Plant Regeneration and Its Functional Analysis within Transgenic Rice of *ASG-1*, an Apomixis-specific Gene Isolated from Apomictic Guinea Grass

Lanzhuang Chen[1*], Liming Guan[2], Daiki Toyomoto[1], Toru Sugita[3], Takuro Hamaguchi[4] and Reiji Okabe[4]

[1]*Graduated School of Horticultural and Food Science, Minami Kyushu University, 3764-1, Tatenocho, Miyakonojo City, Miyazaki, 885-0035, Japan.*
[2]*Faculty of Education and Culture, University of Miyazaki, 1-1, Gakuen Kibanadai, Miyazaki, 889-2192, Japan.*
[3]*Miyazaki Prefectural Agricultural Station, 5805, Shimonaka, Sadowara, Miyazaki, 880-0212, Japan.*
[4]*Miyazaki Prefecture Office, 2-10-1, Tachibanatori-Higashi, Miyazaki, 880-8501, Japan.*

Authors' contributions

This work was carried out in collaboration between all authors. Author LC conceived the project. Authors TS, RO and TH constructed the vectors. Authors LC and LG performed the experiments. Author DT worked on pollen observation of guinea grass. Author LC analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: To analyze the function of the *ASG-1*, an apomixis-specific gene has been isolated from apomictic guinea grass, the combination of *Agrobacterium*-mediated transformation system and pSMA35H2-NG binary vector were used for transformation of *ASG-1* to 'Nipponbare' of rice, and its functional analysis of transgenic rice was carried out.
Study Design: 1) plant regeneration of transgenic rice of *ASG-1* was achieved, and 2) it was

_____

Corresponding author: E-mail: lzchen@nankyudai.ac.jp;

followed by experiments with the obtained non-graining ear in the whole plants (NGEP) using comparative observation in both pollens of guinea grass and the T_0 NGEP with Nomarski differential interference-contrast microscopy (DIC).

Place and Duration of Study: Faculty of Environmental and Horticultural Science, Minami Kyushu University, between September 2012 and October 2015.

Methodology: As a preliminary transformation, a β-glucuronidase (GUS) was used to infect the callus. Plant regeneration of the transgenic rice of *ASG-1* were achieved. DNAs of the transgenic rice were used for detection of *ASG-1* by PCR. For analysis of NGEP, the anthers and ovaries of the transgenic rice and guinea grass were used for comparative observation with DIC.

Results: 1) GUS was successfully expressed in calli of both rice and guinea grass. 2) The transgenic rice plants of *ASG-1* were then obtained from the culture of seed-derived calli. 3) The natures of T_0 callus and T_1 plants were confirmed. 4) A subset (~15%) of T_0 plants showed morphological sterility with NGEP. 5) The ovaries of the NGEP showed identical characteristics to "Nipponbare" and other normal T_1 plants; however, the pollen showed normal rates of <38%. 6) The pollen of guinea grass at anthesis showed rates of normal pollen as low as that of the NGEP rice.

Conclusion: *ASG-1* may play a role in regulating pollen sterility. Comparing these findings with our previous reports, we identified co-localization for *ASG-1* in pollen of both rice and guinea grass, suggesting that the trait of *ASG-1* influencing pollen sterility might be inherited from guinea grass to rice through the transformation process.

1. INTRODUCTION

Apomixis is a unique reproductive mode in plants in which the egg cell does not fuse with the sperm cell and directly develops into the embryo, although the central cell does and forms an endosperm. Through this phenomenon, it is considered that the formation of asexual seeds in plants leads to populations that are genetically uniform maternal clones [1]. As apomixis provides a method for cloning plants through seed and involves the conservation of existing heterozygosis, apomixis is expected to simplify the development of hybrid cultivars and the production of commercial hybrid seed when used in breeding programs [2-4]. The transfer of apomixis to crop plants holds great promise within plant breeding for fixation of hybrid vigor because it would allow the propagation of hybrids over successive generations [5]. However, the use of conventional breeding techniques for the transfer of apomixis to important crops has been largely unsuccessful, as the plants employing apomixis are usually polyploids, and show different types of apomixis, making it difficult to understand the underlying genetic mechanism and to apply it in breeding programs [6]. Therefore, the genetic engineering of apomixis may be a more promising approach to reach this material goal [7]. To reach this goal, understanding the molecular mechanism behind apomixis is an essential initial step. To date, some approaches have been assessed for *Panicum maximum* [6,8], *Hieracium* [9], and *Pennisetum squamulatum* [10]. In *P. maximum*, we initially reported that the fundamental difference between sexual and apomictic accessions of guinea grass is the appearance of the aposporous embryo sac initial cell (AIC) when the megaspore dies or stops its division during the apomict accession, in contrast to sexual accession, which divides into an eight-nucleate embryo sac [11]. We also find that the AICs do not simultaneously appear, but rather appear one by one as the ovary length grows continuously [11]. Based on ovary length as an index for sampling different developmental stages of embryo sac formation in sexual and apomict forms, an AIC stage-specific gene was isolated by using a differential screening method, and was termed apomixis-specific gene-1 (*ASG-1*) [6].

The amino acid sequence of *ASG-1* is related to RD22, a seed-specific and drought-induced gene of *Arabidopsis thaliana* [12]), USP, an unknown seed protein precursor of *Vicia faba* [13], and other important genes of auxin-down-regulated cDNA clone [14], apospory-region marker [15] and polygalacturonase isoenzame I [16]. In addition, *ASG-1* has been identified for its localization in the cells of AIC and AIC-derived

embryo sac by *in situ* hybridization in apomictic Guinea grass [8]). However, the functional analysis of *ASG-1* by gene expression in transformants has not been attempted, as successful transformation procedures and vectors have not been available.

To perform the transformation in guinea grass, some approaches have been conducted using plant regeneration from leaflet culture [17], ovary culture [18], and seed culture [19] However, a practical and efficient procedure for gene transformation in guinea grass has not yet been established. In rice (*Oryza sativa*) of the same family as guinea grass, there have been two reports using *Agrobacterium*-mediated transformation: one using *japonica* rice [20] and the other using *indica* rice [21]. Recently, an efficient *Agrobacterium*-mediated transformation system has been established by Toki et al. [22] using a new binary vector, pSMA35H2-NG. As the initial purpose of the apomixis project was to isolate the putative genes from apomictic guinea grass and to then introduce it to rice for breeding application [6,11,23] *ASG-1* should be assessed for use for transformation of rice.

In the present study, we report that plant regeneration of transformants can be obtained using the combination of an *Agrobacterium*-mediated transformation system for rice (Dr. Toki, NIAS, Japan) and pSMA35H2-NG binary vector (kindly provided by Dr. Ichikawa, NIAS, Japan) specially expressed in the whole plant of rice for transformation of *ASG-1*, and to clarify the function of non-graining ear in the whole plants (NGEP) occurred in T_0 and T_1 transformants, with comparative observation of pollen in transgenic rice. In addition, as a subsidiary analysis for NGEP, a discussion is presented based on the fact that *in situ* hybridization of *ASG-1* in guinea grass provided signals ranging from strong to weak in the pollen developmental process while staged from before the appearance of AIC to the AIC-derived embryo sac formation and matured embryo sac at anthesis.

2. MATERIALS AND METHODS

2.1 The Preliminary Experiment for β-glucuronidase (GUS) Expression in Induced Calli

Seeds of rice (*Oryza sativa*) cultivars "Nipponbare" and "Akenohoshi" (Japanese varieties) were used for callus formation. Surfaces of seeds were firstly sterilized with 70% alcohol for 30 s and 2.5% sodium hypochlorite for 15 min with gentle stirring. After rinsing with sterile distilled water three times, the seeds were placed on filter paper for removing excess water. The seeds were then placed on callus induction medium (N6D) [24]. Seed germination and callus induction protocols of Toki et al. [22] were followed. Four weeks later, the calli were selected for GUS treatment.

As a control, the leaflet tissues of guinea grass (*Panicum maximum*) of sexual accessions N68/96-8-o-5, N68/96-8-o-7, and facultative apomictic accession N68/96-8-o-11, were collected from the farm of Minami Kyusyu University (Miyakonojo city, Japan) and cultured for callus formation according to the procedure previously reported [25]. The calli were selected for GUS treatment in the same manner as that for rice 6–8 weeks later.

Binary vectors pIG121Hm (Fig. 1) and pSMA35H2-NG (Fig. 2) were kindly provided by Dr. Nakamura (Nagoya U. Japan) and Dr. Ichikawa (NIAS, Japan), respectively. These two vectors containing the hygromycin B selectable marker gene (*hpt*), and a GUS, were introduced into *Agrobacterium tumefaciens* strains EH101 [26] and GV3101/pMp90 (kindly provided by Ichikawa), respectively.
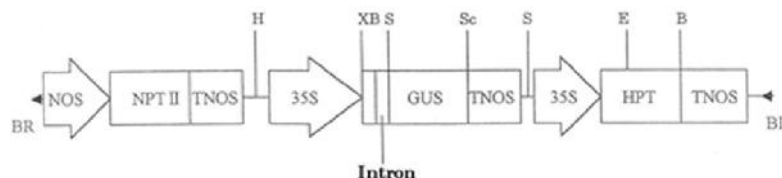

Fig. 1. Diagram of pIG121Hm. LB and BR, T-DNA left- and right-border repeats; NPTII, neomycin phosphotransferase II gene; GUS, ß-glucuronidase; HPT, hygromycin phosphotransferase; NOS, nopalinthyntase; 35S, 35S promotor; TNOS, signal of 3'-nopalinthyntase; T35S, signal of 35S 3'- RNA; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sal*I; Sc, *Sac*I; X, *Xba*

For GUS gene transformation, calli from rice seeds (Fig. 3A, C) and the leaflets of guinea grass (Fig. 3E–G) were used in the present study, and the protocols of infection and removing of *Agrobacterium*, and GUS dyeing were as according to that of rice (Dr. Toki, NIAS, Japan, personal communication).

2.2 Construction of *ASG-1* Gene to pSMA35H2-NG

As the binary vector, pSMA35H2-NG provided infection rates higher than those of pIG121Hm in calli of both guinea grass and rice, according to the preliminary result. The pSMA35H2-NG vector was used for *ASG-1* transformation. The nucleotide sequence of *ASG-1* was referred to the GenBank/EMBL/DDBJ nucleotide sequence database with the accession number AB000809. For the plasmid construction, the PCR products added with *XbaI* and *SacI* recognizable sequences, respectively in 5′ and 3′ sites of sense cDNA region of *ASG-1* was inserted into the sites of *XbaI* and *SacI* of pGEM5 (Promega) cloning vector. Based on the analysis of insert sequences, the clones without variation were selected. The sequences of *ASG-1* cDNA digested with *XbaI* and *SacI* enzymes were then set into pSMA35H2-NG, which was digested with *XbaI* and *SacI*. That is, the cassette of GUS was replaced with *ASG-1* cDNA (Fig. 5). The cassette of *ASG-1* was introduced into *Agrobacterium tumefaciens* GV3101/pMp90 for the transformation experiment.
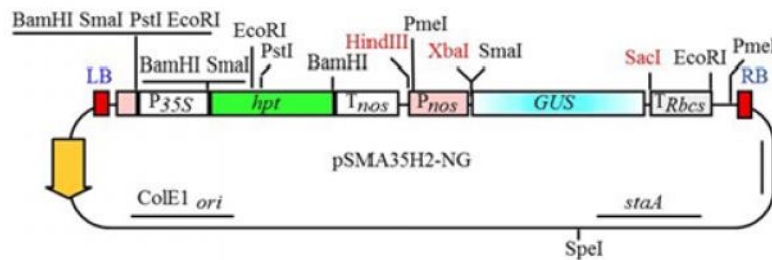

Fig. 2. Diagram of pSMA35H2-NG. *spR*: *Spectinomycin/streptomycin resistance* gene from Tn7*staA*: Region involved in plasmid stability from *Pseudomonas* plasmid pVS1*repA*-HC: *replication protein A* gene from pVS1 (high-copy type) for plasmid maintenance in *Agrobacterium* ; ColE1 *ori*: ColE1 replication origin from pBR322T*RbcS*: Polyadenylation signal from *Arabidopsis RbcS-2B* gene
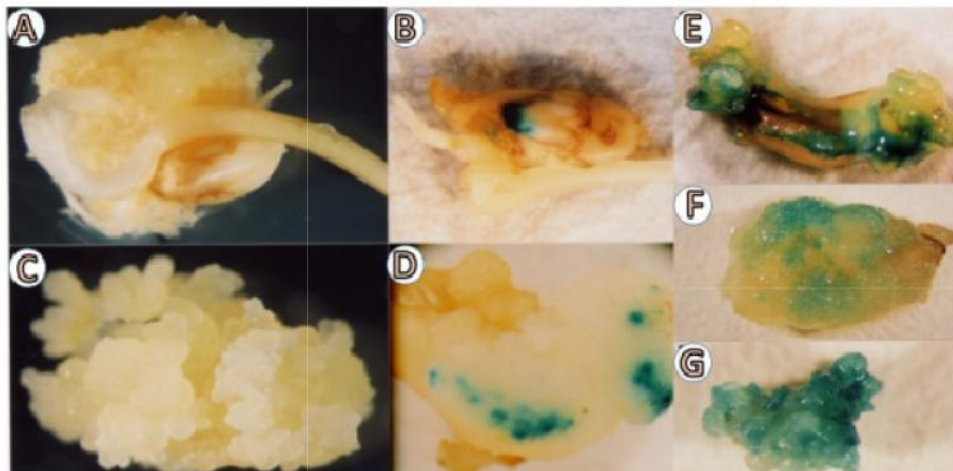

Fig. 3. GUS expression in calli from matured seed culture of rice and leaflets of Guinea grass. A and C: callus and embryogenic callus from seeds of rice; B and D: GUS expression in calli from A and C, respectively; E, F and G: GUS expression in calli in different developmental stages from leaflets of guinea grass, respectively

2.3 Selection and Regeneration of Putative Transgenic Plants

Seeds of "Nipponbare" were used for callus formation according to the protocol conducted in the preliminary experiment (Fig. 4A–G). After co-cultivation for 3 days with GV3101/pMp90, the calli were washed with sterilized water containing 500 mg/L of carbenicillin disodium salt (CDS), and transferred to the selection medium of N6D containing 300 mg/L of CDS and 50 mg/L of hygromycin B twice for the 2 week culture in 30°C and 3,000 lux for 16 h. The selected transgenic calli were transferred to the Regeneration III medium [27] containing 200 mg/L of CDS and 50 mg/L of hygromycin B twice for 3 weeks of culture in the same conditions as those given above. After the shoots and roots were grown to over 1 cm, they were transferred to the same hormone-free medium. The plants grew sufficiently large and were then transported into the pots for grain formation under 28°C and 1×10^4 lux for 16 h (Fig. 4H–M). The grains obtained from T_0 plants were sown to obtain T_1 plants.

2.4 Molecular Analysis of Transgenic Plants

Routine DNA manipulations of leaves from the T_0 and T_1 putative transgenic rice plants and the apomictic guinea grass of N68/96-8-o-11 as a control were performed according to the DNA mini plate method [28]. For PCR, the primers were designed according to the *ASG-1* sequence (Suppl. file) and the hygromycin B sequence (Suppl. file). The PCR conditions were: 95°C, 1 min; 94°C, 30 sec; 54°C, 30 sec; 72°C, 1 min, for 35 cycles; and finally, 72°C, 5 min, then holding at 4°C. The PCR products were electrophoresed in 1.5% agarose gel for determination of specific bands of *ASG-1* and hygromycin B.

2.5 Cytological Analysis of Transgenic Rice Plants

Fifty buds and flowers before and at anthesis, respectively, were collected for embryo sac analysis from T_0 and T_1 putative plants with grain formation and no grain formation, respectively, and non-transgenic plants as the control. Embryo sacs were observed according to the method of Chen and Kozono [11,23]. The buds and flowers were fixed in FPA_{50} (formalin: propionic acid: 50% ethanol = 5:5:90) for 5 to 7 days at 4°C.

Ovaries were then isolated from fixed buds or flowers, cleared in Herr's benzyl-benzoate-four-and-a-half fluid [29] for >2 h at 0–4°C, and observed with Nomarski differential interference-contrast optics (DIC). The anthers of transgenic and non-transgenic plants of T_1 were also observed with DIC.

2.6 Cytological Analysis of Guinea Grass

Fifty buds and flowers of guinea grass were collected before and at anthesis, respectively, from the facultative apomictic accession of N68/96-8-o-11, from which *ASG-1* was isolated. Embryo sacs and anthers were observed according to the same methods as those for rice described above.

3. RESULTS

3.1 Preliminary Experiment of GUS Reporter Gene to Guide Transformation

GUS was used as a reporter gene in the transformation efforts to closely monitor the gene transformation progress. Two kinds of vectors (Fig. 1 and Fig. 2) provided the GUS expression in calli (Fig. 3A–B) and embryogenic calli (Fig. 3C–D) from rice seed culture, respectively. However, for Guinea grass, only the pSMA35H2-NG/GV3101/PMP90 provided GUS expressions in the initial callus formation, normal callus, and embryogenic callus from leaflet culture (Fig. 3E, F, G), but not in pIG120Hm/EHA101. In addition, the expressed rates of the former vector were 37.5–75.0% and 70.7–73.9% in guinea grass and rice, respectively, which were higher than the 0% and 49.3–65.3% expression rate achieved by the latter vector, respectively (Table 1). As the pSMA35H2-NG/GV3101/PMP90 transformation system showed universal application in rice and guinea grass, the system was used in the present study for transformation of the *ASG-1* gene.

3.2 Plant Regeneration of Transgenic rice of *ASG-1* from Culture of Mature Seed

Calli were induced to N6D medium from mature rice seeds after 3–4 weeks of culture (Fig. 4A–D). The calli, of which approximately 20% were white and compact embryogenic calli with numerous embryoids on the surface (Fig. 4E–F), were then transferred onto modified N6D medium for an additional 3 weeks of culture. To further confirm

the efficiency of the transformation system, these calli were partly used for GUS transformation, and as a result, they gradually showed blue spots on the surface (Fig. 4G). The remaining calli were then used for *ASG-1* transformation (Fig. 5), transferred onto N6D regeneration medium, and the shoots emerged growing into complete plants (Fig. 4H–K). Finally, the plants were naturalized and cultured in a growth chamber (Fig. 4L–M).

3.3 Confirmation of the Transgenic Nature of *ASG-1* and Hygromycin B in the Regenerated Rice Plants

Initially, when the primers of A1 and S1 (Suppl. file) and DNA were extracted from calli of T_0 putative transgenic rice and N68/96-8-o-11 were used for analysis of polymerase chain reaction (PCR), the specific bands of *ASG-1* were detected from the samples, respectively, indicating that they included the transgenic natures (Suppl. file).

PCR analysis was then performed on T_1 putative transgenic plants of rice. The various patterns were obtained in 903 bp between S1 and A1, 581 bp between S1 and A2, and 294 bp between S2 and A2 (Fig. 6). The pattern in hygromycin B was 513 bp between HML and HMR (Fig. 7, Suppl. file). These results suggest that the T_1 plants were from independent transformation events and no escape was observed, indicating the tightness of the selection scheme. As a control, when HML and A3 primers (Suppl. file) were used for PCR analysis, different bands were detected (Fig. 7), suggesting that recombinant plasmids were set in the chromosomes of transgenic rice with different patterns.

3.4 Morphology and DIC Analysis of Putative Transgenic Rice Plants

Among the T_0 putative transgenic rice plants, a subset (~15%) showed NGEP, even though cutting and growing to form ears and flowers was repeated three to four times for the plants of NGEP. However, the remaining plants in T_0 and their T_1 generation provided normal graining and seed maturity. To determine the mechanism of NGEP, the buds and flowers of the T_0 and T_1 plants were collected and observed with DIC, as described in the Material and Methods. The female gametophyte, in particular, the embryo sac, showed similar behavior to the normal egg. Synergies, central, and antipodal cells existed in the remaining transgenic plants; however, in the male gametophyte in particular, the pollen showed normal rates <38% (Fig. 4O; Table 2), which was less the rate of >90% of the T_1 plants and un-transgenic plants (Fig. 4N). The difference between the NGEP and the T_1 plants was in the normal rates of pollen.

3.5 Observation of Pollen and Embryo Sac of Apomictic Guinea Grass

To further determine why the sterile transgenic rice plants appeared after *ASG-1* of recombinant plasmid was introduced, and whether it is related to the origin from which *ASG-1* was isolated, the apomict of guinea grass accession N68/96-8-o-11 was used to observe the pollen sterility. The pollen in the anther and out of the anther in the young bud stage before anthesis showed a full spherical shape and filled contents with rich cytoplasm containing starch grains (Fig. 8A-B), whereas those of the anthesis stage of flowers showed a wrinkled shape and empty contents (Fig. 8C–D). The normal rates of pollen in the

Table 1. Comparison of expression efficiencies of two kinds of binary vectors on guinea grass and rice using *Agrobacterium*-mediated method

Materials[1]	pIG120Hm/EHA101		pSMA35H2-NG/GV3101/PMP90	
	No. calli	No. expressed (%)	No. calli	No. expressed (%)
Guinea grass				
N68/96-8-o-7 (S)	67	0 (0)	48	36 (75.0)
N68/96-8 (S)	55	0 (0)	32	12 (37.5)
N68/96-8-o-11 (A)	30	0 (0)	24	17 (70.8)
Rice				
'Nipponbare'	75	37 (49.3)	82	58 (70.7)
'Akenohoshi'	95	62 (65.3)	92	68 (73.9)

[1] *For guinea grass, all are the accessions, and S means sexual and A apomictic accessions. And for rice, they are the varieties*
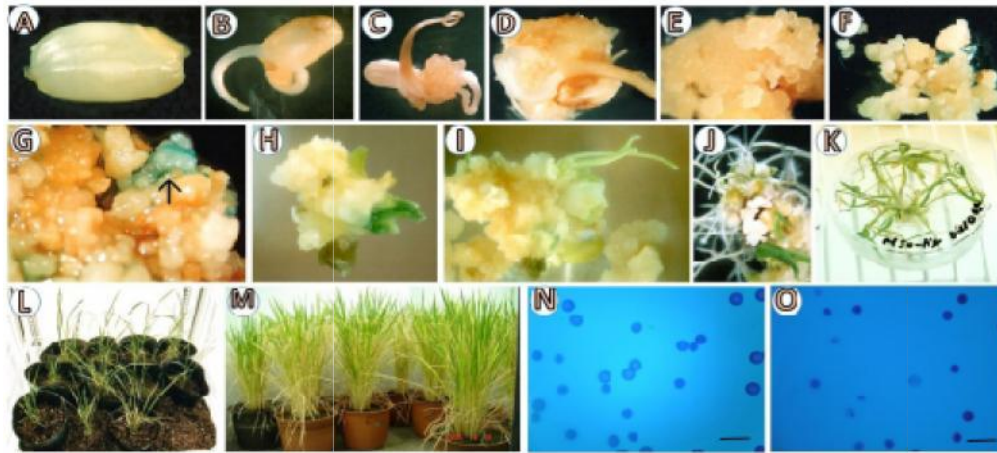
Fig. 4. Plant regeneration and morphologies of transgenic rice of *ASG-1* from matured seeds
A-E: Callus formation from matured rice seeds cultured on N6D medium; F: Calli used for transformation of GUS and *ASG-1*; G: GUS expression in calli; H-J: ASG-1 transgenic rice regenerated on regeneration medium; L-M: Transgenic plants growing and ripening; N-O: Pollens in normal ripened transgenic plants and in un-ripened one, respectively. Bar = 100 μm in N and O
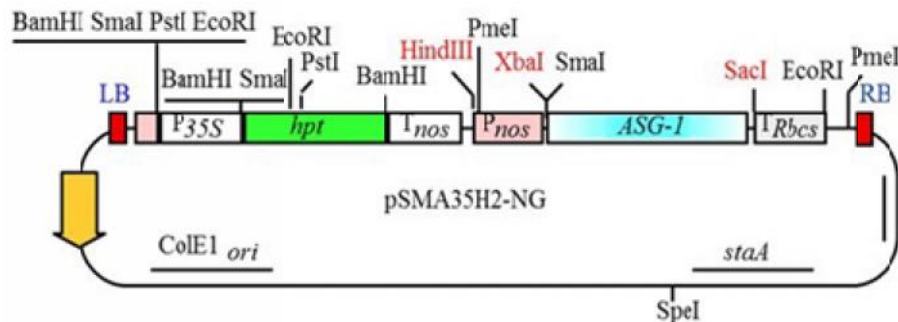

Fig. 5. Construct for *ASG*-1 using binary vector of pSMA35H2-NG
***spR*: *Spectinomycin/streptomycin resistance* gene from Tn7; *staA*: Region involved in plasmid stability from *Pseudomonas* plasmid pVS1; *repA*-HC: *replication protein A* gene from pVS1 (high-copy type) for plasmid maintenance in *Agrobacterium;* ColE1 *ori*: ColE1 replication origin from pBR322; T*RbcS*: Polyadenylation signal from *Arabidopsis RbcS-2B* gene**

anthesis stage were counted as <40%, which was the same as that of sterile NGEP of transgenic rice (Fig. 4N). The changes of morphology of pollen in different developmental stages observed in the present study were compared with those in the previous report of *in situ* hybridization of *ASG-1* in apomictic guinea grass, indicating indirect coincidence between both of the pollen types (Table 2).

4. DISCUSSION

In the present study, we successfully obtained transgenic rice of *ASG-1* using an

Agrobacterium-mediated transformation system and hygromycin B selection, based on the result that performed well in the GUS transformation protocol for all three accessions of guinea grass and two cultivars of rice tested. This is the first time that *ASG-1* was transformed into rice and *ASG-1* was realized as a functional gene to act in the transgenic rice plants.

The *Agrobacterium*-mediated transformation method has mainly been used for dicotyledonous plants. The breakthrough for the application to monocotyledonous plants was achieved by Hiei et al. [20] in *japonica* rice and Rashid et al. [21] in
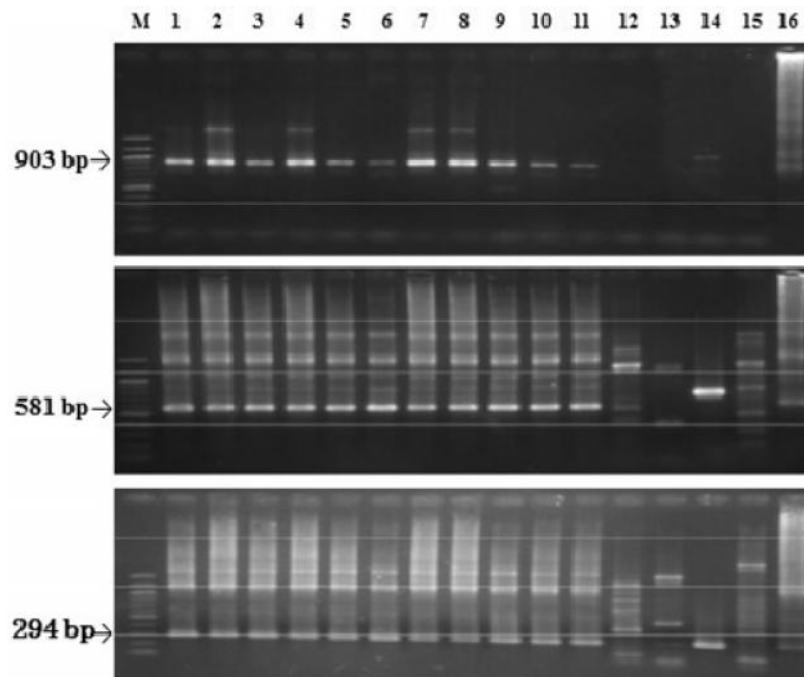
Fig. 6. *ASG-1* specific band patterns in PCR products of transgenic rice plants based on the primers of S1 and A1, S1 and A2, and S2 and A2, respectively (Suppl. file). Lane M: 100 bp ladder; lane 1 to 11: transformant No.; lane 12: guinea grass N68/96-8-4-16; lane13: guinea grass N68/96-8-o-7; lane14: guinea grass N68/96-8-o-11; lane15: guinea grass N68/96-8-o-5; lane16: plasmid (PSMA134S2)

Table 2. Comparative observation analysis of pollens in *ASG-1* transgenic rice with non-graining and graining reported in this study with previously and presently reported pollens in apomictic guinea grass

Methods	Plants	Seed set	Morphologies in pollen developmental stages	
			Pollen in microsporogenesis	Pollen in anthesis
In situ[1]	Guinea grass (N68/96-8-o-11)	Graining	*ASG-1* with strong expression	*ASG-1* with no expression
DIC[2]	Guinea grass (N68/96-8-o-11)	Graining	Near 100% of globular shape with rich cytoplasm	Over 50% of irregular shape with poor or none of cytoplasm
DIC[3]	Transgenic rice (Nipponbare)	Graining	Near 100% of globular shape with rich cytoplasm	Near 100% of globular shape with rich cytoplasm
DIC[4]	Transgenic rice (Nipponbare)	Non-graining	Near 100% of globular shape with rich cytoplasm	Over 60% of irregular shape with poor or none of cytoplasm

[1]*In situ hybridization: Chen et al. 2005;* [2]*DIC (differential interference-contrast microscope): Fig. 8;* [3]*DIC: Fig. 4N;* [4]*DIC: Fig. 4O*

indica rice, respectively. A rapid and efficient *Agrobacterium*-mediated transformation system in rice was then reported by Toki [30] and Toki et al. [22]. In the present study, we followed Toki's protocol to produce the transgenic plants, which performed well in both guinea grass and *japonica* rice.

As a preliminary examination of transformation, two binary vectors of **pIG120Hm/EHA101** and **pSMA35H2-NG/GV3101/PMP90** containing GUS gene were tested for expression rates on calli of both guinea grass and rice. The latter vector provided higher rates than those of the former vector on the two kinds of calli, and the former vector did not work on the three accessions of guinea grass tested (0%), indicating that compatibility existed not only between the binary vector and the donor plant, but also between the binary vectors. The two vectors were usable for the rice transformation system; however, **pSMA35H2-NG/GV3101/PMP90** expressed a

higher efficiency, not only in rice, but also in guinea grass than did **pIG120Hm/EHA101**. This provides important information for further transformation experiments in guinea grass, as up to now, an efficient working protocol for guinea grass has not yet been established for the *Agrobacterium*-mediated system, even though some transformation systems have been working in the other grasses, for example, toll fescue [31].

Similar to the first report, in the present study, the T_0 and T_1 transgenic rice plants of *ASG-1* were successfully achieved, and certified by PCR using the primers designed according to the sequences of *ASG-1*.

However, among T_0 plants, approximately 15% showed NGEP. To date, the low rates of grain formation in transgenic rice were reported in early studies of rice [20,22], mainly due to the antibiotics applied in the recovery process of the culture. In general, the NGEP is considered to be as a consequence of the sterility of female and/or male gametophytes. When we observed the ovary, no abnormal phenomena were evident in embryo sacs at anthesis (data not shown), indicating that the embryo sacs were ready to receive the sperm for fertilization. The female gametophytes are normally formed at anthesis (Fig. 5M) and are not at this point considered to be the factor affecting NGEP in the T_0 plants.

When we continued to observe the male gametophyte of the NGEP, only 38% of the pollen showed a normal nature, which can be described as a sphere shape and containing rich cytoplasm with starch grains. The fact that approximately 62% of pollen was abnormal with irregular and wrinkled shapes and empty cell content without cytoplasm indicates that due to abnormal development, over half of the pollen could not grow sufficiently in size to break out of the anther at anthesis; therefore, the pollen could not exit the anther and reach the stigma for fertilization. The phenomenon of appearance of abnormal pollen is considered to be the reason why the formation of grain was not achieved in the transgenic rice.

To isolate the relationship between the *ASG-1* and NGEP in the transgenic rice, the donor plants of guinea grass accession N68/96-8-o-11, from which *ASG-1* was isolated, were used for observation of male gametophytes. In general, apomictic or facultative apomictic guinea grass is defined as self-sterility [3]. However, a detailed analysis of the sterility has not yet been conducted. We observed the female and male

gametophytes and determined that the normal pollen rates were <40% in the anther at anthesis (Fig. 8), indicating that the <40% of pollen normality may result in its self-sterility in the apomictic guinea grass accession. This result in guinea grass indicates that the same phenomenon appeared in *ASG-1* transgenic rice (Fig. 5N) in the <40% pollen normality (Table 2). An additional report [5] also indirectly found that when *ASG-1* was used as a probe to perform *in situ* hybridization in N68/96-8-o-11, its signals were changed from stronger in the two to four nucleate stage to weaker and/or no signal in the mature pollen stage and/or at anthesis (Table 2). This result may indirectly indicate that *ASG-1* acts functionally in the pollen formation process. The results summarized in Table 2 suggest that the self-sterility in guinea grass might be attributed to pollen abnormality. In addition, it could be considered that the pollen abnormality in the transgenic rice could be due to *ASG-1* playing a role in pollen formation in guinea grass; therefore, during the transformation process, it also acts in the rice and results in abnormal pollen. As a result, the stigma of the ovary in self-pollinating transgenic rice could not receive the pollen from the same flower, meaning that the egg cell could not fuse with the sperm of the pollen, fertilization between the male and female could not be achieved, and the flower would finally become NGEP.

The *ASG-1* gene isolated from facultative apomictic guinea grass (a tetraploid) was transformed into sexual rice (a diploid) showing abnormal pollen, which resulted in NGEP in the present study. In general, whether the gene from a tetraploid, for example *ASG-1*, works normally in a diploid is doubtable. Recently, a new report in apomixis research has emerged concerning the transformation between apomict and sexual plants [10]. When an apospory-specific genomic region (ASGR) isolated from apomict of *Pennisetum squamulatum* grass (4x) was transformed into its sexual form (4x), the gene was expressed in egg cells before fertilization and could induce parthenogenesis and the production of haploid offspring in the transgenic sexual form. This result suggested that the same ploidy level may be necessary for the correct expression of the gene; the gene can work normally in the same ploidy level after transformation. This also hints that the same ploidy level of the gene can work well with normal function. Therefore, the next step is using *ASG-1* on tetraploid sexual plants to examine its function.
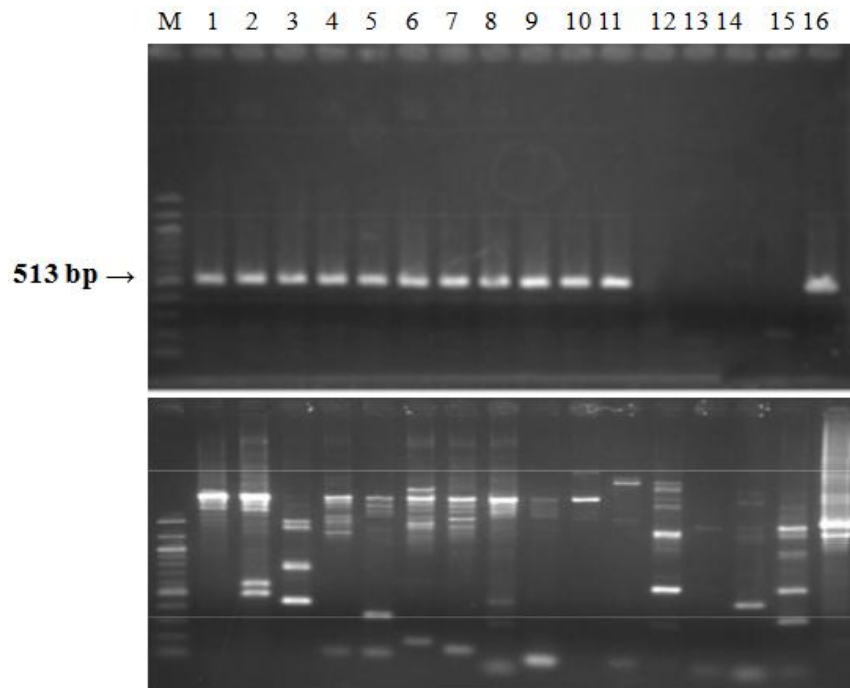
Fig. 7. Hygromycin B specific band patterns, and randomly band patterns in region between Hygromycin B and *ASG-1* in PCR products of transgenic rice plants based on the primers in Suppl. file. Lane M: 100 bp ladder; lane 1 to 11: transformant No.; lane 12: guinea grass N68/96-8-4-16; lane13: guinea grass N68/96-8-o-7; lane14: guinea grass N68/96-8-o-11; lane15: guinea grass N68/96-8-o-5; lane16: plasmid (PSMA134S2)
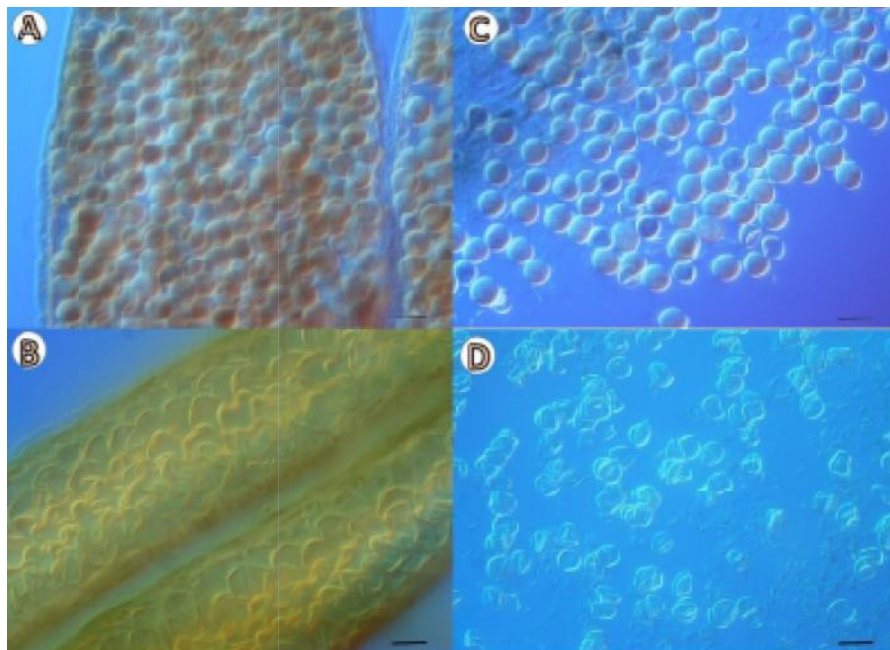

Fig. 8. Observation of pollens of facultative apomictic guinea grass, N68/96-8-o-11. A and B: The pollens in anther and out of the anther 1~2 days before anthesis, respectively; C and D: The pollens in anther and out of the anther at anthesis respectively. Bar = 100 μm in A~D

Another surprising result obtained recently in our laboratory (data not shown) is that when *ASG-1* was transformed into diploid sexual *Arabidopsis*, the apomixis-like phenomenon with multiple embryo sac formation (MESF) in the same ovule appeared. This result suggests that the *ASG-1* gene plays a main role in MESF, and to our knowledge, no study of MESF has as yet been conducted in sexual *Arabidopsis*. According to the above report [10], it can be expected that the *ASG-1* gene will express exactly and provide real morphology when transformed into the same level of tetraploid sexual *Arabidopsis*.

However, as *Arabidopsis* belongs to a different family to that of guinea grass, and is dicotyledonous as opposed to the monocotyledonous form of guinea grass, the *ASG-1* gene should be transformed into the same species of *P. maximum*, namely monocotyledonous, sexual, and tetraploid, for the functional analysis of its gene. To realize this aim, we have successfully established a simple and practical plant regeneration system of guinea grass by using matured seed culture [32]. The project is currently in progress.

5. CONCLUSION

We obtained transgenic rice plants of "Nipponbare" with *ASG-1*, an apomixis-specific gene isolated from apomictic guinea grass, using a combination of the *Agrobacterium*-mediated transformation system and pSMA35H2-NG binary vector. A subset (~15%) of the T_0 *ASG-1* transgenic rice plants with NGEP were determined using DIC, indicating that the plants had higher abnormalities of pollen, showing shapes different from normal and containing empty cytoplasm and rates of <38%, in contrast to normal, and the anther could not be opened normally to allow pollen out. Furthermore, to identify the relationship between the rice and guinea grass, pollen of facultative apomictic guinea grass of N68/96-8-o-11 was also observed, and the same high abnormality of pollen was confirmed, suggesting that *ASG-1* may play a role in pollen development in both rice and guinea grass. As a reference, the previous report showing that *in situ* hybridization of *ASG-1* in N68/96-8-o-11 provided signals gradually changing from strong to weak and no signals in the pollen developmental process also indirectly supports the above result. Comparing these findings with previously reported pollen observations in relation to *ASG-1*, we identified a coincidence for *ASG-1* in pollen of both rice and

guinea grass (Table 2), suggesting that the function of *ASG-1* was transferred from guinea grass to rice through the transformation process. This result indicates that *ASG-1* may play a role in pollen development and may also find applications in the synthesis of artificial male gametogenesis lines to reduce the time and cost to produce F_1 in breeding programs.

NOTE

The other data can be found in attached supplementary files.

ACKNOWLEDGEMENTS

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFRENCES

1. Ravi M, Marimuthu MPA, Siddiqi I. Gamete formation without meiosis in *Arabidopsis*. Nature. 2008;451:1121-1124.
2. Asker SE. Progress in apomixis research. Hereditas. 1979;91:231-240.
3. Nakajima K, Mochizuki N. Degrees of sexuality in sexual plants of guineagrass by the simplified embryo sac analysis. Jpn J Breed. 1983;33:45-54.
4. Nogler GA. Gametophytic apomixis. In: Johri BM (ed): Embryology of angiosperm. Springer-Verlag, Berlin. 1984;475-518.
5. Bicknell RA, Koltunow AM. Understanding apomixis: Recent advances and remaining conundrums. Plant Cell. 2004;16:S228-S245.
6. Chen LZ, Miyazaki C, Kojima A, Saito A, Adachi T. Isolation and characterization of a gene expressed during the period of aposporous embryo sac initial cell appearance in guineagrass (*Panicum maximum* Jacq.). J Plant Physio. 1999; 154:55-62.

7. Chen LZ, Xu CT, Du ZS, Hamaguchi T, Sugita T, Ichikawa H, Guan LM. 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. British Biotech J. 2013;3:458-470.

8. Chen LZ, Guan LM, Seo K, Hoffmann F, Adachi T. Developmental expression of *ASG-1* during gametogenesis in apomictic guinea grass (*Panicum maximum*). J Plant Physio. 2005;162: 1141-1148.

9. Guerin J, Rossel JB, Robert S, Tsuchiya T, Koltunow A. A *DEFICIENS* homologue is down-regulated during apomictic initiation in ovules of *Hieracium*. Planta. 2000;210: 914–920.

10. Conner JA, Mookkan M, Huo HQ, Chae K, Ozias-Akins P. A parthegenesis gene of apomict origin elicits embryo formation from unfertilized eggs in a sexual plant. Proc Nat Acad Sci USA. 2015;112:11205-11210.

11. Chen LZ, Kozono T. Cytology and quantitative analysis of aposporous embryo sac development in guineagrass (*Panicum maximum* Jacq.). Cytologia. 1994a;59:253-260.

12. Yamaguchi-Shinozaki K, Shinozaki K. The plant hormone abscisic acid mediates the drought-induced expression but not the seed-specific expression of rd22, a gene responsive to dehydration stress in *Arabidopsis thaliana*. Mol Gen Genet. 1993;238:17-25.

13. Baumlein H, Boerjan W, Bassuner R, van Montagu M, Inze D, Wobus U. A novel seed protein gene form *Vicia fava* is developmentally regulated in transgenic tobacco and *Arabidopsis* plants. Mol Gen Genet. 1991;25:459-467.

14. Datta N, LaFayette PR, Kroner PA, Nagao RT, Key JL. Isolation and characterization of three families of auxin down-regulated cDNA clones. Plant Mol Biol. 1993;21:859-869.

15. Pessino SC, Evans C, Ortiz JPA, Armstead I, Valle CB, Hayward MD. A genetic map of the apospory-region in Brachiaria hybrids: Identification of the two markers closely associated with the trait. Hereditas. 1998; 128:153-158.

16. Zhang L, Hupel RC, DellaPenna D. The beta subunit of tomato fruit polygalacturonase isoenzame i: Isolation,

characterization and identification of unique structural features. Plant Cell. 1992;4:1147-1156.

17. Chen LZ, Okabe R, Guan LM, Adachi T. A simple and efficient culture of leaflets for plant regeneration in guineagrass (*Panicum maximum*). Plant Biotech. 2002; 19(1):63-68.

18. Chen LZ, Okabe R, Hamaguchi T, Guan LM, Adachi T. Effect of harvest seasons on the efficiency of ovary culture in *Panicum maximum*. Plant Biotech. 2002;19:173-179.

19. Chen LZ, Nishimura Y, Umeki K, Zhang J, Xu CT. Establishment of a Simple Plant Regeneration System Using Callus from Apomictic and Sexual Seeds of Guinea Grass (*Panicum maximum*). British Biotech J. 2015;7:183-190.

20. Hiei Y, Ohta S, Komari T, Kumashiro T. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 1994;6:271-282.

21. Rashid H, Yokoi S, Toriyama K, Hinata K. Transgenic plant production mediated by *Agrobacterium* in *indica* rice. Plant Cell Reports. 1996;15:727-730.

22. Toki S, Hara N, Ono K, Onodera H, Tajiri A, Oka S, Tanaka H. Early infection of scutellum tissue with *Agrobacterium* allows high-speed transformation of rice. Plant J. 2006;47:969-976.

23. Chen LZ, Kozono T. Cytological evidence of seed-forming embryo development in polyembryonic ovules of facultatively apomictic guineagrass (*Panicum maximum* Jacq.). Cytologia. 1994b;59:351-359.

24. Chu CC, Wang CS, Sun CC, Hsu C, Yin KC, Chu CY. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sinica, 1975;18:659-668.

25. Chen LZ, Anami E, Guan LM, Adachi T. Somatic embryogenesis and plant regeneration from leaflets of "Nanou" bahiagrass. Plant Biotech. 2001;18:119-123.

26. Hood EE, Helmer GL, Fraley RT, Chilton MD. The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J Bac. 1986; 168:1291-1301.

27. Murashige S, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant. 1962; 15:473-497.

28. Gotou Y. Extraction method of DNA and RNA for Arabidopsis. In: Okada T, Shimamoto K, Tabata T (eds.): Protocols for model plants (Rice, *Arabidopsis* and Legume). Shujunsha. 2005;90-92. (in Japanese)

29. Herr JM Jr. An analysis of methods for permanently mounting ovules cleared in four-and-a-half type clearing fluids. Stain. Technol. 1982;57:161-169.

30. Toki S. Rapid and efficient *Agrobacterium*-mediated transformation in rice. Plant Molecular Biology Reporter. 1997;15:16–21.

31. Wang ZY. Toll fescue for the twenty-first century (Fribourg HA et al., eds.), Agronomy Monograph. 2009;53:398-406.

32. Nishimura Y, Umeki K, Zhang J, Xu CT, Chen LZ. The functional analysis of apomixis specific genes: Establishment of plant regeneration system using callus induced from seeds of guineagrass (*Panicum maximum*). Bull Minami Kyushu U. 2015;45:9-16.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/16563