Establishing a Rice Calli Subculture System With Long-term Morphogenic Potential

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Summary

This paper describes a subculture system of rice calli (cultivar J-104) that maintains the potential for long-term regeneration. We examined the effects of 2.4-D (1.0, 2.0, 3.0, 4.0 and 5.0 mg/L) and time of subculture on the morphogenesis and regeneration potential. Calli were subcultured for two years on semisolid medium, in the dark. During this time, calli with similar weight were monthly transferred to the regeneration medium, under photoperiod regime. Plant regeneration took place through two morphogenic pathways: indirect somatic embryogenesis and indirect organogenesis. The concentration of 2.4-D used in the callogenesis determined the prevalence of one path or another. In the first ten months of subculture, embryogenesis prevailed over organogenesis. However, from this point to the final stages, the shoots were organogenic. Callus lines retained their regeneration potential by organogenesis until the end of the second year, without presenting a significant number of abnormal plants. These results indicate that it is possible to maintain for two years J-104 rice callus lines potentially morphogenic, which can be applied to a plastid transformation protocol, specifically to the design of an efficient and long-stage selection, for reaching of homoplasmy during the callus period, before the regeneration of the plants.

Keywords: 2.4-D, regeneration, shoots, subculture

Resumen

Establecimiento de un sistema de subcultivos de callos de arroz con potencial morfogénico a largo plazo

Este trabajo describe un sistema de subcultivos de callos de arroz (variedad J-104) que mantienen su potencial de regeneración a largo plazo. Se evaluó el efecto de cinco concentraciones de 2.4-D (1,0; 2,0; 3,0; 4,0 y 5,0 mg/L) y del tiempo de subcultivo sobre la morfogénesis y la regeneración. Los callos fueron subcultivados dos años sobre medio sólido, en la oscuridad. Durante ese tiempo, los callos con peso similar fueron mensualmente transferidos a medio de regeneración bajo régimen de fotoperíodo. La regeneración de plantas ocurrió por dos rutas morfogénicas: embriogénesis somática y organogénesis indirectas. La concentración de 2,4-D en la fase de callos determinó la prevalencia de una ruta u otra. En los diez primeros meses de subcultivos, la embriogénesis predominó sobre la organogénesis. Sin embargo, en las etapas finales, los brotes fueron organogénicos. Las líneas de callos mantuvieron su potencial de regeneración por organogénesis hasta el final del segundo año, sin presentar un número significativo de brotes anormales. Estos resultados indican que es posible mantener por dos años líneas de callos de arroz J-104 potencialmente morfogénicas, lo que puede aplicarse a un protocolo de transformación de plastidios, específicamente para el diseño de una etapa de selección larga y eficiente que permita alcanzar la homoplasmia durante la fase de callos, antes de la regeneración de las plantas.

Palabras clave: 2,4-D, regeneración, brotes, subcultivos

Introduction

In vitro culture is an important component of any genetic transformation protocol, because it provides sources of materials that can be used as recipients of introduced foreign genes. The ability of the targeted plant cell to regenerate into plantlet, and its subsequently developing into a mature plant, is a prerequisite for plant transformation (Evangelista *et al*., 2009). A refined *in vitro culture* system is required for plastid transformation, specifically in rice, which is quite inefficient compared to dicotyledonous plant species. The first reason is the lack of subculture methods with sustained regeneration capability for transplastomic plants. Second, undeveloped plastids (proplastids) are used as the transformation target, rather than chloroplasts. Transcription and translation levels are lower in proplastids than in chloroplasts (Silhavy and Maliga, 1998). The other major difficulty in rice plastid transformation involves the acquisition of homoplasmy, which is of critical importance with regard to genetic stability.

Khan and Maliga (1999) previously reported plastid transformation of rice, demonstrating stable transgene integration in the rice plastome. But the transmission of this transgene to subsequent generations could not be achieved. Lee *et al.* (2006) generated transplastomic rice plants from calli, with stable integration and expression of the *aadA* and *sgfp* transgenes in their plastids. However, it was impossible to convert the transplastomic rice plants to homoplasmy, even after two generations of continuous selection.

To date, the application of selection pressure after the transformed callus has been regenerated into a plantlet, may not be effective for the achievement of homoplasmy. Lee *et al.* (2006) have shown that subculturing process can not be applied to cereal crops for prolonged periods under light conditions, due to the rapid reduction of regenerability in the transformed tissues.

The type and concentration of plant growth regulators in culture medium affect the successful induction of embryogenic tissues. In monocotyledons, it is possible to induce indirect somatic embryogenesis using auxins as promoters of cell undifferentiating. Among these 2.4-dichlorephenoxyacetic acid (2.4-D) is one of the most commonly used growth regulators to obtain embryogenic calli in cereals, at concentrations between 1mg/l and 5mg/l (Mitsuoka *et al.,* 1994; Rueb *et al*., 1994). In the specific case of rice, indirect somatic embryogenesis from mature embryos is induced by the auxin 2.4-D at concentrations between 4.52 and 9.05 M under darkness conditions.

Several plant growth regulators have been used in Cuban rice cultivars (Pérez Bernal *et al.,* 2007, 2008, 2009) and among them 2.4-D has been the most effective auxin for embryogenic callus induction. Nevertheless, the optimum amount of 2.4-D required for long-term callus culture, without affecting the morphogenic response, has not been studied for J-104 cultivar. In addition, there are not previous reports that describe how long can last the morphogenic capacity of these calli.

The goal of this research is to establish a subculture system, applicable to plastid transformation, to obtain rice calli with long-term morphogenic potential for plant regeneration. With this system it could be possible to maintain a large period of selection pressure of calli, for reaching of homoplasmy before the obtaining of the plants. We examined the effects of 2.4-D (1.0, 2.0, 3.0, 4.0 and 5.0 mg/L) and culture duration on the morphogenesis and regeneration potential. Calli were subcultured during two years on callus induction medium with the appropriate concentration of 2.4-D. Callus masses were periodically transferred to the regeneration medium, containing a mixture of cytokinins and auxins, to evaluate the morphogenic response.

Materials and Methods

Seed source

Mature rice seeds, of J-104 rice variety (indica type rice), a commercial Cuban cultivar, which was provided by Rice Experimental Station «Sur del Jíbaro» from La Sierpe, Sancti Spiritus, Cuba.

Sterilization procedure

Selected healthy seeds were manually dehulled and were washed with water, following surface sterilization with a brief (30 s) rinse with 70 % ethanol. The seeds were then rinsed with 2.5 % active chlorine solution for 25 min, in a sterilized flask. The seeds were thoroughly washed six times with sterilized distilled water, and were shifted separately to sterilized filter papers for drying.

Callus culture

The callus induction medium (CIM) consisted of salts and vitamins from N6 (Chu *et al*., 1975), 30 g/L sucrose, 1.0 g/L casein hydrolysate, 2.0 g/L Phytagel, and five concentrations of 2.4-D were proven: 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L. The culture medium was poured into Petri dishes, with 10 seeds per dish. Cultures were maintained in darkness at 28 °C. After 30 days the calli were excised from the seeds and continued growing on CIM with different concentrations of 2.4-D. Every month, 2.0 g of calli were passed to

regeneration medium, and the rest of them continued on fresh CIM.

Before monthly transfer of subcultured calli to regeneration medium, we counted the number of calli with globular embryos, necrosed calli, calli with roots and non-embryogenic calli.

Plant regeneration

The plant regeneration from calli was assessed using MS (Murashige and Skoog, 1962) salts and vitamins, 30 g/L maltose, 3 mg/L kinetin, 1 mg/L NAA, 0.5 mg/L 6-BAP and 4.5 g/L Phytagel. Cultures were maintained in environmentally controlled room under illumination of 1500 lux emitted by fluorescent tubes. Temperature was kept at 25 ± 2 ºC all through the regeneration period.

The average of calli with green spots, calli forming shoots, not responding calli, and average of shoots per callus, were quantified during the first six months, in order to evaluate the effects of 2.4-D concentration on morphogenic potential of calli. From the seventh month onwards, the calli were subcultured on CIM with the optimal concentration of 2.4-D. Regenerated shoots were classified into bipolar (with stem and roots), single-pole (without roots) and abnormal (albinos and/or with atypical forms). Shoots were transferred to flasks containing MS medium (Murashige and Skoog, 1962), 3 % sucrose and 3 g/L Phytagel.

Statistical analysis

All the experiments were conducted in triplicates. The data were analyzed in a completely randomized design using the version 11.5 of Statistical Package for Social Sciences (SPSS). In addition, the comparison of means was carried out using the Student-Newman-Keuls test at 0.05 level of probability.

Results and Discussion

Callus culture

Callus initiation started on the scutellum region of the seed embryo within one week of culture. After 20 days of culture, the calli with an intense yellow colour, small size, and compact and globular form, were recognized as embryogenic calli. While the calli which were loose and wet in texture and white in colour were identified as non-embryogenic calli. Depending on the 2.4-D supplemented on the culture medium, different callus types and callus traits were identified.

The globular embryos average was variable among treatments with 2.4-D. An increase was observed in relation to the augment of 2.4-D concentration from 1 to 2 mg/L (Table 1). The highest average of globular calli resulted from using 2 mg/L of 2.4-D. But the average decreased when the 2.4-D concentration was augmented. The use of 5 mg/L of the auxin caused poor formation of globular embryos, and the maximum amount of roots and non-embryogenic calli.

The presence of roots in the calli was detected in all treatments (Table 1). But the proliferation of rooting was observed as a direct effect of the increase of 2.4-D concentration: calli were not able to sustain cell division and mostly tended to differentiate roots. It is expected that high concentration of auxin cause this unfavourable process during callogenesis.

The use of different concentrations of 2.4-D did not induce the necrosis in calli. Only small parts of incubated calli turned brown a few days before the change to fresh culture medium. Considering all the treatments evaluated, significant differences in the average of necrosis were not observed (Table 1).

The level of 2.4-D supplemented in the callus culture medium significantly affected the type and quality of calli

Table 1. Average of calli with globular embryos, roots, necrosis and non-embryogenic calli at the end of the callus phase. The table shows the means obtained for six months.

2.4-D (mg/L)	Globular embryos	Roots	Necrosis	Non-embryogenic calli
1	19 (a)	6 (a)	5 (a)	1 (a)
2	30 (b)	8 (a)	3 (a)	1 (a)
3	17 (a)	23 (b)	4 (a)	3 (a)
4	20 (a)	27 (c)	5 (a)	3 (a)
5	7 (c)	31 (d)	5 (a)	14 (b)

Means within columns followed by the same letters are not significantly different at 95 % level of confidence by Student Newman Keuls test.

formed. The disadvantage of increasing 2.4-D levels was evident from the negative correlation that was obtained between the 2.4-D concentration and the average of globular embryos. High levels of 2.4-D may cause inhibition of cell division within the explant (Oggema *et al*., 2007). Radhakrishnan *et al*. (2001) reported that cells used up 2.4-D amounts as required, and any excess began to actively show the herbicidal effects, therefore slowing down the callus induction process. Silvertand *et al.* (1996) described that high 2.4-D levels gave wet and soft callus, while low 2.4-D gave compact and embryogenic callus, coinciding with our results. The ability to form embryogenic structure of calli is an important consideration in transformation studies, because it is an indicator of the capacity of the callus to form somatic embryos and regenerate complete plantlets (Evangelista *et al*. 2009).

Regeneration of calli subcultured six months on CIM with different concentrations of 2.4-D

Green spot formation initiated 15 days after incubation of calli on regeneration medium, and shoot differentiation did not occur in a synchronized way. The presence of shoots at different developmental stages at the same time indicates asynchrony of the regeneration process. This event is often reported by Pérez Bernal *et al.* (2007, 2009) for Cuban rice varieties.

Evangelista *et al*. (2009) affirmed that the totipotent cells of the calli follow different pathways of development to regenerate entire plants. Some of them develop plants through somatic embryogenesis, which means that the calli form somatic embryos to generate entire plants. Others plants regenerate by organogenesis, which means that plant organs emerge from the totipotent cells, and develop complete plantlets after the transferring of shoots to root-forming medium. In this study, regeneration took place through two morphogenic pathways: indirect somatic embryogenesis and indirect organogenesis. In the initial phase of regeneration, small shoots emerged out of the somatic embryos and after two weeks of shoot differentiation; root began to bulge out in the form of fine threads which later became prominent and distinguishable. These plantlets had a bipolar morphology, representative of germinated from somatic embryos (Figure 1). Regenerated shoots from non-embryogenic calli had single-pole morphology and they were fused to the maternal tissue, typical of organogenesis (Figure 1).

The best results in regeneration stage, throughout the first six months of subculture on CIM, were obtained in calli from 2 mg/L 2.4-D, an average of 76 of them had green spots, and 72 formed shoots, with seven shoots per calli. After eight weeks on regeneration medium, the percentage of bipolar shoots were higher in respect to single-pole and abnormal shoots (Table 2). In the calli cultivated at concentrations of 2.4-D higher than 3 mg/L, the regeneration process was deprived. Morphogenic response appeared only in small sectors of the compact calli. Calli with green spots and calli forming shoots diminished radically in calli with 5 mg/L 2.4-D, and we obtained an average of 75 not responding calli (Table 2). In this case, the organogenesis prevailed over embryogenesis. Single-pole shoots emerged with difficulty from the masses. In some cases the growth of shoots,

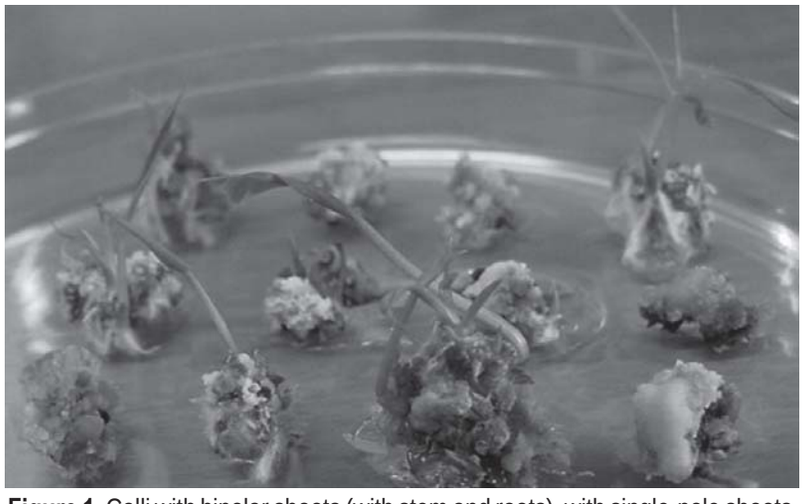

Figure 1. Calli with bipolar shoots (with stem and roots), with single-pole shoots (without roots) and not-responding calli. Time of culture on solid regeneration medium: 30 days.

Table 2. Average of calli with green spots (GS), callus forming shoots (FS), not responding calli (NR), average of shoots per calli (S/C) and shoot morphology (percentage of bipolar, single-pole or abnormal shoots) after eight weeks on regeneration medium. The table showsthe results obtained from calli subcultured six months.

2.4-D in callus subculture	GS	FS	NR	S/C	Bipolar	Single-pole	Abnormal
1	76	72	24	7	61	39	0
2	89	85	11	11	77	23	0
3	58	54	42	4	49	51	0
4	42	37	58	2	33	60	2
5	25	16	75	1	18	66	16

especially those atypically organized, was inhibited. The abnormal shoots were albinos or with strange forms of leaves. The amount of abnormal shoots was notable, probably due to the mutagenic effect of high level of 2.4-D during callus phase.

Mitsuoka *et al.* (1994) affirmed that callus regeneration is produced slowly, since this process initiates as the amount of intercellular 2.4-D in the calli decreases. The auxin is necessary for the formation of rice embryogenic calli, but it negatively affects their redifferentiation process. Rueb *et al.* (1994) informed that the use of high 2.4-D concentrations are necessary to induce somatic embryogenesis in rice, but at the same time they exert an inhibitory effect in shoot regeneration, because regulator residues remain within the embryo cells. After six months, we did not find shoots in callus cultured at concentrations higher than 2 mg/L 2.4-D.

The study was continued over time with calli subcultured with 2 mg/L 2, 4-D, to see how far their morphogenic potential could maintain.

Maintenance and loss of morphogenic potential in calli subcultured two years on CIM with 2 mg/L 2.4-D

The aptitude for morphogenesis from *in vitro* cultures very commonly decreases as the tissues are serially subcultured. The reasons for the loss of regenerative ability may vary under different circumstances: genetic variation in the cell population, epigenetic changes, disappearance of an organogenesis-promoting substance, and the progressive and eventually irreversible loss of totipotency (Gaspar *et al*., 2000). Changes in various metabolic and catabolic reactions in the cells, which are becoming old, are other reasons for losing the ability of proliferation with nodular calli under more passages (Amarasinghe and Yang, 2005).

In the present study, the time of callus subculture on CIM with 2 mg/L 2.4-D was extended until two years. It was observed a homogeneous tendency to decrease in the percentage of calli with green spots, calli forming shoots and average of shoot per callus throughout the months (Figures 2 and 3). Nevertheless, this study has demonstrated that calli maintained their morphogenic ability during two years, despite the well-known tendency for degeneration and disturbances of callus tissue if the culture period is extended. Zhang *et al*. (2000) reported that the majority of transformed wheat callus lines retained regeneration potential for 11 months without neither albinism nor fertility problems. Redway *et al.* (1990) described a similar situation for regenerated wheat plants, with prolonged culture of 3 to 14-month-old scutellum-derived callus lines, from 18 of 39 genotypes of winter wheat.

We observed a distinctive behavior in the morphology of regenerated shoots. In the first ten months of subculture, embryogenesis prevailed over organogenesis, with the regeneration of the majority of bipolar shoots. However, from this point to the last stages, the most parts of shoots had single-pole morphology (Figure 4). Consequently, organogenesis prevails over embryogenesis if the culture period is longer.

The regeneration of bipolar shoots finished at 18 months, and the organogenesis was increased until the 17 month, but then the gradual declination of the percentage of shoots with single-pole morphology started. Finally, in the calli subcultured 24 months on CIM the regeneration did not occur (Figure 4). The continuous use of 2.4-D in the subcultures is necessary for callus proliferation, but at the same time, the prolonged use causes an inhibitory effect in regeneration, probably because regulator residues remain within the cells.

Amarasinghe and Yang (2005) reported that *in vitro* culture responses of old calli of japonica and indica varieties, declined after the second subculture of calli, indicating the loss of plant regeneration from long-term cultured calli. The authors restored the *in vitro* responses by using pre-culture media containing high level of bencilaminopurine, abcisic acid, naphthalene acetic acid, and different kinds of carbon sources before transference to regeneration medium.

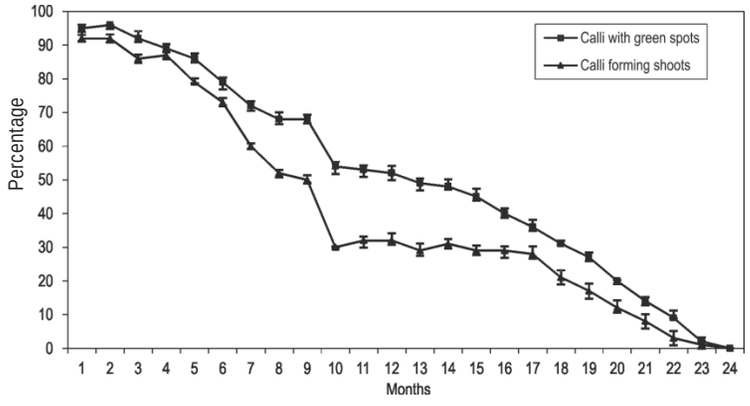

Figure 2. Effect of subculture time of rice calli on callus induction medium with 2 mg/L 2.4-D, on the percentages of calli with green spots and calli forming shoots.

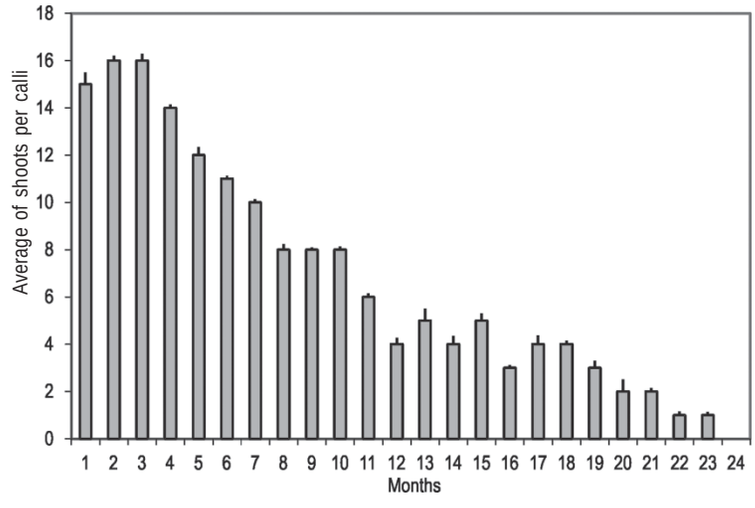

Figure 3. Effect of subculture time of rice calli on callus induction medium with 2 mg/L 2.4-D, on the average of shoots per callus in regeneration stage.

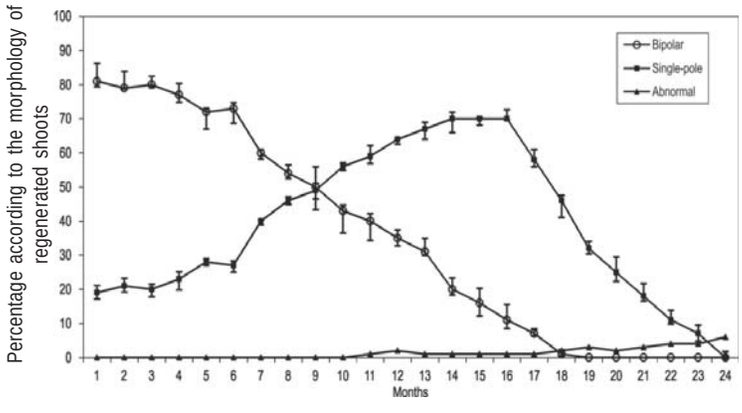

Figure 4. Effect of subculture time of rice calli on callus induction medium with 2 mg/L 2.4-D, on the percentages of shoots according to their morphology (bipolar, single-pole or abnormal shoots).

The low amount of abnormal shoots observed throughout the months indicated that 2 mg/L 2.4-D used in callus culture is not significantly toxic or mutagenic for the cells.

Bipolar shoots and shoots with single-pole morphology were transferred to rooting medium, where they appeared morphologically uniform, with normal roots and leaves, and typical growth pattern (Figure 5). The plants were transplanted to soil under natural conditions, and all of them grew without problems of fertility.

There are some reports about the *in vitro* morphological development of Cuban indica rice (Pérez Bernal *et al.,* 2007, 2008, 2009) describing the somatic embryogenesis from callus and the organogenesis from shoot apical meristems. Taking into account the importance of J-104 as commercial

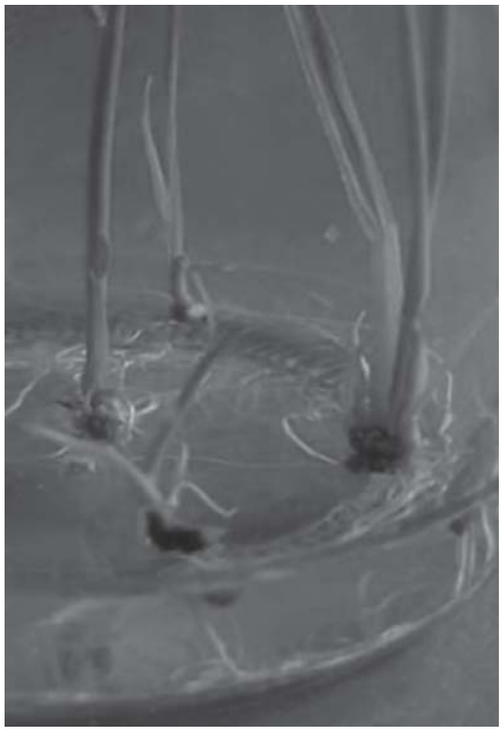

Figure 5. Regenerated plantlets with single-pole morphology growing on rooting medium MS (Murashige and Skoog, 1962).

variety in Cuba, the present work has provided additional data on morphogenic routes in calli of J-104 during 24 months, as well as the characteristic of the regenerated plantlets.

As conclusion, we have achieved a subculture system to obtain rice calli with long-term morphogenic potential for plant regeneration, applicable to rice plastid transformation. Most calli lines retained regeneration potential, in spite of the prolonged period on callus induction medium. These calli are valuable explants for the application of extensive selection process, for reaching of homoplasmy before the obtaining of the transplastomic plants.

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