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Establishment of Efficient System for Isolation and Manipulation of Single Protoplasts Containing Aposporous Embryo Sac Initial Cell in Guinea Grass (*Panicum maximum*)

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Authors' contributions

This work was carried out in collaboration by the authors. Authors LC and CX designed the study and author LC wrote the first draft of the manuscript. Authors YN and LC performed the enzyme treatments and data management. Author CX managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: In order to do the molecular analysis of mechanism of aposporous embryo sac initial cell (AIC) appearance, as the first step, we attempted to establish the system of isolating and manipulating single cells containing AIC using different methods in guinea grass (*Panicum maximum*).

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Study Design: At first, single protoplasts were isolated from the ovaries staged in different developmental stages using different pre-treatments and different concentrations of enzyme solutions on ovaries; Based on it, the single protoplasts containing AIC were manipulated with ultra particle electronic syringe picopipet (UPESP) machine set onto microscope.
Place and Duration of Study: Faculty of Environmental and Horticultural Science, Minami Kyushu University, between June 2012 and December 2013.
Methodology: The ovary of facultatively apomictic guinea grass (*Panicum maximum*) was used as materials in this study for single protoplast isolation. The ovaries were classified and collected from young buds and flowers staged in different developmental stages, according to the colors of stigma and the ovary length. And then, the ovaries were pre-treated with needle into different shapes, and treated with different kinds of enzyme solution with different concentrations of mannitol to increase efficiency of protoplast isolation. In final, the single protoplasts containing AIC isolated from different stages of ovaries were manipulated by handle control.
Results: 1) The ovaries in different stages of before AIC appearance, AIC appearance, and AIC-derived embryo sac formation were collected successfully and respectively, indicating that the stigma colors and length of ovaries are proportionate to stages of ovary mature. And single protoplasts containing AIC were isolated from the ovaries staged in white to yellow color. 2) The ovaries be flooded in enzyme solution, were pre-treated with needle in 4 types, that is, (1) Cut in micropylar end; (2) Cut in chalazal end; (3) Cut in middle part; (4) after 1hr of (3) treatment, cut in micropylar end. As a result, the efficiency of protoplast isolation of (3) and (4) was 1-2 hrs shorter than that of (1) and (2). 12%, 11%, 10% and 9% were proper enzyme concentrations for obtaining perfect shingle protoplasts from the ovaries with white, yellow, peach and purple colors, respectively. 3) The single protoplasts containing AIC were collected and manipulated with UPESP in the performance of controlled aspiration and spit.
Conclusion: In this study, in order to do molecular analysis of the mechanism of AIC appearance, we focus on that, the key points were to isolate AIC single protoplasts from apomictic guinea grass using different methods, and then to establish the method of controlling a single protoplast using UPESP machine. These results obtained in this study will be a useful tool for molecular analysis of AIC, and provide important information for clarification of apomixis reproductive mode.

Keywords: Apomixis; aposporous embryo sac initial cell (AIC); enzyme solution; guinea grass (Panicum maximum); isolation and manipulation of AIC, pre-treatment of ovary.

1. INTRODUCTION

Apomixisis a reproductive mode that only maternal genotype descends to its progeny.it is expected that apomixis can not only fix the hybrids to cost down the seed-production fee [1,2], but also make it possible producing seeds from the vegetative plant, indicating its supreme economic effects in agriculture even over that of “green revolution” [3,4]. The authors have reported that the most different event between the sexual and apomictic plants was the appearing of aposporous embryo sac initial cell (AIC) following the degeneration of the mature haploid megaspore in guineagrass (*Panicum maximum*). This was observed by Normarski differential interference-contrast microscopy (DIC), and transmission electron microscopy (TEM) [5-8]. And according to the above results, the authors have obtained the

apomixis-specific gene, named as *ASG-1*, from facultative apomict of guineagrass, using the differential screening method based on the ovary length as an index [9,10]. Now, we are doing the functional analysis of *ASG-1* using model plant of *Arabidopsis* for gene transformation [4].

On the other hand, it is considered as the important thing to clarify the mechanism of AIC appearance for understanding the apomixis phenomenon. Based on the obtaining of *ASG-1*, it is essential to seek the relation between the *ASG-1* and AIC appearance at molecular level. In this study, we focus on that as the first step to obtain single protoplast containing AIC, the isolation method of AIC was established based on that the ovaries were classified and collected from young buds and flowers staged in different developmental stages, according to the colors of stigma and the ovary length; and then, the ovaries were pre-treated with needle into different shapes, and treated with different kinds of enzyme solution with different concentrations of mannitol to increase efficiency of protoplast isolation; in final, the protoplasts isolated from different stages of ovaries were manipulated in the performance of controlled aspiration and spit with ultra particle electronic syringe picopipet (UPESP) machine set onto microscope.

2. MATERIALS AND METHODS

2.1 Plant Material

Guinea grass (*Panicum maximum* Jacq.) of facultative apomict, N68/96-8-o-11 (apomixis rate, 94%) and variety “Natsukaze” (apomixis rate, 90%) [6], were used in this study. These plants were cultivated in field center of Faculty of Environmental and Horticultural Science, Minami Kyushu University (Miyakonojo, Japan). The young buds and flowers were collected for protoplast isolation.

2.2 Ovary Sampling from Young Buds and Flowers in Different Developmental Stages

After measurement of length and width of young buds and flowers staged in different developmental stages, the ovaries were cut out (Fig. 1). The ovaries were classified and collected, according to the colors of stigma (white, yellow, peach and purple) (Table 1), and the ovary length was measured. Combining the ovary length andthe data reported by Chen and Kozono [5] the developmental stages of ovary were estimated (Table 1).

2.3 Pre-treatment and enzyme treatment of ovary for protoplast isolation

Three kinds of enzyme solutions were used for protoplast isolation of ovary, that is, I (0.5%CellulaseOnozuka RS, 0.25% PectolyaseY-23, 0.75%Pectinase), II (0.5% Cellulase Onozuka RS, 0.1% PectolyaseY-23), III (1%CellulaseOnozuka RS, 0.5% Pectinase, 0.5% Dricellase) with 9% mannitol, respectively [11,12]. The ovaries be flooded in enzyme solution were deaired with vacuum pump, and then, kept in 100 rpm among 1 to 4 hrs, and in 60 rpm after 4^{th}hr, under 25ºC. During enzyme treatment, the ovaries be flooded were pre-treated with cuttings of ovary (Fig. 3), in order to promote the absorbance of the solution and protoplast isolation. The isolation process was observed under microscope every hour.

Table 1. Comparative observations among colors of stigma and sizes of young buds and ovary in facultative apomictic guinea grass

Accessions	Colors of stigma	Young buds (μm)		Ovary μm)		Ovule developmental stages
		Length	Width	Length	Width	(Chen and Kozono)[1)] [7]
N68/96-8-o-11	White	2,558.8±56	696.3±43	360.3±33	203.3±29	Functional megaspore ～1st AIC
(Apomict)	Yellow	2778.9±78	899.3±55	415.8±31	229.8±40	1st AIC ～2nd AIC
	Peach	2,718.6±43	995.7±33	471.9±28	244.1±39	3rd AIC ～4th AIC
	Purple	2,764.1±97	965.3±27	534.2±35	280.1±43	4th AIC and/or over 5th AIC～
	(Anthesis)	2,845.4±101	1,26.4±51	737.1±41	318.6±48	Mature 4-nucleate embryo sac
"Natsukaze"	White	2,782.8±87	823.2±28	322.3±30	190.1±34	Megaspore ～Functional megaspore
(Apomict)	Yellow	2,848.4±98	932.6±44	405.1±34	226.1±41	1st AIC ～ 2nd AIC
	Peach	2,980.1±86	1,112.3±59	438.0±65	233.9±45	2nd AIC ～3rd AIC
	Purple	3,015.0±120	1,091.4±67	532.5±41	274.8±61	3rd AIC ～4th AIC
	(Anthesis)	-	-	649.1±59	333.5±57	Mature 4-nucleate embryo sac

1) AIC: Aposporous embryo sac initial cell. -: no examination

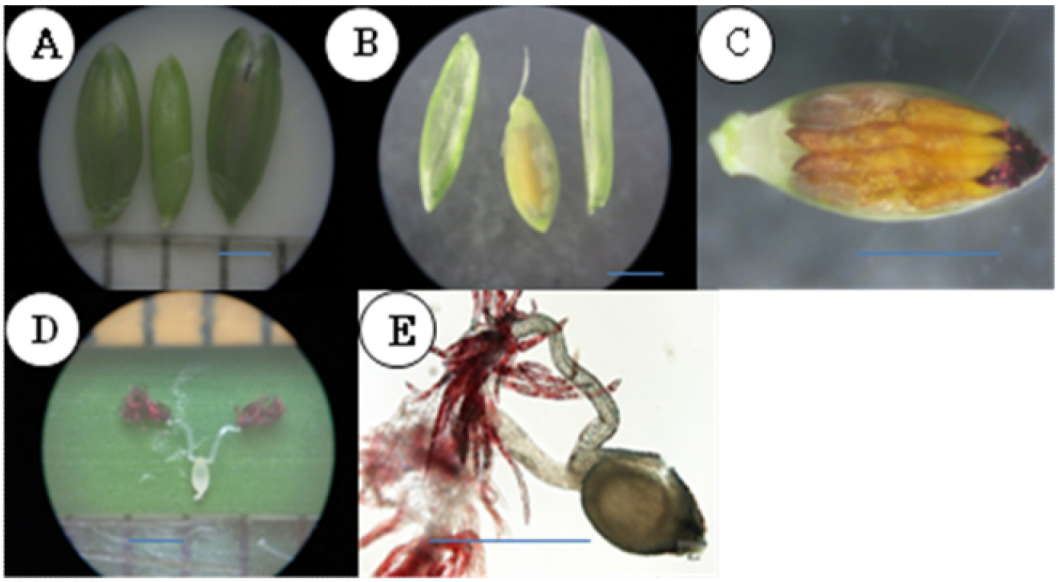

Fig. 1. The process of ovary cutting out from young buds of apomictic guineagrass for enzyme treatment. A) Young budsin different stages; B) Young bud cutting out; C) Ovary (left)and anther (middle) and stigma (right); D) Cut out whole pistil with ovary (lower), style (middle), stigma (upper); E) Ovary beflooded in enzyme solution (being brown color). Bar=1mm

2.4 Single Protoplast Manipulation with UPESP Machine

The isolated single protoplasts containing AIC were collected into a 6 cm dish, and the dish was set in LeicaDMI6000B, and then, the UPESP machine was set onto the same microscope formanipulation by handle control.

3. RESULTS AND DISCUSSION

3.1 Ovary Sampling from Young Buds and Flowers in Different Developmental Stages

Chen and Kozono [5] have reported firstly that to clarify the reproduction mode of facultative apomictic guinea grass, the ovaries in different developmental stages were observed using DIC and method of embryo sac clearing. And during the observation, the ovary length was also measured, so that it was possible to estimate the developmental stage of embryo sac using ovary length. In this study, according to the relation between the ovary length and stage of embryo sac development in N68.96-8-o-11 and “Natsukaze” [5], the method of ovaries cutting out from young buds and flowers was established to isolate AIC exactly. That is, based on the measurement of the sizes of the young buds that contain ovaries with different colors being able to view with naked eyes, and the ovary length (Fig. 2), and the data of stages of embryo sac development [5] (Table 1), the ovaries in different developmental stages were cut out from young buds and flowers of apomict of guinea grass, N68/96-8-o-11 (Table 1). The sizes of ovary and young bud in apomict were 350μm/2,000μm at stage of before AIC, 380–600μm/2,100-3,500μm at stage of AIC to

matured AIC-derived embryo sac, respectively [5,6,9,10]. According to those data, the relation between the length of young buds and length of ovaries was verified based on the colors of ovaries cut out as an index. As a result, it was clear that the length of young buds and the length of ovaries were proportion to the mature period of ovary (Fig. 2). And from the result, it was possible that the mature period of embryo sac could be estimated based on the colors of ovary. From the above results, it was considered that based on the standards showed in Table 1, the ovaries in different developmental stages could be sampled according to the colors of ovary as an index. Therefore, it was indicated that using this method showed here, embryo sac, ovule and ovary in different developmental stages could be sampled.

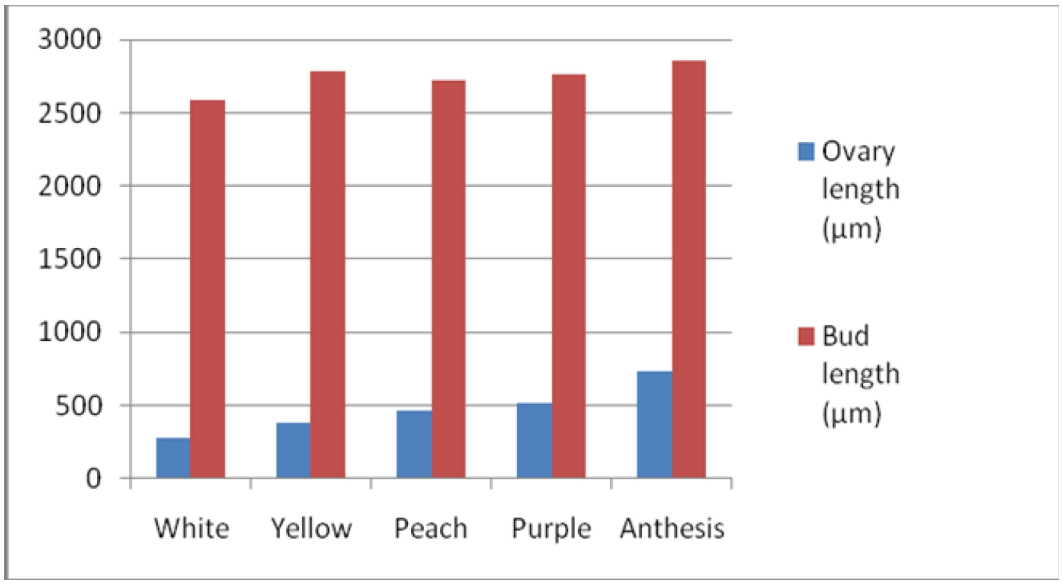

Fig. 2. The relations between the lengths of young budsand ovaries in the maturing stages with different colorsof ovaries. (The numbers of young buds and ovaries were 15, with 4 repetitions, respectively)

As the purpose of this study is to isolate single protoplasts containing the AIC, based on the data of the colors of ovary and stages of embryo sac showed in Table 1, the ovary with white color to yellow color was considered as that containing AIC, and these colors of ovaries were cut out and used for enzyme treatment.

3.2 Pre-Treatment and Enzyme Treatment of Ovary for Protoplast Isolation

It is difficult to isolate 1 to few cells from ovary. We planned to pre-treatment of ovary to isolate AIC exactly. As the pre-treatment, the ovaries cut out were flooded into enzyme solution and then, the treatments were done as following: (1) Cut in micropylar end (Fig. 3A); (2) cut in chalazal end (Fig. 3B); (3) cut in middle part (Fig. 3C and D); (4) after 1 hr of (3) treatment, cut in micropylar end (Fig. 3E). The mass of cell and single protoplasts were observed after 2, 4 and 6 hrs, respectively (Fig. 3F, G, and H). As a result, the efficiency of protoplast isolation of (3) and (4) was 1-2 hrs shorter than that of (1) and (2), even though protoplasts were isolated in either treatment. And the protoplasts containing AIC were also isolated (Fig. 3H, arrow). From these results, it is indicated that the protoplasts could be

isolated from regional tissues. Here, the 3 kinds of enzyme solutions used in this study were same to that of tomato [12] and that of reproductive protoplasts of apomictic guinea grass [13]. However, the single protoplasts were isolated successfully here only in the use of ovaries at anthesis. In our study, it was observed that when the ovary younger than anthesis stage was used, the protoplasts ruptured soon though they could be isolated. As the reason, it was considered that the un-coincidence of the osmotic pressure between the isolated protoplast and the enzyme solution causing the rupture.

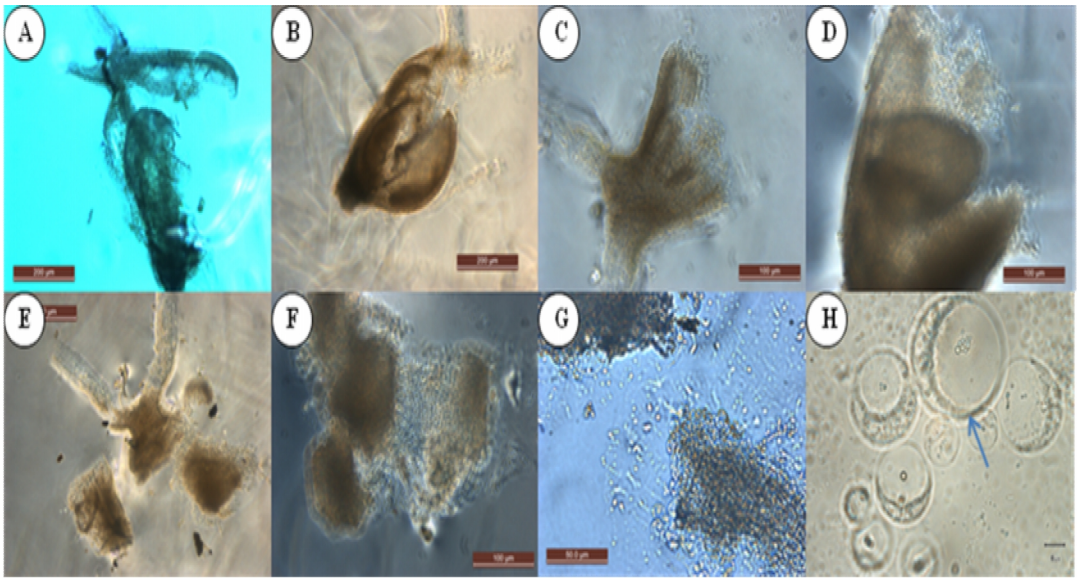

Fig. 3. The process of protoplast isolation from the ovaries under the different pre-treatments in enzyme solution, in different developmental stages of facultatively apomictic guineagrass. A) the ovary with cutting in micropylar end; B) the ovary with cutting in chalazal end; C) and D) ovary with cutting in middle part (the upper part, C) and (the lower part, D); E) after one hour of the ovary cutting in middle part (C and D), and then, the part of micropylar end (D) was cut lengthways; F), G), H) the mass of cells or tissue (F) was become to single cells (G), and then, the single protoplasts were observed (H), after 2nd hour, 4th hour and 6th hour enzyme treatments, respectively. →: the biggest protoplast was considered as the AIC

To seek the proper concentrations of enzyme solution, the osmotic pressure was adjusted for different stages of ovaries. We firstly clarifies the colors of stigma into 4 kinds of white, yellow, peach and purple, and more, adjusted the osmotic pressure with mannitol in 12%, 11%, 10% and 9%. As the 3 kinds of the enzyme solutions used in this study were reported in previous papers [11,12], showing successful protoplast isolation, they all gave some degrees of protoplasts isolated. Even though the protoplasts were isolated from the 4 kinds of ovaries in all of the enzymes used, the rates of protoplasts isolated were different (Table 2). To make it clear that which one is the best concentration of enzyme for protoplast isolation, we, here, used the survived rates of protoplasts as the index. For example, after 1 hour of enzyme treatment, over 70% of protoplasts survived (not ruptured protoplasts compared with that showed before 1 hour by counting the ratio under microscope) was called as + (the best isolation of protoplasts), and under 70% called as – (the bad isolation of protoplasts). As a result, it was clear that 12%, 11%, 10% and 9% were proper enzyme

concentrations for the ovaries with white, yellow, peach and purple colors, respectively. From these results, it is considered that the successful protoplast isolation containing AIC was essential to adjust proper concentration of enzyme solution. That is, the younger the developmental stage of ovary is, the higher the concentration of enzyme solution should be. And these results also provide important information for the experiments of protoplast isolation and cell fusion from different developmental stages of plants.

3.2 Establishment of Single Protoplast Collection Method

The single protoplasts were isolated from the ovaries in different stages classified with colors and sizes of the ovary as described as above. And more, the AIC as the target cell was isolated from the ovaries with white and yellow colors as successful as expected. For the molecular analysis of mechanism of AIC appearance, it is considered in the future that the ovaries of *ASG-1*transformants is used to isolate the single protoplast in different developmental stages, especially the AIC stage, and then, to observe the *ASG-1* expression. Therefore, it is necessary that at first, to manipulate the shingle protoplasts at controlled performance. In this study, we verified whether the shingle protoplasts can be collected successfully by using the UPESP machine set onto microscope. The shingle protoplasts isolated from ovaries (Fig. 4A, arrow) were successfully aspirated (Fig. 4B, arrow), and spitted out (Fig. 4C, arrow). Using the method described here, the single protoplasts containing AIC could be collected. Based on the establishment of the systems of 1) estimation of ovary mature period according to the sizes and colors of stigmas, 2) pre-treatments of ovaries before enzyme treatment, and 3) ovaries in different developmental stages should be treated with different concentrations of enzyme solution, the protoplasts can be isolated and collected from the target cells containing AIC, and that be able to become to the AIC, and now, the *ASG-1* transformation experiments using the combining of AIC and microinjection method is in progress. With this system, by using the transient expression of *ASG-1* to establish the method of verifying AIC cells exactly, we aim on the functional analysis of *ASG-1*, an apomixis-specific gene isolated from facultatively apomictic *Panicum maximum*.

Table 2. The relation between the concentrations of enzyme solutions and maturing stages of pistil

Enzyme			Ovary colors	
Concentrations (%)	**White**	**Yellow**	**Peach**	**Purple**
12	+	—	—	—
11	—	+	—	—
10	—	—	+	—
9	—	—	—	+

1) The colors of stigma) for protoplast isolation (the numbers of ovaries; were 15, with 4 repetitions, respectively);2) +: best isolation of protoplasts; —: bad isolation of protoplasts (refer tothe text)

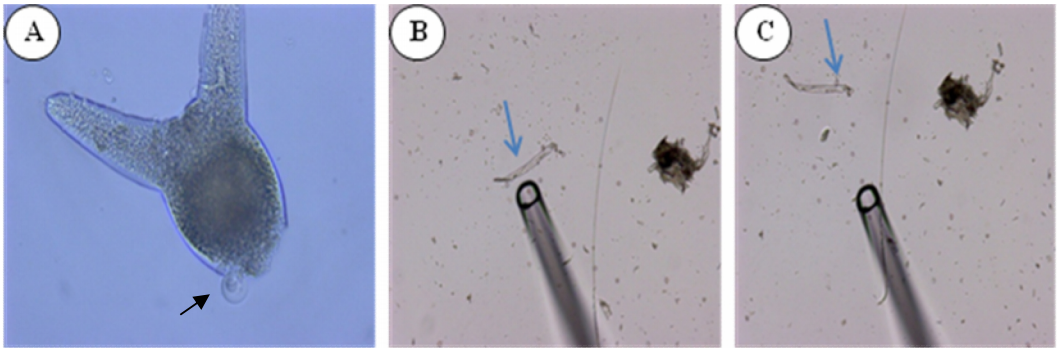

Fig. 4. The process of manipulation of single protoplasts using the UPESP machine. A) The single protoplast (arrow) isolated from white ovary; B) The single protoplasts aspirated by picopipet with controlled UPESP machine. →: as a mark, the ruin of ovary enzyme-treated was aspirated; C) the single protoplasts spitted out by picopipet with controlled UPESP machine. →: as a mark, the ruin of ovary enzyme-treated was spitted out

4. CONCLUSION

In this study, in order to do molecular analysis of the mechanism of AIC appearance, we focus on that, the key points were to isolate AIC single protoplasts from apomictic guinea grass using different methods, and then to establish the method of controlling a single protoplast using UPESP machine. These results obtained in this study will be a useful tool for molecular analysis of AIC, and provide important information for clarification of apomixis reproductive mode.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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