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Establishment of *Agrobacterium*-Mediated Transformation System in Sweet Potato (*Ipomoea batatas*) by Culture of Leaf Segments for Functional Analysis of *ASG*-1, an Apomixis-Specific Gene

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Authors' contributions

This work was carried out in collaboration by the authors. Authors LC and CX designed the study, and LC wrote the first draft of the manuscript. Authors ZD and LG wrote the protocol for plant regeneration, authors TH and TS performed, and HI managed the plasmid construction and transformation of the plasmid into Agrobacterium. Author LC performed transformation of sweetpotato, and author CX managed the literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: In order to do the functional analysis of apomixis-specific gene (*ASG*-1), which was

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isolated from apomictic guineagrass, the sweet potato was used to establish an *Agrobacterium*-mediated transformation system.
Study Design: At first, plant regeneration was achieved from the culture of leaf segments of sweet potato. Based on it, a binary vector pSMA35H2-NG for transformation of *ASG*-1 was used for establishment of a suitable procedure for plant regeneration of transformants.
Place and Duration of Study: Faculty of Environmental and Horticultural Science, Minami Kyushu University, between June 2009 and December 2012.
Methodology: The leaf segments were used for somatic embryogenesis and plantlets regeneration. For the preliminary transformation, a *GUS* gene set in pSMA35H2-NG was introduced into the *Agrobacterium* strain GV3101/PMP9, and the *Agrobacterium* was used to infect the callus derived from leaf segments of sweet potato "Miyazakibeni" and the callus derived from seeds of rice "Nipponbare". For the plasmid construction, the *GUS* was replaced by *ASG*-1, named as pSMA35H2/ASG1. The resultant plasmid was mobilized into *Agrobacterium* strain GV3101/PMP9 for transformation. For detection of *ASG*-1, DNAs of the transgenic plantlets were used for PCR, using the primers designed according to *ASG*-1 and hygromycin, respectively.
Results: 1) When the leaf segments were sterilized with sodium hypochlorite solution of 0.3% and 0.4% for 15 min, 100% of surviving rates was achieved. And the segments cultured on Murashige and Skoog (1962) gave 100% of callus formation rates. 2) When the calli were placed onto Komamine and Nomura (1998) medium for differentiation, somatic embryogenesis was obtained with white color and grain-like tissue, and plantlets with multiple shoot-like tissues were obtained from the somatic embryo. 3) For the preliminary transformation, the calli showed *GUS* blue spots gradually on the surface. 4) When the pSMA35H2/ASG1 was used to the transformation of the embryogenic calli, the plantlets were developed through multiple shoots. 5) The specific bands of *ASG*-1 and hygromycin were observed from the PCR products of the plantlets' DNAs, respectively.
Conclusion: Overall the above results, the procedure using the binary vector pSMA35H2/ASG1 containing *ASG*-1 revealed, as the first case, that *Agrobacterium*-mediated transformation system in sweet potato was established using the culture of leaf segments in this study.

Keywords: Apomixis; ASG-1 gene; GUS gene; Ipomoea batatas (L.) Lam.; leaf segment; multiple shoot; somatic embryogenesis; transformation.

1. INTRODUCTION

Apomixis bypasses female meiosis and syngamy to produce embryo genetically identical to the maternal parent [1], and it is expected that apomixis could not only fix the hybrids to cost down the seed-production fee [2], but also make it possible producing seeds from the vegetative plant [3], indicating its supreme economic effects in agriculture. The authors have obtained the apomixis-specific gene, named as *ASG*-1, from facultatively apomict of guinea grass [4,5]. In order to clarify the function of *ASG*-1 and seek its application, it is important to establish a practical protocol of *Agrobacterium*-mediated transformation system, especially, using a vegetative plant, like sweet potato.

As an important crop in tropical, subtropical and temperate areas, and an efficient biomass-producing plant for starch, especially for bio-alcohol, recently, sweet potato plays a key role and has become attracted target [6,7]. As known as that, in general, the propagation of sweet potato is kept by vegetative organism, like tubers used as "seed-tuber", so that the

usage of “seed-tuber” not only reduces yields but also costs a lot for its storage in real production. On the other hand, sweet potato blooms in warmer area or in the case of grafting with morning glory, which are used in breeding program [8, 9]. However, as there contained self-incompatibility in sweet potato [10], the seeds obtained by cross hybridization were hetero-type [11]. And when the seeds are sowed they give variety in next generation instead of uniformed morphology [12]. Therefore, even the seeds are achieved but they cannot be used in production.

International Potato Center (CIP), where the sweet potato genetic resources collected from the world were kept and conserved, holds several varieties which were found to be resistant to the russet crack disease [13]. Recently, Okada et al. [14] reported that they had produced transgenic sweet potato with virus resistance of SPFMV-S, using culture of mesophyll protoplasts and electroporation method.

As described above, either the shoot apex culture [15,16] for virus-free plants or mesophyll protoplasts culture [17] for plant regeneration or transgenic plants [14], or shoot meristem culture for transgenic plants [18], needs higher handy techniques to get the materials for starting culture.

Today, as the techniques for construction and expression of foreign transgenic genes have been established, any genes isolated from any organism could be operated perfectly. However, the problem is that whether the basic culture for simple and efficient plant regeneration system can be provided or not. Recently, some approaches have been conducted in transformation of sweet potato by using *Agrobacterium* mediated method via organogenesis from leaf segment culture [19], and via callus formation from leaf explants culture [20], and by using electroporation and particle bombardment mediated transformation via embryogenic callus formation from leaf segment-derived protoplast culture [21]. In this study, as the preliminary step to get transgenic plants, we have made the approach to establishment of a simple and efficient plant regeneration system via somatic embryogenesis from culture of leaf segments that can be collected in any season, at any time, and then be used to produce transgenic plants for use of the functional analysis of *ASG*-1.

2. MATERIALS AND METHODS

2.1 Plant Material

Sweet potato, *Ipomoea batatas* (L.) Lam. cv. “Miyazakibeni”, cultivated in southern Kyushu in Japan was provided kindly from Horticulture branch, the Miyazaki Prefectural Agricultural Experimental Station (Miyokonojo, Miyazaki, Japan). The tubers were planted in vermiculite sterilized under 120ºC, 15 min. and grown in a growth chamber at 25ºC, 3.3 μmol $m^{-2}s^{-1}$ on 16 h photoperiod.

2.2 Leaf Segment Culture

After the 3rd to 5th expanded leaves far from the growing point (meristem) of the young runner were cut and treated with 0.2~0.8% concentration of sodium hypochlorite solution for 15 min, they were washed with sterilized water. Then, the leaves were put onto the glass laboratory dish, and after the edges and veins were carefully removed with a knife and tweezers, the remains of the leaves were cut into segments in size of about 0.5~0.7x0.3~0.5

cm. For callus formation, the leaf segments were cultured on MS [22] and KN [23] media supplemented with 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) with different concentrations, respectively, in dark condition. For embryogenesis and germination of somatic embryo, the calli formed on MS and KN media were transferred onto KN medium supplemented with indoleacetic acid (IAA), 2,4-D, NAA, kinetin (Kin) and BAP with different combinations, in a growth chamber of 25ºC, 3.3 µmol $m^{-2}s^{-1}$ on 16 h photoperiod. The ultrastructures of the somatic embryos were observed with scanning electron microscope (SEM) as described by Chen et al. [24].

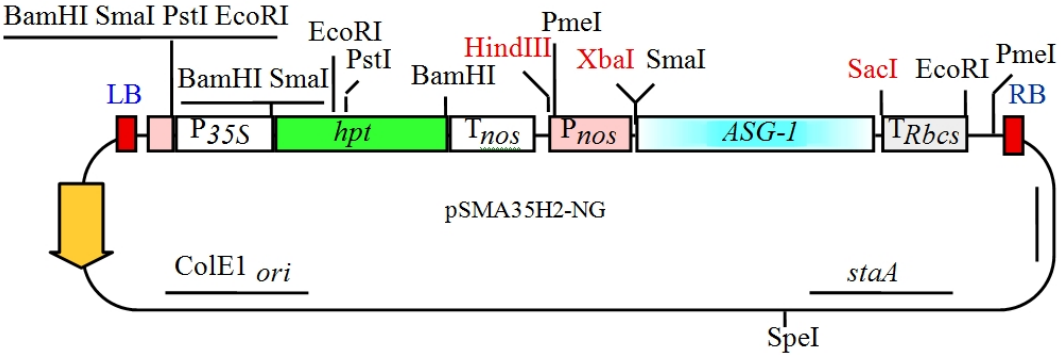

Fig. 1. Construct for *ASG*-1 using binary vector of pSMA35H2-NG

spR: *Spectinomycin/streptomycin resistance* gene from Tn7 ; *staA*: Region involved in plasmid stability from *Pseudomonas* plasmid pVS1 ; *repA*-HC: *replication protein A* gene from pVS1 (high-copy type) for plasmid maintenance in *Agrobacterium* ; ColE1 *ori*: ColE1 replication origin from pBR322 ; T*RbcS*: Polyadenylation signal from *Arabidopsis RbcS-2B* gene

2.3 Plasmid Construction

The nucleotide sequence of the *ASG*-1 was referred to the GenBank/EMBL/DDBJ nucleotide sequence database with the accession number AB000809. For the plasmid construction, at first, cDNA region of *ASG*-1 was joined with 35S promoter in sense direction, and cloning vector (pMCS5, MoBitec) was used for plasmid construction. The cDNA region of *ASG*-1 was introduced into multi-sites of *Xba*I and *Kpn*I in pMCS5. A shuttle vector of pSMA35H2-NG containing 35S promoter, Getamycin-resistant gene, Rifampicin-resistant gene and Spetinocin-resistant gene [25] was cut in *Sac*I site, and then, it was done smoothly with T4 DNA Polymerase (TAKARA, Japan). Moreover, after it was cut with *Xba*I, and β-glucuronidase (*GUS*) sequences in pSMA35H2-NG were replaced by *ASG*-1, and it was connected with Nopaline synthase (NOS) promoter in sense direction, named as pSMA35H2/ASG1 (Fig. 1). The resultant plasmid was mobilized into *Agrobacterium tumefaciens* strain GV3101/pMp90 through electroporation. Getamycin and Rifampicin at 50 mg/l were used as the selective agent for GV3101/pMp90.

2.4 *Agrobacterium*-Mediated Transformation

For *GUS* gene transformation, calli from the culture of leaf segments, a *GUS* gene set in pSMA35H2-NG and an *Agrobacterium* strain GV3101/PMP9 were used in this study. For the

protocol of infection and removing of *Agrobacterium*, and *GUS* dyeing was according to that of rice (Dr. Toki, NIAS, Japan, personal communication). For the pSMA35H2/ASG1 transformation, it is also according to that of rice.

2.5 Polymerase Chain Reaction (PCR)

To confirm *ASG*-1 existed in the transgenic plants in this study, we used plasmid pSMA35H/ASG1, and apomictic guinea grass N68/96-8-o-11, which *ASG*-1 was isolated from, as positive control, and sexual guinea grass N68/96-8-4-16, N68/96-8-o-7 and N68/96-8-o-5 as negative control, for DNA extraction, and templates for PCR. Manipulations of routine DNA from the selected and the normal plants of the regenerated plantlets were performed according to DNA mini plate method [26]. For PCR, the primers were designed according to the *ASG*-1 sequence (Fig. 2), as follows:

S1: ATGGCATTCGTGATGGGA,
S2: GGGTAAAACCTTCCCCATG,
S3: GTTCTAGCCCCGTCGATTC,
A1: CCTCTTGCCAAAGATCACG,
A2: ATCGACGAGGCTAGAACCT,
A3: AGGTTTTACCCTCGAGCACA.

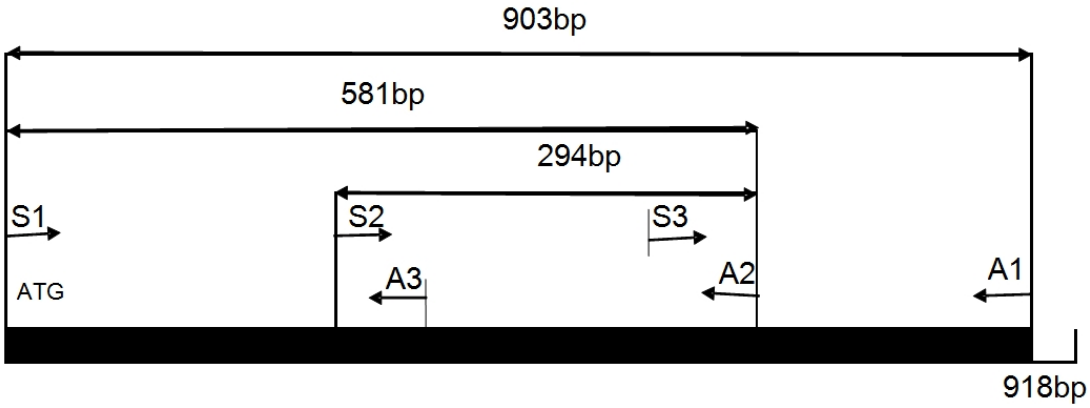

Fig. 2. Locations of the primers designed according to *ASG*-1 gene. The primer sequences of A1, A2, A3, S1, S2, and S3 referred from M & M

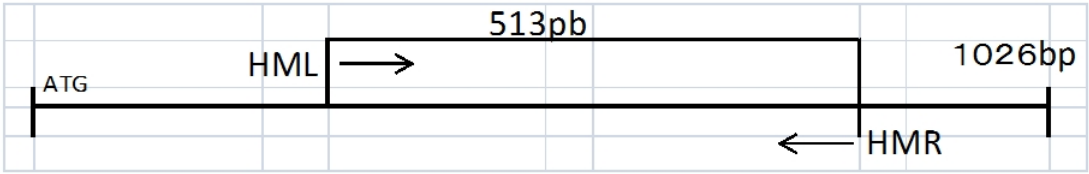

Fig. 3. Locations of the primers designed according to hygromycin B gene. The primer sequences of HML and HMR referred from M & M

And the other primers were according to hygromycin B sequence (Fig. 3) as follows: HML: CGCAAGGAATCGGTCAATAC, HMR: TTTGTGTACGCCCGACAGT. The PCR conditions were followed as 95ºC, 1 min; 94ºC, 30 sec; 52ºC, 30 sec; 72ºC, 1 min, for 35 cycles; in final, 72ºC, 5 min, 4ºC ∞. The PCR products were electrophoresed in 1.5% agarose gel for determination of specific bands of *ASG*-1 and hygromycin B.

3. RESULTS AND DISCUSSION

3.1 Effect of the Concentration of Sodium Hypochlorite Solution for Surviving Rates of Leaf Segments

Up to now, there have been many methods established concerning the shoot apex culture [15,27,28], petiole protoplast culture [29] and mesophyll and cell suspension protoplast culture [17], in sweet potato. However, even though there were some papers reported concerning leaf segment culture for plant regeneration [30,31], they could not repeated by the same protocols reported. Therefore, a practical and detailed procedure is needed to bridge the blank of leaf segment culture, as it is very simple and valuable culture system for propagation and gene transformation in sweet potato. Here, we firstly checked the effect of sodium hypochlorite solution concentration on leaf segment culture (Table 1). Among the range of 0.2 ~ 0.8% for 15 min, surviving rates of 100% were achieved in the treatments at 0.3% and 0.4%. From the result, it is clear on leaf segment culture that the treatment at a 0.3% or 0.4% sodium hypochlorite solution for 15 min. can stop the infection of unwanted bacteria perfectly when using the young runner cultivated in growth chamber.

3.2 Callus Formation on Different Media with Different Combinations of Phytohormones

We compared the effects of MS and KN media supplemented with 2, 4-D, BAP and NAA on callus formation (Table 2). Calli always occurred around the leaf segments of 0.7cm x 0.5 cm in size (Fig. 4B, C) at culturing (Fig. 4A) of two weeks. When we measured the size of callus at interval of two weeks, the sizes were 1.4x1.0 cm, 2.0 x 1.4 cm and 2.5 x 1.9 cm, respectively.

Table 1. Effects of sterilizing concentrations of sodium hypochlorite solution on culture of leaf segments of sweet potato (Observed after culture of two weeks, sterilizing time is 15 min. each treatment)

	Sterilizing concentration (%)				
	0.2	0.3	0.4	0.6	0.8
No. of explants	30	30	30	30	30
Surviving rates (%)	90±5	100±0	100±0	0±0	0±0

The callus formation rates of 100% were obtained from all of the treatments used (Table 2). In general, there were no differences between the media and combinations of phytohormones. Phytohormones were needed for callus formation from leaf segments, as there was nothing formed in leaf segment culture when phytohormone-free MS and KN media were used.

3.3 Somatic Embryogenesis and Germination of Somatic Embryo

After 2 times of subcultures of calli on the same medium, they were placed onto KN medium with different phytohormone combinations for somatic embryogenesis. After two weeks of culture in the KN medium, somatic embryogenesis, firstly, occurred on the surface of the original callus placed, with white color (Fig. 4D). At the same medium, the culture was continued for 4 weeks, and grain-like somatic embryos were differentiated around the original callus. The ultrastructures of the somatic embryos were also observed with SEM as shown in Fig. 4E. Embryogenic structures with nodular tissues, and somatic embryo with scutellum (SC), coleoptiles (CO) and germinated plumule (P) were observed. These structures were similar to those previously reported in *Panicum maximum* [32, 33], *Paspalum notatum* [34, 24]. Additionally, the recovery of chemical- or radiation-induced mutations or genetically transformed plants should be possible using this technique since somatic embryos are generally believed to arise from single cell [35].

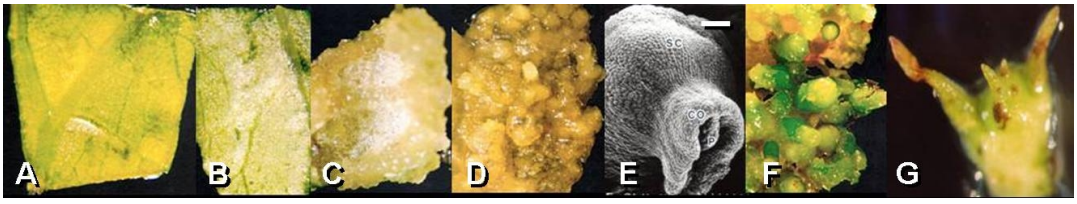

Fig. 4. Callus formation, somatic embryogenesis and germination of somatic embryos from culture of leaf segments of sweet potato. A) Leaf segment was cut and cultured on MS media; B) Initial callus like white frost observed on rear surface of the leaf segment, after three days of culture; C) Callus formation after two weeks of culture; D) Somatic embryogenesis with white color from callus derived from leaf segments after subcultured onto KN medium; E) Scanning electron microscope of different stages of somatic embryos with scutellum (SC), coleoptiles (CO) and germination of plumule (P), bar = 100µm; F) Somatic embryos with green color after another two weeks of culture; G) Germination of somatic embryo with elongation of leaf-like tissue, which initiated plantlet, cultured on modified KN medium

Table 2. Effects of concentrations of phytohormones on callus formation derived from *in vivo* the leaf segments of sweet potato in different media (Observed after four weeks of culture)

No. explants	Media	Combinations of phytohormones (mg/l)			Rates of callus
		2, 4-D	BAP	NAA	(%)
30	MS	1	0.5	0.01	100
30	MS	2	0.5	0.01	100
30	KN	1	0.5	0.01	100
30	KN	2	0.4	0.01	100
30	KN	2	0.5	0.01	100

NAA: 1- naphthaleneacetic acid; BAP: 6-benzylaminopurine; 2, 4-D: 2,4-dichlorophenoxyacetic acid

And then, the calli with white color were transferred onto the same medium, and after two weeks of the culture, the somatic embryos with green color were differentiated (Fig. 4F).

The rates of 100% were obtained for somatic embryogenesis on KN medium supplemented with 1.0 mg/l IAA and 1.0 mg/l Kin, and 0.01 mg/l NAA and 2.0 mg/l Kin, respectively (Table 3). On the other hand, no somatic embryogenic calli were obtained on KN medium supplemented with 1.0 mg/l IAA, 1.0 mg/l BAP and 2.0 mg/l 2, 4-D, and 1.0 mg/l IAA and 4.0 mg/l 2, 4-D, respectively. From the result of somatic embryo differentiation, it is considered that the phytohormone combination affects the rates of somatic embryogenesis.

Table 3. Effects of concentrations of photohormones on somatic embryogenesis in calli derived from leaf segments of sweet potato (Observed in three weeks after culture on KN medium)

No. callus cultured	Combinations of photohormones(mg/l)					No. somatic embryogenesis
	BAP	**IAA**	**NAA**	**2, 4-D**	**Kin**	**(%)**
40	--	1.0	--	--	1.0	40 (100)
40	--	--	0.01	--	2.0	40 (100)
48	1.0	1.0	--	2.0	--	48 (0)
48	--	1.0	--	4.0	--	48 (0)

ZR: zeatin riboside; NAA: 1- naphthaleneacetic acid; BAP: 6-benzylaminopurine; 2, 4-D: 2,4-dichlorophenoxyacetic acid; IAA: indoleacetic acid; GA3: giberellic acid; Kin: kinetin; BA: benzyladenine

Germination of somatic embryo was observed on the green callus derived from leaf segments after subcultured on modified LS regeneration medium with half sucrose (15%) for the culture of two weeks (Fig. 4F). The shoot-like or leaf-like tissues were regenerated from the germinated somatic embryos after another two weeks culture. Now the plantlets with shoot-like tissues were kept by subculture on the same medium. The difficult point is that shoot-like or leaf-like tissues could not grow into complete plant. However, media and phytohormone combinations are being used for overcoming the difficult point in our laboratory.

3.4 Examination for *GUS* Gene Expression in Callus Transformed with *Agrobacterium*

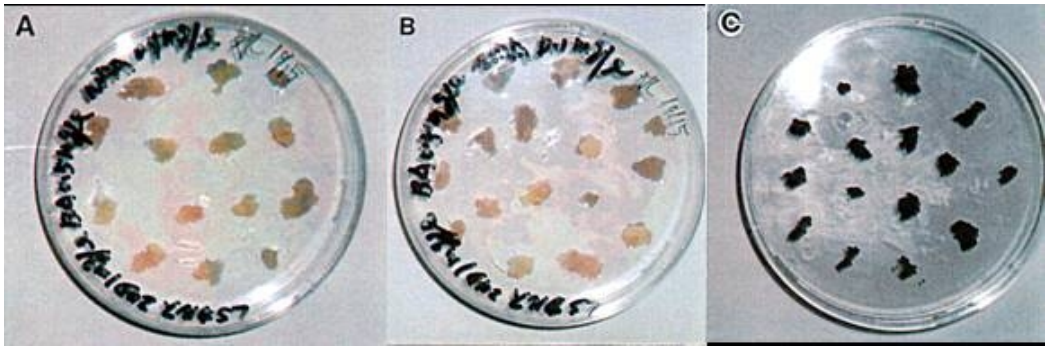

Fig. 5. *GUS* gene expression in callus of sweet potato after co-cultivation with *Agrobacterium tumefaciens* strain GV3101/PMP90 (pSMA35H2-NG Hm). A) Calli derived from leaf segments before *GUS* staining; B) Calli derived from rice seeds of "Nipponbare" before *GUS* staining; C) Calli with blue color from leaf segments (Upper) and those from rice seeds (Lower), after *GUS* staining

A *GUS* gene set in pSMA35H2-NG was introduced into the *Agrobacterium* strain GV3101/PMP9, and a suspension solution of the bacterium was used to infect the calli derived from leaf segments of “Miyazakibeni” (Fig. 5A) and rice calli of “Nipponbare” (Fig. 5B). After three days of co-culture of calli and *Agrobacterium* at 28ºC in dark, a *GUS* dyeing solution was added onto the collected calli, and incubated for 4 h at 37ºC. The calli showed blue spots gradually on the surface. As the time past, the blue spots were increased, until the whole callus was dyed with blue color after 12 h (Fig. 5C). As the control, rice calli also dyed with blue color. From the above results, it is suggested that this transformation system described in this study is usable and practical for transformation of sweet potato, when the system of plant regeneration is established from leaf segment culture.

Based on *GUS* gene expression done successfully in calli transformation with *Agrobacterium*, the same method was used for pSMAH2/ASG1, and the transgenic plantlets were obtained as shown in Fig. 4G.

3.5 *ASG*-1 Detection by PCR

DNAs extracted from the transgenic plantlets were used as templates, and 8 kinds of the primers designed according to *ASG*-1 gene (Fig. 2), and hygromycin B (Fig. 3), respectively, were used for *ASG*-1 and hygromycin detection by PCR. The results of electrophoresis of PCR products showed that the *ASG*-1 gene specific bands were confirmed in the 4 primer combinations of S1-A1, S1-A3, S2-A2 and S3-A1. As shown in Fig. 6 and Fig. 7, the specific bands of *ASG*-1 and hygromycin B from PCR were detected in S2-A2 and HML-HMR primers, respectively.

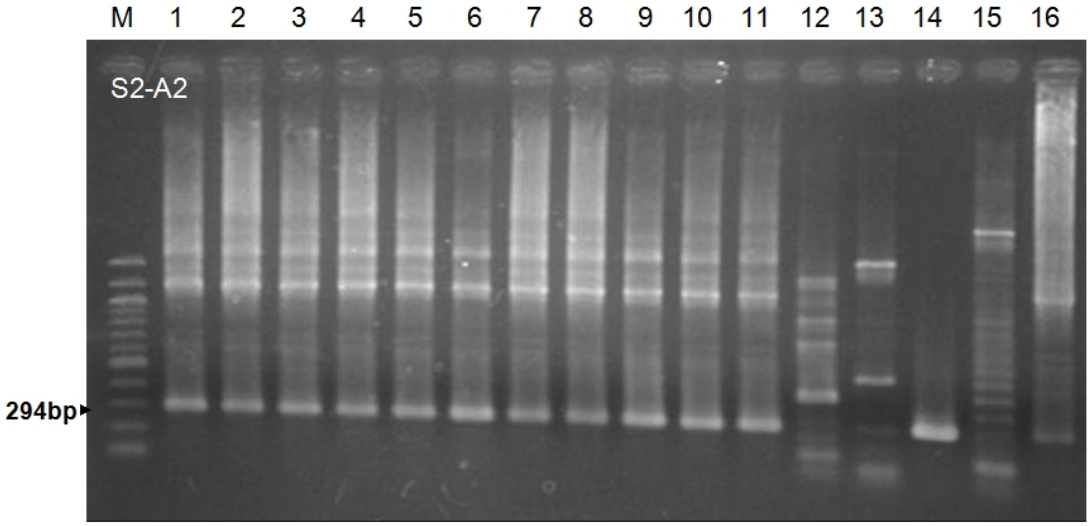

Fig. 6. *ASG*-1 detected from PCR products. M: 100bp; 1 to 11: Transformants of sweet potato; 12 to 13: sexual guinea grass N68/96-8-4-16 and N68/96-8-o-7; 14: apomict N68/96-8-o-11; 15: sexual N68/96-8-o-5; 16: plasmid pSMA35H/ASG1. ►: *ASG*-1 specific bands; The primers were S2-A2

For detection of foreign gene transformation, Southern hybridization [36, 37] and PCR [37] were usually used to reveal its presence in genome. In this study, we have used positive and negative controls, respectively, as materials for DNA exaction and templates, and tried to use the primers designed based on the sequences from hygromycin and target gene, to detect the *ASG*-1. That the expected specific bands were detected in the plantlets of sweet potato (Fig. 6 and Fig. 7) means that the unit of pSMA35H2/ASG1 containing hygromycin and *ASG*-1 gene was introduced into the genome of the transgenic plants by *Agrobacterium*-mediated transformation. At the same time, it has been clear that the positive controls showed expected specific bands same to that of *ASG*-1, and the negative controls gave nothing same to that of *ASG*-1 in Fig. 6. As a same reason, positive and negative controls gave nothing same to the specific band of hygromycin B in Fig. 7, except plasmid pSMA35H/ASG1.

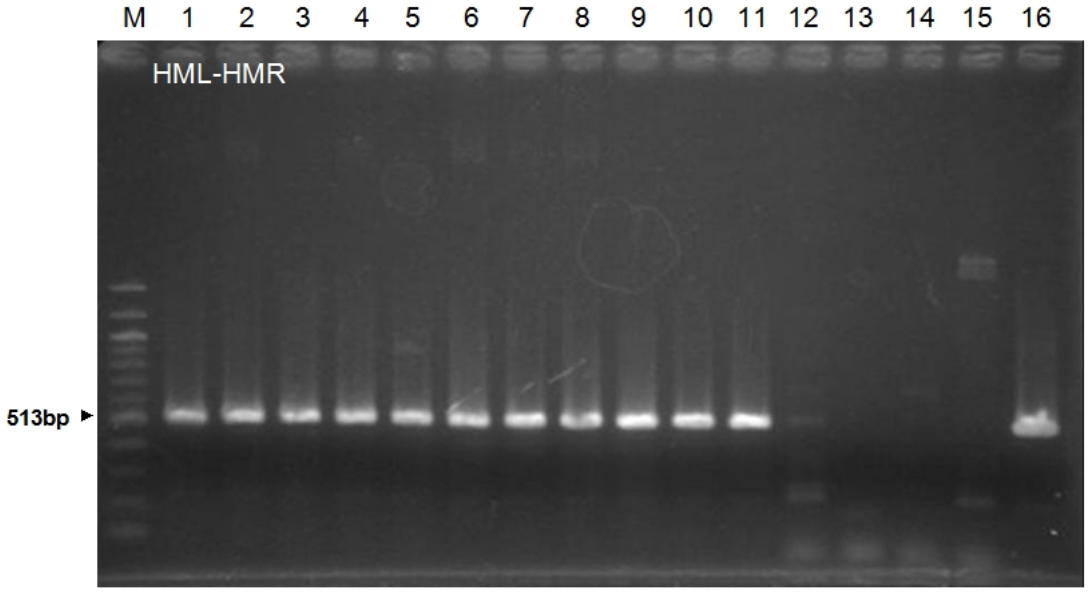

Fig. 7. Hygromycin B detected from PCR products. M: 100bp; 1 to 11: Transformants of sweet potato; 12 to 13: sexual guinea grass N68/96-8-4-16 and N68/96-8-o-7; 14: apomict N68/96-8-o-11; 15: sexual N68/96-8-o-5; 16: plasmid pSMA35H/ASG1. ►: Hygromycin B specific bands; the primers were HML and HMR

4. CONCLUSION

Overall, we established, as the first case, an efficient and vital *Agrobacterium*-mediated transformation system of pSMA35H2/ASG1 containing hygromycin B and *ASG*-1 gene, an apomixis specific gene for sweet potato by using the combination of leaf segment culture and *Agrobacterium*-mediated transformation methods. It will provide a strong tool for functional analysis of *ASG*-1. Now, this project is in progress.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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