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RESEARCH ARTICLE

Expression Analysis of miRNA 164c During Rice Regeneration in Different *indica* Rice Genotypes

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Abstract

A highly efficient and reproducible tissue culture regeneration system is an important prerequisite for achieving successful transformation. However, most *indica* rice varieties remain recalcitrant to genetic transformation and require refinement of the tissue culture protocol for the generation of embryogenic calli or regeneration of fertile plants from transformed cells. In this study, standardized regeneration system for *indica* rice cultivars was carried out. Further the expression of miR164c was investigated in the different rice genotypes. The expression of miR164c was proportional to the percentage of regeneration of the rice genotypes. These results provide interesting insights that will significantly improve our understanding of direct regeneration of rice from callus tissue.

Keywords: miRNA, tissue culture, Rice regeneration, Genotypes.

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INTRODUCTION

In vitro tissue culture is widely applied for experimental breeding and biotechnological and genetic manipulation of plants in basic and applied research. Plants can be readily regenerated from cultured tissue; this characteristic is termed totipotency and is a robust tool for genetic engineering (Larkin and Scowcroft, 1981). Although tissue culture techniques are well known with respect to monocotyledonous plants, Graminae species are recalcitrant during *in vitro* manipulation and hence cannot be cultured easily. Moreover, the competency of tissues or cells for regenerating a whole plant is a prerequisite of *in vitro* culture.

Fujiwara and Ojima (1955) cultured excised roots and immature embryos (Amemiya *et al*., 1956, Li-na *et al*., 2010) for the *in vitro* culture of rice. Furthermore, different explants were cultured in different nutrient media augmented with different concentrations of phytohormones (Vasil and Vasil, 1982, Vasil, 1988, and Pipatpanukul *et al*., 2004). As a result, rice became the first cereal that was regenerated into a whole plant (Vasil, 1983). Strikingly, the tissue culture of the japonica variety of rice showed a higher callus yield and frequency of regeneration than those of *indica* (Abe and Futstuhara, 1986).

Embryogenesis is initiated by altered development during the *in vitro* regeneration of plant tissues effectuated by differential expression of genes in somatic cells. However, only a few such molecular pathways have been established in rice. Thus, a map-based cloning strategy was utilized to isolate a major quantitative trait locus (QTL) that encoded a ferredoxin-nitrite reductase, which estimated the regeneration ability in rice (Nishimura *et al*., 2005). This regeneration ability was enhanced by introgression of QTL (Takeuchi *et al*., 2000 and Kwon *et al*., 2001& 2002), *indica*ting the requirement of key loci or genes. *Oryza sativa* L., or rice, has two ecotypes, *indica* and japonica. Hitherto, plant regeneration has been reproducible in *japonica* rice. Although a few *indica* varieties have also been reported to be regenerated, this ecotype is primarily recalcitrant to genetic manipulation (Ignacimuthu *et al*., 2000). Both rice cultivars are regenerated as follows. Briefly, 2,4-D medium is used for the inoculation of dehusked mature seeds, which helps in the formation of embryogenic calli from the scutellum within 3-4 weeks. The culture is supplemented with auxin and cytokinin and incubated in the light. Subsequently, the calli differentiate into green sectors that later form into shoot bud primordia. Additionally, plant regeneration and genotype-dependent variations in *in vitro* callus formation have been detailed (Ge *et al*., 2006). The transcripts that are produced by members of the MIR gene family are processed to the identical or almost identical mature miRNA molecules. Different members of the MIR gene family are expressed in a developmental and tissue-specific manner and in response to various biotic and abiotic stimuli (Zhao *et al*., 2007, 2011; Moldovan *et al*., 2010; Kruszka *et al*., 2014).

Present study was aimed to standardize the callus induction and regeneration protocols for the selected *indica* rice varieties. Additional experiments were carried out to identify the expression pattern of miRNA 164C key molecular regulator underlying the regeneration of rice plants.

MATERIALS AND METHODS

Plant materials used

Rice varieties Pusa Basmati 1, IR64, IRBB60, N22, Lalat, and Basmati 370 were used to carry out tissue culture experiments.

Establishment of culture and explant preparation

The dehusked rice seeds were washed with sterile distilled water to remove the dust and other visible floating particles, followed by surface sterilization with 70% (v/v) ethanol 3 min, rinsed with sterile distilled water 3 times, resterilized with 2% NaOCl for 20 min, and finally rinsed again with sterile distilled water 5 times.

Subsequently, approximately 20–25 seeds were dried using sterile filter papers and inoculated on callus induction medium such that half the embryo was in contact with the media while the other half was exposed to air. The seeds plated on Petri plates (90 mm diameter x 16 mm height) were incubated in either the dark or light at 26°C for 21 days.

In vitro regeneration of plantlets from calli

The diverse combinations of auxin and cytokinins in the MS basal medium would increase the efficiency of the *in vitro* regeneration of rice varieties. The cytokinin BAP (3 mg/L), the synthetic auxin naphthalene acetic acid (NAA) (0.5

mg/L), sucrose (30 g/L), phytagel (0.4%), casein hydrolysate (500 mg/L), and L-proline (500 mg/L) at concentrations comprised the media, which were adjusted for pH and sterilized.

The embryogenic calli were placed in regeneration media and incubated in the dark at 26 ± 2°C for 4-5 days and under fluorescent light at the same temperature for 3-4 weeks. The regeneration efficiency was calculated as follows:

$$\text{Regeneration efficiency} = \frac{\text{No. of calli placed in regeneration media}}{\text{Total No. of plantlets regenerated}} \times 100$$

RNA isolation

Dehusked and sterilized rice seeds were grown on 2,4-D medium for 2 weeks. Subsequently, the calli were transferred to the regeneration medium. The callus and regeneration tissue samples were collected and pooled from four independent experiments and stored at -80°C until RNA isolation. Total RNA was isolated using the Spectrum™ Plant Total RNA Kit according to the manufacturer's instructions (Sigma, USA). DNase treatment of the samples removed any remaining trace DNA. The purity of the RNA was evaluated on 1.2% denatured agarose gels and quantified by a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA).

Expression analysis using semiquantitative RT-PCR

After subculturing once on the maintenance medium for 15 days, the embryogenic calli were transferred to the regeneration medium to initiate differentiation and harvested after 14 days. Total RNA was extracted, and 5 µg was synthesized into cDNA using the Superscript III cDNA Synthesis Kit (Invitrogen, USA). RT-PCR was performed in a 20 µL reaction for the selected genes using Kappa PCR MasterMix (Kappa Biosystems, USA). The primers (Sigma, USA) are listed in Table 1. For miRNA primers were designed according to Wan *et al*., 2010. PCR was performed as follows: initial denaturation at 94°C for 2 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72ºC for 30 s; and a final extension at 72°C for 5 min. The PCR products were analyzed by 2.5% agarose gel electrophoresis, and images were captured using a gel documentation system (Alpha Innotech, USA).

RESULTS AND DISCUSSION

Most *indica* rice varieties are recalcitrant to hereditary control. Accordingly, enhanced tissue culture is a standard practice for embryogenic calli or for the recovery of fruitful plants from a changed cell. Although MS medium is widely used, factors such as explant, carbon source, development controllers, natural enhancements, and gelling operators are necessary for callus growth and plant recovery, particularly in *indica* rice varieties. In addition, a widespread culture medium is not characterized for a random genotype/assortment. Therefore, in this study, we refined the medium by changing the carbon source and expanding the development controllers, amino acids, and gelling operator to produce embryogenic calli for *indica* rice cultivars.

Seed-derived embryogenic calli were transferred to the regeneration medium and maintained under fluorescent light. Consequently, small shoots were produced within 10 days, and after 2-3 weeks, calli were observed to induce a large number of green shoots (Figure 1 and Table 2). The mean frequency of regeneration was found to be highest 82% in the case of Pusa Basmati 1 and lowest 34.3 in lalat variety. Based on the above findings, it can be deduced that an efficient regeneration system is imperative before undertaking any transformation study. A majority of regeneration studies have been conducted in *japonica* because *indica* cultivars are not conducive to tissue engineering (Pandey *et al*., 1994, Seraj *et*

Table 1. List of primers used in the study

S.No.	Primer Name	Sequence
1	U6 forward	5' CGATAAAATTGGAACGATACAGA3'
2	U6 reverse	5' ATTTGGACCATTTCTCGATTTGT3'
3	Mir164c RT	5' CACCGTTCCCCGCCGdUCGGTGGCACGT3'
4	Mir164c qrt F	5' CCCGCCTGGAGAAGCAGG3'
5	Mir164c qrt R	5' GCCGTCGGTGTGCACGTAC3'

Table 2. Percentage of Regeneration in different *indica* rice genotypes

Variety	% of regeneration
IRBB60	59.4±1.84
IR64	64.2±0.95
N22	72.3±1.91
Basmati 370	73±1.78
Pusa Basmati 1	82±1.97
Lalat	34.3±1.8

Values are means of five replications. ± is SE

al., 1997 and Nishimura *et al*., 2005). Additionally, the optimal media composition and culture conditions for efficient plant regeneration are currently under intensive research focus (Khanna and Raina, 1997, Datta *et al*., 1992, Kishore *et al*., 1999 and Lin and Zhang, 2005).

The present results suggest that the regeneration of rice is dependent on the type of calli and the concentrations of auxin and cytokinin in the media (Jain, 1997, Saharan *et al*., 2004, Aygun and Dumanoglu, 2015, Saha *et al*., 2017, Bekircan 2018 and Stevens and Pijut, 2018). The strategies for improving the frequency of plant regeneration in rice have steadily evolved for two decades (Kyozuka *et al*., 1988 and Datta *et al*., 1992). Reportedly, partial desiccation of calli is beneficial for embryogenesis and regeneration in several plant species, including rice.

MIRNA (*MIR*) genes play a vital role in the regulation of the expression of the transcription factor miRNAs. miRNAs are single-stranded RNA molecules 21–24 nucleotides long that regulate the expression of the genes involved in plant development (Bartel, 2009 and Rubio-Somoza and Weigel, 2011). To study the role of miRNA 164c in rice regeneration semiquantitative RT-PCR and realtime PCR were used. RNA from different rice regenerating tissues of different genotypes were analysed for miRNA 164c expression levels. Subsequently, the expression of miR164c was found to be directly proportional to the regeneration percentage of different rice genotypes (Figure 2).

In conclusion the differential regulation of miR164c is effecting the percentage of rice regeneration from callus tissue. These results provide interesting insights that will significantly improve our understanding of direct regeneration in rice from callus tissue.

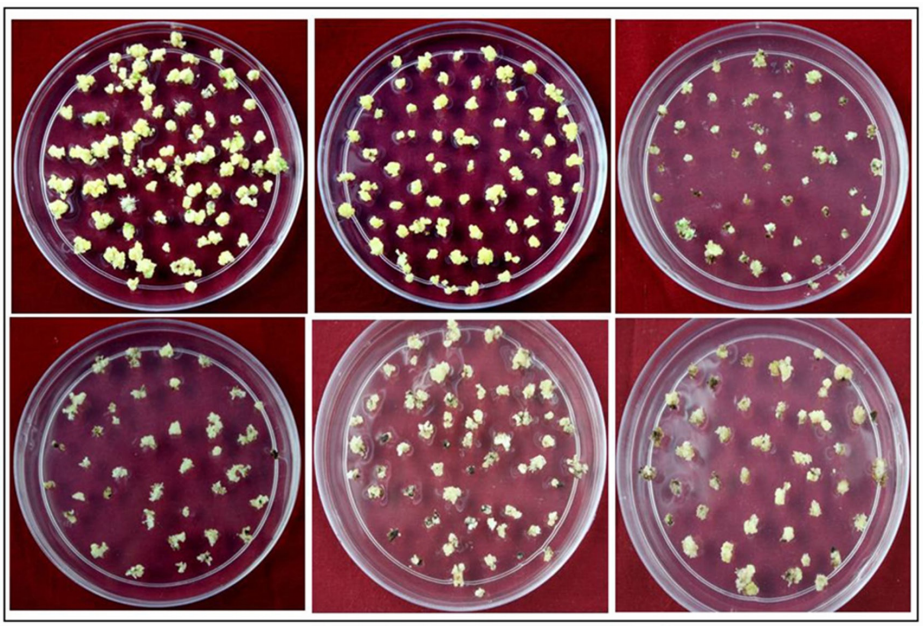

Fig. 1. Rice Regeneration images of different *indica* genotypes A)Pusa basmati 1, B) N22, C) Lalat, D) IRBB60, E) Basmati 370, F) IR64

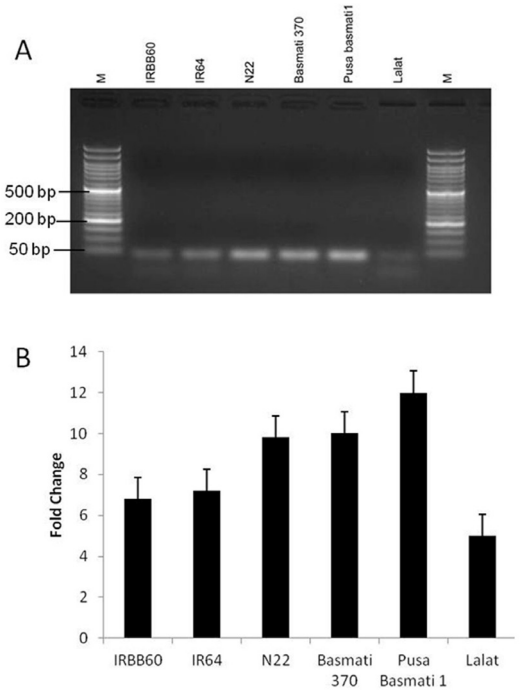

Fig. 2. expression of miRNA 164c in regenerating tissue. A) semiquantitative RT-PCR, B) Realtime PCR analysis

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