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Attempt to Establish Direct Gene Transformation System to Seeds of Sweet Potato (*Ipomoea batatas*) Using Electroporation Method

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LC and CX conceived the study and performed the statistical analysis. Author LC wrote the protocol and wrote the first draft of the manuscript. Authors SM, TS, YT and LC worked on grafting. Authors SM, YN and LC worked on electroporation. Author CX worked on literature searching. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The purpose of this study is to get transformants by using the seeds of sweet potato and the instrument of NEPA21, an electroporation method.

Study Design: As the first step, we used grafting method to get seeds from the combinations of *Ipomoea crassicaulis* cv. [Kidachiasagao] as stock and *I. batatas* (L.) Lam varieties as graft. Then, we used an electroporation method with instrument of NEPA21 to get the GUS transformants by using direct gene introduction into the seeds.

Place and Duration of Study: Faculty of Environmental and Horticultural Science, Minami Kyushu University, between September 2014 and December 2016.

Methodology: The materials for grafting between *Ipomoea crassicaulis* var. [Kidachiasagao] as stock and *I. batatas* (L.) varieties as graft to get seeds, were provided by Kyushu-Okinawa

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Agricultural Research Center (KOARC), and they were cultivated in growth chambers at Minami Kyusyu U. For the judgment of crossing incompatibility (CI) among the varieties of sweet potato, the standard of KOARC was adopted. For crossing experiment of sweet potato, the flowers of any two varieties were used for pollination, and the performance was stopped before 10 am. The seeds were harvested when they matured.
For the transformation of GUS into sweet potato, the seeds obtained from [Koganesengan] were used in this study. As the seeds were wrapped with hard skin, they were damaged with knife to absorb water easily. Then, the seeds were placed onto wetted filter papers laid in the dishes for 3~4 days. When the seeds germinated, they were used for transformation according to the procedures of NEPA21. After the process was finished, the container was placed in dark condition for 2 days at 25℃, and then, dyed with GUS dyeing solution.

Results: 1) According to the standard of selection for varieties having crossing ability based on the result of CI conducted by KOARC, we selected 3 varieties of [Koganesengan], [Narutokintoki] and [Beniazuma] out from 6 ones held; 2) According to the standard of selection suitable to be used as either for seed or pollen we have obtained mature seeds successfully from the 3 varieties; 3) Using the sweet potato seeds collected from [Koganesengan] and electroporation have made GUS transformant of [Koganesengan] with GUS gene expression, for the first time, detected by GUS dyeing with blue color which appeared on the surfaces of the seeds and young buds of the transformants.

Conclusion: The results obtained in this study, provide a practical sweet potato transformation system by two steps: 1) Using the grafting between [Kidachiasagao] and sweet potato can consistently obtain mature seeds of sweet potato according to the judgmental standard for CI provided by KOARC, and 2) Using the combination of seeds of sweet potato and electroporation instrument of NEPA21, can get GUS transformants of sweet potato by using the direct gene transformation system. The establishment of GUS direct gene transformation system in seeds of sweet potato can provide a powerful tool for the *ASG-1* gene transformation into sweet potato, which is considered as a crop, difficult to be cultured for plant regeneration. Furthermore, the system can be expected to open the way for all of the seed-set crops with a simplified and practical method to produce transformants.

Keywords: Electroporation; grafting method; GUS transformant; Ipomoea crassicaulis; seeds of sweet potato (Ipomoea batatas (L.) Lam.).

1. INTRODUCTION

Sweet potato (*Ipomoea batatas* (L.) Lam) is a vegetative reproductive crop and belongs to family of Convolvulaceae. Its origin lies in the region from Mexico to Peru of central-southern America. Sweet potato has come to Japan since 1600's, by different routes from China and Okinawa region. It has played an important role as a famine-relief crop during its long history, and has recently been reevaluated as a health-promoting food. In Japan, the yield of sweet potato is about one million tons, and about 50% of it is from southern Kyushu [1]. The sweet potato is used for a wide range of applications, such as vegetables [2], processed foods, alcohol and starch production [3,4]. In recent years, sweet potato has being researched as the main crop at molecular level to enhance the application, such as construction of the linkage map [5], and genome sequence [1].

Sweet potato is an outcrossing hexaploid, and every one of the organs, such as seeds, vines, and root tubers, can be used for propagation [6]. Sweet potato breeders utilize it in breeding process, such as grafting for flower induction and the identification of incompatibility groups before crossing to solve the problems unique for sweet potato. The phenotypes of the progeny obtained by seed propagation shows diversity due to 1) its heterozygous characteristics, and 2) its cross incompatibility (CI). Today's production of sweet potato is still dependent only upon the vegetative propagation by using the organs, even the seeds can be produced [4,7]. However, the vegetative propagation of sweet potato needs root tuber or vines derived from the tubers. As the cultivated area becomes bigger, there appear the problems that the yield of the root tubers will be reduced as the tubers are used for vine production, and the cost will be expensive when the tubers require air-conditioned storage space for nearly half a year [8].

On the other hand, apomixis is a reproductive mode which bypasses meiosis and syngamy to produce seed genetically identical to maternal

parent. So it is considered to bring economic benefits over the "Green Revolution" by using it to fix heterozygous crops in agriculture [9]. In our laboratory, apomixis-specific gene (*ASG-1*), has been isolated and characterized successfully from guinea grass (*Panicum maximum* Jacq.), a facultative apomict using differential screening method based on ovary length as an index [10,11,12]. In order to conduct functional analysis of the *ASG-1*, we have been carrying out studies on gene transformation of *ASG-1* not only in sexual plants of guinea grass [13,14], rice [15] and *Arabidopsis* [16], but also in sweet potato, a vegetative reproductive crop [17,18]. Our goal is to produce *ASG-1* transformants of sweet potato and to exchange the vegetative propagation for seed propagation. If the apomictic seeds were produced from sweet potato, all of the above problems will be resolved. So the significance of the project will be expected to surpass that of "the Green Revolution".

Up to now, transformation of sweet potato has been mainly carried out by using the combination of tissue culture of the organs and *Agrobacterium* mediation [19], and the most successful case study in this research area has been reported by Otani et al. [19] in Japan. However, the case is too special to be applicable to other situations [17,18]. In recent years, a new successful attempt different from the one before has been reported that the mature-seeds of rice and guinea grass [20] were used for direct gene transformation by using the electroporation instrument developed by NEPA Gene company (Japan). In particular, the NEPA instrument has being reported in animals with the newest gene editing method [21].

In this study, we reported how to get GUS transformants by using the combination of seeds of sweet potato and the instrument of NEPA21, an electroporation method. As the first step, we used *Ipomoea crassicaulis* var. [Kidachiasagao] as the stock and *I. batatas* (L.) varieties as the graft to get seeds. Then, the GUS gene was introduced directly into the seeds of sweet potato to get the transformants by using the instrument of NEPA21.

2. MATERIALS AND METHODS

2.1 The Production of Sweet Potato Seeds by Grafting Method

The experimental materials used in this study were provided by Kyushu-Okinawa Agricultural Research Center (KOARC), and they were cultivated in growth chambers at Minami Kyusyu U. The procedures of [Kidachiasagao] and sweet potato for cultivation followed those of KOARC. For the judgment of CI among the varieties of sweet potato, the standard of KOARC was adopted (Table 1).

2.2 Transformation of GUS into Sweet Potato Seeds

For the transformation of GUS into sweet potato, the seeds obtained from [Koganesengan] were used in this study. As the seeds were wrapped with hard skin, they were ddamaged with knife to absorb water easily. The seeds were placed onto the wetted filter papers laid in the dishes for 3~4 days. When the seeds germinated, they were used for transformation according to the procedures of NEPA21.

3. RESULTS AND DISCUSSION

3.1 The Production of Sweet Potato Seeds by Grafting Method

According to the judgment of CI among the varieties of sweet potato (Table 1), and the standard of KOARC, 3 varieties of [Koganesengan], [Narutokintoki] and [Beniazuma] were selected from 6 ones (Table 2), from which the seeds can be expected by crossings each other.

To get seeds of sweet potato using grafting method, 1) the seeds of [Kidachiasagao] was sowed and cultivated for usage of stock according to the practice of KOARC, and 2) the vines of the 3 varieties were used as graft (Fig. 1 A, B), and the [Kidachiasagao] as stock (Fig. 1C). For crossing experiment of sweet potato, the flowers of any two varieties were used for pollination (Fig. 1D, E), and the performance was stopped before 10 am. The seeds were harvested when they matured (Fig. 1F).

As the result, the seeds were obtained from all of the 3 varieties in different crossing combinations, except the [N] x [B] (Table 3). Among the 3 varieties of reciprocal crosses, it is clear that [Narutokintoki] showed lower seed set rates when used as the maternal parent compared with the others. Even it showed similar numbers of blooming flowers as the others, the flower was observed with very few pollens, and their flowers used for crossing were very few compared with the other two (Table 3).

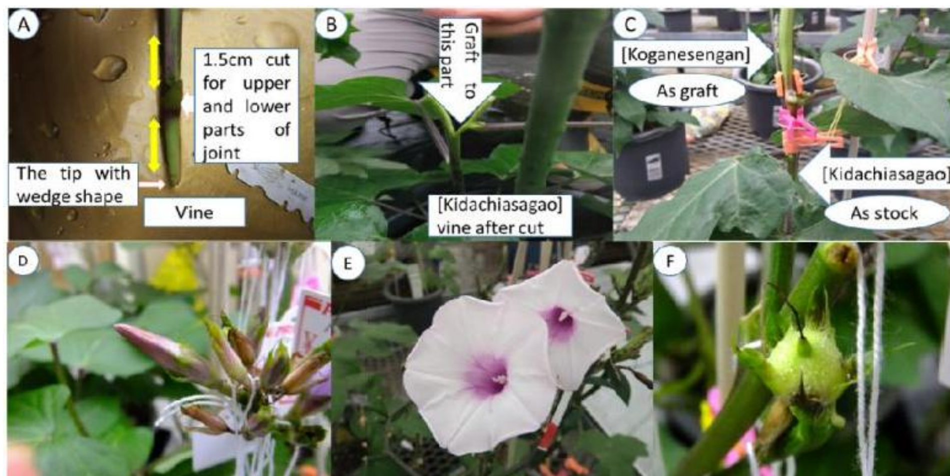

Fig. 1. Grafting process between [Kidachiasagao] and sweet potato [Koganesengan] and their flowering and seed formation in the graft of [Kidachiasagao] and three varieties. A. Graft making of [Koganesengan]; B. Stock making of [Kidachiasagao]; C. Mounting graft in stock; D. Buds of [Beniazuma] staged at one day before anthesis for pollination; E. Flower of [Narutokintoki] at anthesis; F. Fruiting of [Koganesengan]

According to the report from Murata [22], the lower seed set rate resulted in the abnormal embryo sac formation within which the normal female gamete could not be formed. On the other hand, in general, the rate of pollen germination in almost all varieties of sweet germination are not considered as the direct reason for the lower seed set rate [22]. In this study, it is considered according to the report of Murata [22] that the [Narutokintoki] used as maternal parent gave only 0 ~ 2% seed set rates, as the female gamete may be stopped during their developmental process. However, the [Narutokintoki] gave the similar seed set rates (63.9 ~ 52.4%) compared with the others (46.1 ~ 60.0%) when used as pollen parent, indicating that the few pollens may not affect their own seed set rate. In addition, C/B gave the values from 0 to 2.4 in all of the combinations (Table 3), and the number of seeds/fruit obtained in this study was similar to that of OKARC (by personal commutation).

3.2 Transformation of GUS into Sweet Potato Seeds

In brief, the transformation procedure is described as follows. The seeds were placed in the buffer solution (Ultrapure water 1,100 µl, PVP 500 µl, 10%Tween20 30 µl, 0.1 M spermidine solution100 µl, pWI-GUS plasmids DNA and pWI-H5K solution 200 µl, 2.5 M CaCl2 solution 100 µl) in a plastic container (Fig. 2A), and were treated with supersonic wave (Intensity 3 W/cm_2, Duty 50%, On 5, Off 5 sec.) for 3 mins, to be followed with reducing pressure for 90 mins using vacuum pomp (Fig. 2B). After that, the seeds were treated with NEPA21 under different treatment conditions (Fig. 2; Tables 4, 5). After the process was finished, the container was placed in dark condition for 2 days at 25°C, and then, GUS assay was applied (Table 6).

As the results, 1) GUS expression has been observed under different conditions carried out in this study (Tables 4, 5), with the seeds or buds of young seedlings stained with blue color (Fig. 2D); 2) With water treatment beforehand (Table 4), [Koganesengan] gave GUS expression with 20~25% in No. 1~3, and 0% in No. 4 (Table 7); 3) Without water treatment beforehand (Table 5), it gave the rates in No. 5~9 with 10~83.3%, No. 10, 11 with 0%, and No. 6 and 7 with 66.7% and 83.3% (Table 7).

From the results above, it is clear that between the two conditions with and without water treatment beforehand, the GUS expression rates obtained in the former were lower and higher in the latter, indicating that seeds with water treatment beforehand may intercept the absorption of the buffer solution which contains pWI-GUS plasmids DNA and pWI-H5K solution necessary for GUS expression. Therefore, the conditions like No. 6 and 7 giving higher GUS expression in this study can be expected to be applied in *ASG-1* transformation in seeds of sweet potato using electroporation with NEPA21 instrument.

Table 1. The standard of judgement of CI between varieties of sweet potato (based on the results of KOARC)

A (Anthesis, based on seed parent)		B (Fertility, based on seed parent)		C (Pollen, based on fertility rate when used as pollen parent)		D (or based on pollen volume when the new one used for judgement of CI	
The number of flowering per pot		Fertility rate		Fertility rate		Fertility rate	
<10	x	<2%	x	<2%	x	Non	x
10~<20	▲	2%~<5%	▲	2%~<5%	▲	Little	△
20~<40	△	5%~<10%	△	5%~<10%	△	Middle (CI group A, C, E)	○
40~<70	○	10%~<20%	○	10%~<20%	○	Many (CI group B, D)	◎
>70	◎	>20%	◎	>20%	◎		

**B: If designed pots have enough numbers, the part increased numbers of crossing should be the part increased with good fertility (in this case, no relation to the numbers of flowers should be considered, and just it is the priority for fertility). D: It should be removed from male parent when the pollens are not good*

Table 2. Estimation of ability of crossing compatibility among the varieties and test of their CI based the results of KOARC for seed production in 2015

Materials		Test of CI		Record	Characteristics of seed setting			
No.	Names	CI groups	Past	Old names	Anthesis	Fertility	Pollen	Remarks
N1	Simon	(E)			X			Simon No.1
N2	White star	B			Many			Ref.
N3	Genki	A			○	x	△	
N4	Koganesengan	B		Kyushu No. 55; C95-2	○	◎	◎	
N5	Narutokintoki	(E)			▲	○	○	Koukei No.14
N6	Beniazuma	A	1982	Kantou No.91; Senkei No.3	○	△	○	
N7	Benimasari	BF	1997	Kyushu No.130; Kyukei No.191	△	△	△	

**N1 is referred to data of Simon No.1, which belongs to (E) group of CI; N2 is based on the test of CI, but no data from crossing; N5 is referred to data of Koukei No. 14, which belongs to (E) group of CI*

Table 3. Fertility rates of different combinations between the varieties of sweet potato grafting

Cross combinations	No. of pollinated flowers (A)	No. of fruits (B)	B/A (%)	No. of seeds (C)	C/A	C/B
[B] x [K]	137	63	46.1	100	0.7	1.6
[B] x [N]	36	23	63.9	32	0.9	1.4
[N] x [K]	7	2	28.6	2.0	0.2	1.0
[N] x [B]	5	0	0.0	0.0	0.0	0.0
[B] x [B]	85	51	60.0	82	0.9	1.6
[K] x [N]	21	11	52.4	26	1.2	2.4

**[B]: [Beniazuma]; [K]: [Koganesengan]; [N]: [Narutokintoki]; B/A (%): Fruiting rates; C/A: Seeds per pollinated flower; C/B: Seeds per fruit*

Table 4. Conditions of electroporation for GUS transformation to seeds of sweet potato with water treatment beforehand

No. of treatments	Set values for transformation process											
	Poring pulse (Pp)						Transfer pulse (Tp)					
	V	Pr	Pi	F	Dr	P	V	Pr	Pi	F	Dr	P
1	50	5	50	5	10%	+	10	50	50	15	10	+/-
2	100	5	50	5	10%	+	10	50	50	15	10	+/-
3	150	5	50	5	10%	+	10	50	50	15	10	+/-
4	250	5	50	5	10%	+	10	50	50	15	10	+/-

**V: Voltage; Pr: Pulse range; Pi: Pulse interval; F: Frequency; Dr: Decay rate; P: Polarity*

Table 5. Conditions of electroporation for GUS transformation to seeds of sweet potato with water treatment beforehand

No. of treatments	Set values for transformation process											
	Poring pulse (Pp)						Transfer pulse (Tp)					
	V	Pr	Pi	F	Dr	P	V	Pr	Pi	F	Dr	P
5	50	5	50	5	10%	+	10	50	50	15	10	+/-
6	100	5	50	5	10%	+	10	50	50	15	10	+/-
7	150	5	50	5	10%	+	10	50	50	15	10	+/-
8	200	5	50	5	10%	+	10	50	50	15	10	+/-
9	250	5	50	5	10%	+	10	50	50	15	10	+/-
10	250	5	50	5	10%	+	20	50	50	15	10	+/-
11	250	5	50	5	10%	+	75	50	75	25	0	+/-

**V: Voltage; Pr: Pulse range; Pi: Pulse interval; F: Frequency; Dr: Decay rate; P: Polarity*

Table 6. Composition of GUS dyeing solution

Ingredient	Volume or weight	Final concentration
100 mM Phosphate buffer (pH 7.0)*	50.0 ml	50 mM
Methanol	5.0 ml	5%
X-Gluc	25 mg	1 mM
Distilled water	44.975 ml	
Silwet L-77** (Detergent)	0.025 ml	0.025%
Total	100 ml	

**1 M Na_2HPO_4: 57.7 ml; 1 M NaH_2PO_4: 42.3 ml; H_2O: 900 ml.*
***Alternatively, Triton x-100 or Tween 20 could be used*

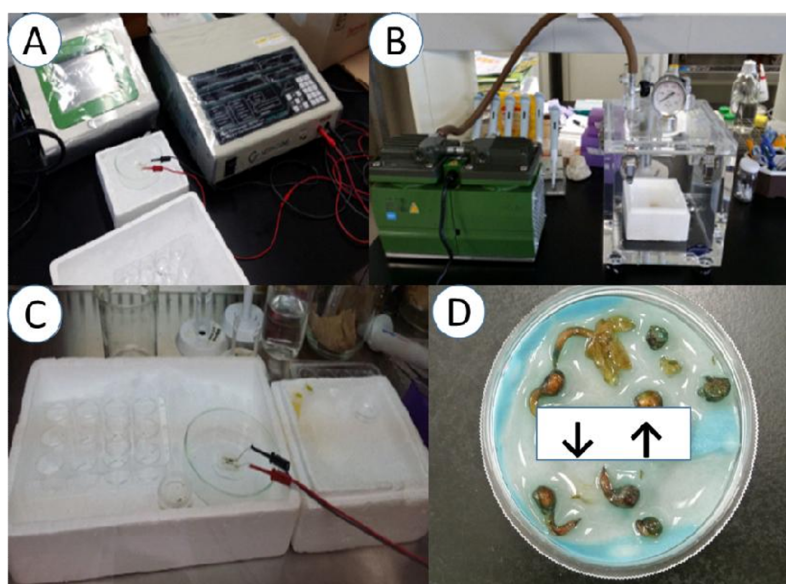

Fig. 2. The process of direct gene introduction of GUS into sweet potato seeds of [Koganesengan] by using electroporation method with NEP21. A. The sseeds placed in dish (Lower right) treated with supersonic instrument (Center), and NEP21 instrument (Upper right); B. The dish containing seeds in vacuum pump for air suction; C. Direct gene introduction of the seeds by using electroporation instrument; D. The seeds and seedlings undergone direct gene introduction showed GUS expression with blue colors after one day dyeing (↓indicating germinated seeds and seeds;↑indicating seedlings and seeds)

Table 7. GUS expression in sweet potato seeds after electroporation in different treatment conditions

Materials	Conditions no. [1)]	Seed no. treated (A)[2)]	Seeds average no. with GUS expression (B)	% (B/A)
[Koganesengan]	1	10	2.0	20
	2	10	2.5	25
	3	10	2.5	25
	4	10	0.0	0
	5	10	2.0	20
	6	10	6.5	65
	7	10	8.4	84
	8	10	4.5	45
	9	10	1.0	10
	10	10	0.0	0
	11	10	0.0	0

[1)] The No. referred to Table 4 and 5; [2)] Three times repeated

4. CONCLUSION

The results obtained in this study provide a practical sweet potato transformation system in two steps: 1) using the grafting between [Kidachiasagao] and sweet potato can obtain consistently matured seeds of sweet potato according to the judgmental standard for crossing compatibility provided by KOARC, and 2) using the combination of seeds of sweet potato and electroporation instrument of NEPA21, can get GUS transformants of sweet potato by using the direct gene transformation system. The establishment of GUS direct gene transformation system in seeds of sweet potato can provide a powerful tool for the *ASG-1* gene transformation into sweet potato, which is considered as a hard-to-culture crop for plant regeneration. In addition, the system can be expected to open the way for all of the seed set crops with a simplified and practical method to produce transformants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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