Note

Isolation and identification of oryzamutaic acids A and D from an *Oryza sativa* spontaneous mutant with yellow endosperm

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Oryzamutaic acids A and D, alkaloids with a novel type of nitrogen-containing heterocyclic ring system, which had previously been isolated only from the brown rice (husked rice) of Hatsuyamabuki, an *Oryza sativa* artificial mutant cultivar with yellow endosperm, were isolated from the brown rice of Musashino 20, an *Oryza sativa* spontaneous mutant line with yellow endosperm. The concentrations of oryzamutaic acids A and D in the brown rice of Musashino 20 were similar to those of Hatsuyamabuki, respectively. These results suggest that oryzamutaic acid A may play an important role in the yellow color of the brown rice of Musashino 20.

Key Words: *Oryza sativa*, gramineae, alkaloid, oryzamutaic acids A and D.

Introduction

Rice (*Oryza sativa*) is the most important crop in Asia. Artificial mutation by means of radiation and chemicals has been used to improve the yield and quality of rice (Amano 2001). The rare rice cultivar Hatsuyamabuki, which has yellow endosperm, was selected from the progeny obtained by treatment of the rice cultivar Kinuhikari with γ -rays at the National Agricultural Research Center for Kyushu Okinawa Region (Kaji *et al.* 2010). In previous study, we isolated oryzamutaic acids A–J, alkaloids with a novel nitrogen-containing heterocyclic ring system, from the yellow endosperm (polished rice) of Hatsuyamabuki (Nakano *et al.* 2009, 2010a, 2010b). Oryzamutaic acids A–C are yellow, but oryzamutaic acids D–J are colorless. Among oryzamutaic acids present in the endosperm of Hatsuyamabuki, oryzamutaic acids A and D are the most prevalent.

Recently, Musashino 20, a spontaneous mutant rice line that also has yellow endosperm, was selected from the progeny of the rice cultivar Sainokagayaki at the Saitama Prefectural Agriculture and Forestry Research Center (Ooka *et al.* 2010). However, the yellow pigments from Musashino 20 have not yet been isolated. In this study, we focused on the yellow pigments in the brown rice of Musashino 20.

Materials and Methods

General experimental procedures

HRESIMS spectra were recorded on a Bruker MicroTOF spectrometer.

Plant materials

The rice (*Oryza sativa*) grains of a line Musashino 20 and a cultivar Sainokagayaki were obtained from the Saitama Agricultural Research Center in 2007. The rice grains of cultivars Hatsuyamabuki and Kinuhikari were obtained from the National Agricultural Research Center for Kyushu Okinawa Region in 2007. All of the grains obtained from these research centers were husked.

Extraction and isolation

The brown rice (100 g) of Musashino 20 was extracted with 300 mL of aq. MeOH (MeOH/H₂O, 1 : 9) for 5 days at 25°C. The extract (4 g) was subjected to C₁₈ column chromatography (MeOH/H₂O, 0 : 1 → 1 : 0) followed by C₁₈ HPLC to yield oryzamutaic acids A (0.1 mg, 0.0001% yield) and D (0.1 mg, 0.0001% yield) as yellow and colorless powder, respectively.

Determination of the concentrations of oryzamutaic acids A and D

The brown grain (5 g) was extracted with 25 mL of aq. MeOH (MeOH/H₂O, 1 : 4) for 1 day at 25°C, and then was homogenized and extracted for 1 day at 25°C. Fifty mL of

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aq. MeOH (MeOH/H₂O, 1 : 4) was added to the solution, which was then centrifuged at 3,500 g for 10 min at 25°C. The supernatant was filtered and concentrated. Seventy five mL of aq. MeOH (MeOH/H₂O, 4 : 1) was added to the concentrate, which was centrifuged at 3,500 g for 10 min at 25°C. The supernatant was filtered and concentrated. One mL of H₂O was added to the concentrate, which was then subjected to C₁₈ HPLC (ODS-80Ts, Tosoh, 4.6 × 250 mm; eluent MeOH/ H₂O, 0 : 1 → 3 : 7; flow rate 0.8 mL/min; UV detection at 395 and 360 nm). The amounts of oryzamutaic acids A and D were calculated from standard curves by measurement of the areas of the peaks for them. This experiment was replicated three times.

Results and Discussion

We compared HPLC chromatograms of the brown rice extracts of Musashino 20 and Sainokagayaki (Fig. 1). In the extract of Musashino 20, two major peaks were detected, one at 395 nm and the other at 360 nm. However, in Sainokagayaki, no peaks were detected at either wavelength. The compounds corresponding to the two peaks Musashino 20 were isolated. The compound corresponding to the peak at 395 nm, which had the same HPLC retention time as oryzamutaic acid A (Fig. 2), had the molecular formula C₂₃H₃₂N₄O₆, established by HRESIMS [*m/z* 459.2253 (M-H)⁻, Δ +1.5 mmu], which is the same as that of oryzamutaic acid A (Nakano *et al.* 2009). The compound corresponding to the peak at 360 nm, which had the same HPLC retention time as oryzamutaic acid D (Fig. 2), had the molecular formula, C₁₇H₂₅N₃O₄, established by HRESIMS [*m/z* 334.1805 (M-H)⁻, Δ +4.4 mmu], which is the same as those of oryzamutaic acids D–G (Nakano *et al.* 2010a). Oryzamutaic acids E–G had shorter retention times than oryzamutaic acid D. Among oryzamutaic acids present in the endosperm of Hatsuyamabuki, oryzamutaic acids A and D are the most prevalent (Nakano *et al.* 2009, 2010a, 2010b). These results indicate that the brown rice of Musashino 20, like that of Hatsuyamabuki, contained oryzamutaic acids A and D.

We determined the concentrations of oryzamutaic acids A and D (Fig. 2) in the brown rice of Musashino 20 and Hatsuyamabuki (Table 1). By subjecting the brown rice extracts Musashino 20 and Hatsuyamabuki to HPLC and measuring the peak area of the chromatograms, we estimated the concentrations of oryzamutaic acid A in Musashino 20 and Hatsuyamabuki to be 2.54 and 2.97 mg/kg, respectively, and the concentrations of oryzamutaic acid D to be 3.37 and 3.26 mg/kg, respectively; that is, the concentrations of oryzamutaic acids A and D in the brown rice of Musashino 20 were similar to those of Hatsuyamabuki, respectively. These results suggest that oryzamutaic acid A may play an important role in the yellow color of the brown rice of Musashino 20 as well as Hatsuyamabuki.

Kaji *et al.* (2010) reported that the spectrophotometer “b*” value representing the level of yellow is only slightly higher in the brown rice of Hatsuyamabuki (19.17) than in

Fig. 1. HPLC chromatograms of brown rice extracts of Musashino 20 and Sainokagayaki. Arrows indicate major peaks.

that of Kinuhikari (17.69), but the value is much higher in the endosperm of Hatsuyamabuki (19.48) than in that of Kinuhikari (9.61). Thus, although distinguishing the difference in endosperm color between Musashino 20 and Sainokagayaki and between Hatsuyamabuki and Kinuhikari could be expected to be easy, distinguishing the color difference in the brown rice of these pairs of cultivars might be difficult. In the initial selection stage of rice breeding, although many progeny of various cultivars and lines are selected, the endosperm color of their progeny is hardly examined. Therefore, the mutation with yellow endosperm except for Musashino 20 and Hatsuyamabuki might have occurred spontaneously or artificially over the course of a long rice

Fig. 2. Structure of oryzamutaic acid A (top) and D (bottom).

Table 1. Concentrations of oryzamutaic acids A and D in the brown rice of Musashino 20 and Hatsuyamabuki

Line or cultivar	oryzamutaic acid A	oryzamutaic acid D
	mg/kg	
Musashino20	2.54 ± 0.03	3.37 ± 0.22
Hatsuyamabuki	2.97 ± 0.10	3.26 ± 0.04

Means ± SE from three replicates.

breeding history but have gone undetected. Linkage analysis of loci and identification of genes controlling the biogenetic pathway of oryzamutaic acids in these mutants should be conducted.

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