperature was initially 60°C with an isothermal increase of 15°C min^{-1} to 200°C, 5°C min^{-1} to 250°C, and 10°C min^{-1} up to 280°C. Helium gas was used at a flow rate of 38 cm sec^{-1}. Quantification of the ABA was based on the peak area ratio of endogenous and deuterated ABA, *m/z* 190 and 194, respectively.

Extraction and analysis of batatasins

For the analysis of batatasins by GC-MS-SIM (some modification of Hasegawa and Hashimoto, 1973; Wallstedt *et al.*, 1997), the lyophilized tubers (1 g, DW) and the bulbils (1 g, DW) were homogenized in 50 ml of cold acetone and filtered. The acetone extract was evaporated to dryness and chromatographed on TLC system [Silica gel: chloroform-methanol (97:7, v/v)] and the band corresponding to each of batatasin I, III, IV, and V were scrapped and eluted with ethylacetate. The eluate of each band was evaporated to dryness, derivatized at 70°C for 30 min with 30 μl of BSTFA in 25 μl of pyridine containing 10 ng μl^{-1} of 1,3-dihydroxynaphthalene as an instrumental internal standard.

Finnigan GCQ Mat equipped with a fused silica DB-5 column (30 m×0.25 mm ID, film thickness 0.25 μm, J & Scientific) was used for the quantification of each batatasin in the samples. The column temperature was initially 150°C 7°C with a linear increase of 7 min^{-1} up to 285°C. Helium gas was used as carrier gas at a flow rate of 38 cm sec^{-1}. The each sample was injected to 1 μl. Identification of each batatasin I, III, and V was determined with retention time and ion spectrum (*m/z* 356, 388, 360 at B-I, B-III, and B-V) with their standards (a gift from Professor, Tohru Hashimoto, Dept. of Life Sciences, Kobe Women's University, Kobe, Japan).

For quantification of these compounds, a standard calibration curve was drawn by plotting the area ratio between batatasins I, III, and V and 1, 3-dihydroxynaphthalene for eight different concentrations of (10-600 ng μl^{-1}). The correlations for batatasins I, III, and V were determined to be 0.9947, 0.9985, and 0.9956, respectively.

Extraction and analysis of sugars

For the extraction of sugars (sucrose, fructose, and glucose) by some modification (Sinniah *et al.*, 1998), the lyophilized tubers (1 g, DW) and the bulbils (1 g, DW) were homogenized in 50 ml of 80% ethanol and sonicated at 30°C for 1 h. Homogenate was filtered and supernatant was passed through a C_{18} Sep-Pak (Waters Associates, USA), the first milliliter was discarded and the next 2.5 ml was collected. A standard calibration curve was prepared by injecting 2, 6, 10, 14 and 18 μl of the standard solution. For the analysis of sugars by HPLC, A 30 μl of each aliquot was subjected to HPLC (Waters 510) analysis using a differential refractometer.

The separation of sugars was achieved by Carbohydrates column (4.6 mm × 250 mm) heated to 80 μl. The mobile phase was a solution (acetonitrile:water, 75:25, v/v). Flow rate of mobile phase was 1.2 ml min^{-1}.

Identification of sugars was by comparison of retention times with known standards (Sigma Chemical Co.). Quantification was achieved by integration of elution peak areas and comparison with known amounts of external standards.

RESULTS AND DISCUSSION

Quantitative changes of endogenous ABA in stored tubers and bulbils

Dormancy of reproductive organs, tubers and bulbils, in

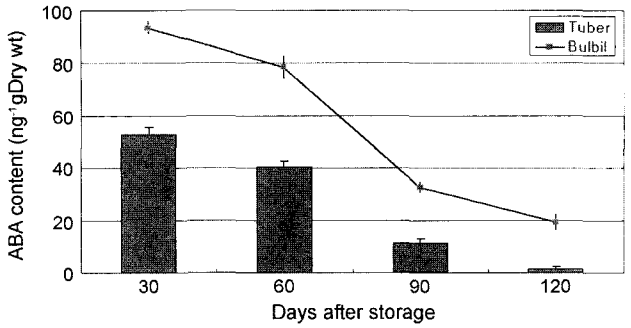

Fig. 1. Quantitative changes of endogenous ABA in tuber and bulbil of Chinese yam, *Dioscorea opposita* Thunb. cv. Tsukune during storage. Ripened tubers and matured bulbils were harvested on November 22 and then stored in a temperature-controlled room in the dark at 4 ± 0.5°C at an approximate relative humidity of 85 ± 3%. Vertical bars represented are mean values of three replicates ± SE.

Table 1. Quantitative changes of batatasins in the stored tubers and bulbils of *Dioscorea opposita.*

Organs	Days after storage†	Batatasin content (mg · g^{-1}, Dry wt.)		
		B-I‡	B-III	B-IV
Tubers	30	12.9 ± 1.3	8.6 ± 1.7	7.5 ± 0.5
	60	8.6 ± 1.1	10.2 ± 0.8	6.1 ± 0.4
	90	9.5 ± 1.0	4.2 ± 1.4	3.9 ± 0.7
	120	3.2 ± 1.3	2.5 ± 0.8	1.1 ± 0.4
Bulbils	30	18.4 ± 1.0	15.9 ± 1.8	15.3 ± 0.9
	60	14.7 ± 1.3	20.7 ± 0.3	10.7 ± 0.6
	90	12.1 ± 1.3	14.9 ± 2.0	8.2 ± 1.5
	120	6.3 ± 1.6	7.8 ± 0.2	4.3 ± 1.4

†Tubers and bulbils were harvested on November 22 and stored in a temperature-controlled room in the dark at 4 ± 0.5°C at an approximate relative humidity of 85 ± 3%.
‡B-I, batatasin-I; B-I, batatasin-III; B-V, batatasin-V. Data presented are mean values of three replicates ± SE.

Dioscorea opposita was easily induced by exogenously applied gibberellins, GA_1, GA_3, and GA_4 (Okagami and Nagao 1971). And also, application of gibberellin increases the batatasin contents although the gibberellin action varied depending on the batatasin composition and pretreatment of the tuber and bulbil in Chinese yams, in addition, the level of ABA in the bulbils was not affected by GA_3 (Hasegawa and Hashimoto, 1974; Hasegawa and Hashimoto, 1975). Recently, we have evaluated the quantitative changes of endogenous ABA and several batatasin contents in *Dioscorea opposita* Thunb. cv. Tsukune during postharvest storage periods.

Endogenous ABA content in tubers and bulbils was rapidly decreased as storage period prolonged (Fig. 1). In particular, a dramatic decrease of endogenous ABA at the 90 days after storage showed that the amounts of ABA in tuber and bulbil were 11.4 and 32.8 ng per g (DW), respectively. In addition, ABA content of bulbils was always higher than that of tuber during storage periods. Furthermore, in sprouting test, sprouting rates of two reproductive organs at the 120 days after storage transferred to 23°C not showed a significant difference (data not shown). In other species of Chinese yam, *Dioscorea opposita* cv. 'Naga' showed that ABA content was slightly decreased and thereafter remained constant up to the end of the 5-month storage period (Hasegawa and Hashimoto, 1975).

Therefore, among *Dioscorea opposita* species including *D. japonica*, *D. opposita*, and *D. bulbifera*, the ABA alteration in tubers and bulbils during dormancy periods may depends on the genetic character of these species.

Quantitative changes of three batatasins in stored tubers and bulbils

Batatasins (batatasin-I, batatasin-II, and batatasin-III) were first identified and isolated in yam bulbils (Hashimoto et al., 1972). These compounds, which are produced *de novo* or show increased production on elicitation, together with batatasins I-V have been implicated as dormancy inducing compounds in *D. opposita* (Ei-Olemy and Reisch 1979; Hashimoto *et al.*, 1972). In the Chinese yam, in particular, batatasin-III is closely related to dormancy-inducing compound.

Table 1 shows quantitative changes of three batatasins in the stored tubers and bulbils during storage periods. All batatasins at the 30 days after storage decreased steadily compared to 120 days after storage, as storage period prolonged, and the content of batatasin-I and batatasin-IV at the 90 days after storage increased to some extent, thereafter decreased three-fold at 120 days after storage. On the contrary, batatasin-III was only decreased at 60 days after storage. In quantitative changes of batatasins in the stored bulbils of Chinese yam, the content of batatsin-I and batatasin-V was gradually decreased, otherwise, the content of batatasin-III was only increased at 60 days after storage.

Quantitative changes of sugars in stored tubers and bulbils

We also evaluated the quantitative changes of sugars in tubers and bulbils during post-harvest storage (Table 2). Sucrose content in stored tubers and builbils increased as storage period was prolonged, glucose and fructose contents in stored tubers and bulbils increased steadily during storage period. Sucrose content in the stored tubers was always higher than that of the stored bulbils at all analyzed intervals. In this state, the dormant period of bulbils stored for 120 days after harvest in sprouting tests was 30 days much longer than that of the bulbils (data not shown). Finally, in

Table 2. Quantitative changes of sugars in the stored tubers and bulbils of *Dioscorea opposita*.

Organs	Days after storage†	Sugar content (μg · g^{-1}, Dry wt.)		
		Suc‡	Glu	Fru
Tubers	30	8.5 ± 1.1	11.2 ± 2.6	10.3 ± 1.6
	60	13.8 ± 2.3	16.0 ± 1.3	17.3 ± 2.3
	90	22.1 ± 0.5	18.7 ± 2.1	24.0 ± 0.3
	120	32.6 ± 1.8	22.0 ± 0.5	24.1 ± 1.2
Bulbils	30	5.7 ± 1.1	6.8 ± 1.5	4.9 ± 0.8
	60	9.8 ± 0.9	9.5 ± 0.5	9.5 ± 1.6
	90	13.6 ± 0.7	11.2 ± 1.0	15.1 ± 0.3
	120	24.1 ± 1.3	16.5 ± 0.9	14.9 ± 0.8

†Tubers and bulbils were harvested on November 22 and stored in a temperature-controlled room in the dark at 4± 0.5°C at an approximate relative humidity of 85 ± 3%.
‡Suc, sucrose; Glu, glucose; Fru, fructose. Data presented are mean values of three replicates ± SE.

comparison of the batatasin and ABA content responsible for dormancy of the Chinese yam, batatasins may be closely related to dormancy-inducing compound rather than ABA, although its biological activity of single compound by exogenous application was not determined.

Thus, the dormancy of Chinese yam, *Dioscorea opposita* Thunb. cv. 'Tsukune' like *D. japonica*, *D. alata*, and *D. bulbifera*, might be controlled by endogenous batatasins and ABA. Further studies are in progress to determine the critical concentrations of these biological activity in batatasin and ABA actions during tuber and bulbil dormancy in the Chinese yam.

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