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ENHANCEMENT OF PLANT REGENERATION EFFICIENCY FROM MATURE GRAINS OF THAI *INDICA* RICE (*ORYZA SATIVA* L. CV. KDML105)

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Abstract

In vitro protocol for efficient plant regeneration has been developed from Thai *indica* rice cultivar (*Oryza sativa* L. cv. KDML105). Embryogenic callus was initiated from mature seeds on NBMI medium. The highest frequency (96.00%) of callus induction was obtained on NBMI1 medium [2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) without kinetin]. These calli were sub-cultured on NBM medium supplemented with various concentrations of BA or combination with supporting materials for plant regeneration. Plant regeneration from embryogenic callus was increased in the medium supplemented with 5 g L^{-1} Phytagel® in the regeneration medium (NBMR9 medium; 30.00%). In addition, the best regenerated frequency in this experiment was obtained by culturing the embryogenic callus to 4:4 ratios of Gro-lux tubes to fluorescent light tubes at 1000 lux light intensity (56.72%). The regenerated plants were successfully transplanted to soil. The results indicated that manipulation of medium supplements and the cultured condition leads to increase plant regeneration efficiency in KDML105 cultivar for genetic transformation in the future.

Introduction

Rice (*Oryza sativa* L.) is the most important food crop and a primary food source for more than 50% of the world population (Khush & Virk, 2000). The demand for rice is continuously growing with the increasing population, thus genetic improvements of important rice varieties have been targeted. A Thai *indica* rice cultivar, Jasmine rice (Khao Dawk Mali 105; KDML105) is the commercially important rice varieties of Thailand. KDML105 has been very popular in South East Asia and has recently gained wider acceptance in Europe and USA (Leohakunjit & Kerdchoechuen, 2007). Moreover aromatic scent, KDML105 rice has preferable grain characteristics. Although rice grains of KDML105 variety have the high qualities but rice plants are pathogen susceptible phenotypes thus this variety should be improved.

KDML105 is an *indica* variety which has been reported as the recalcitrant varieties for plant regeneration in tissue culture (Khanna & Raina, 1998; Hoque & Mansfield, 2004). However, high efficient protocols of rice regeneration from calli are required for genetic improvement using transformation method. Rice regeneration has been reported from different explants, such as root (Hoque & Mansfield, 2004), coleoptile (Sahrawat & Chand, 2001), anther (Guzman & Arias, 2000), leaf (Oinam & Kothari, 1995; Afrasiab & Jafar, 2011) and mature embryo (Nhut *et al.*, 2000; Islam *et al.*, 2005; Noor *et al.*, 2011). Mature embryo from dry seed has been commonly used as primary explants for callus induction in regeneration process (Karthikeyan *et al.*, 2009). The use of mature embryos in monocotyledons is easy for the manipulation in tissue culture but the low regeneration efficiency has been reported (Sharma *et al.*, 2005). The callus induction and plant regeneration frequencies of explants are influenced by various factors such as the culture methods, the media, the culture conditions (Kyungsoon *et al.*, 2002; Saharan *et al.*, 2004; Ilahi *et al.*, 2005; Rafique *et al.*, 2011). The efficient protocols of rice regeneration should be specifically developed for the particular explants and varieties.

The purpose of this research attempts to develop a reproducible plant regeneration system from mature seed explants. The effects of different type and concentrations of plant growth regulator and supporting materials in media on the regeneration process of mature rice embryo were determined. Moreover, the effects of different light conditions on the culture were observed.

Materials and Methods

Plant materials: Mature seeds of Thai *indica* rice (*Oryza sativa* L.) cv. Khao Dawk Mali 105 (KDML105) were obtained from Pathumthani Rice Research Center, Rice Research Institute, Department of Agriculture and Cooperative, Thailand. Seeds were dehusked and surfaces were sterilized in 70% ethanol for 2-3 min., 5% (v/v) commercial bleach (5.25% sodium hypochloride) for 40 min and 30% (v/v) commercial bleach for 30 min. Then seeds were washed 5 times with sterilized water. In these experiments, rice explants were incubated on NB modified medium [NBM; NB medium (Li *et al.*, 1993) supplemented with 500 mg L^{-1} proline, 3% (w/v) sucrose and 8% (w/v) agar] and incubated at 25±2°C, 85-90% relative humidity for all experiments.

Effect of kinetin additive on callus induction: Embryogenic calli were induced from sterilized mature seeds by culturing on NBM medium containing 500 mg L^{-1} glutamine, 2 mg L^{-1} 2,4-D and without or with 0.5 mg L^{-1} kinetin (NBMI1 and NBMI2 media respectively). The cultures were incubated under dark condition for 4 weeks. Four-week-old callus were determined the percentage of callus induction, size, fresh weight (FW) and dry weight (DW). For DW, callus was dried in 70°C for 24 h and then weight was recorded.

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of seeds produced calli}}{\text{No. of seeds cultured}} \times 100$$

Differentiated calli were subjected to Scanning Electron Microscopy (SEM) to confirm its embryogenic morphology. Callus was fixed in FAA (formalin: ethanol: glacial acetic acid, 1:18:1) for 48 h. The processing started in 50% ethanol and continued through 75%, 95% and 100% ethanol, with 10 min at each step. Samples were then placed in fresh 100% ethanol overnight at 4°C. Samples were brought back to room temperature and critical point dried. The samples were viewed on a Hitachi S-3400N scanning electron microscope (Tokyo, Japan).

Effect of partial desiccation on plant regeneration: Four-week-old calli derived from NBMI1 medium were directly transferred to NBM medium supplemented with 1000 mg L^{-1} yeast extract, 4 mg L^{-1} BA and 1 mg L^{-1} IAA (NBMR2 medium) (non-desiccated callus treatment) or desiccated by transferring to the sterilized Petri dishes containing sterilized Whatman-1 filter papers. The Petri dishes were sealed with parafilm and incubated under dark condition for 7 days. After desiccation, calli were transferred to NBR2 medium. Both treatments were incubated under 16 h photoperiod with 500 lux fluorescent light intensity.

Effect of BA concentration on plant regeneration: Desiccated calli from NBMI1 medium were transferred to NBM medium supplemented with 1000 mg L^{-1} yeast extract, 1 mg L^{-1} IAA and various concentration of BA [NBMR1 (3 mg L^{-1}), NBMR2 (4 mg L^{-1}), NBMR3 (5 mg L^{-1}) and NBMR4 (6 mg L^{-1})] and incubated under 16 h photoperiod with 500 lux fluorescent light intensity.

Effect of supporting materials on plant regeneration: Desiccated calli from NBMI1 medium were transferred to NBM medium supplemented with 1000 mg L^{-1} yeast extract, 1 mg L^{-1} IAA, 5 mg L^{-1} BA using various types or concentrations of supporting materials [NBMR3 (8 g L^{-1} agar), NBMR5 (12 g L^{-1} agar), NBMR6 (16 g L^{-1} agar), NBMR7 (18 g L^{-1} agar), NBMR8 (4 g L^{-1} Phytagel®), NBMR9 (5 g L^{-1} Phytagel®), NBMR10 (6 g L^{-1} Phytagel®) and NBMR11 medium (7 g L^{-1} Phytagel®)] and incubated under 16 h photoperiod with 500 lux fluorescent light intensity.

Effect of light intensity and light quality on plant regeneration: Desiccated calli from NBMI1 medium were transferred to NBMR9 medium and incubated under 16 h photoperiod with different light intensities (500, 800 or 1000 lux) of light fluorescent tubes. For the light quality experiment, the regeneration cultures were incubated under 16 h photoperiod with different light sources using the ratio of Gro-lux tubes to fluorescent light tubes (2:6, 4:4 and 6:2) with 1000 lux light intensity.

$$\text{Plant regeneration frequency (\%)} = \frac{\text{No. of callus produced plants (shoot bud formation)}}{\text{No. of seeds cultured}} \times 100$$

Statistical analysis: The results of each experiment correspond to the average of 4 replications and were recorded every 2 weeks. The mean values were compared by Duncan's New Multiple Range Test (DMRT) or *t*-test and analyzed by SPSS software (SPSS for Windows version 15, SPSS Inc., USA).

Results and Discussion

Effect of kinetin additive on callus induction: To investigate the effect of kinetin additive on callus induction frequency of KDML105 mature seeds, media containing 2,4-D without (NBMI1) or with low concentration of kinetin (NBMI2) were used. In both treatments, the developments of yellow and compact calli were distinct at 5-7 days after being cultured on the media (Fig. 1A). Scanning electron micrograph revealed the calli cultured on the NBMI1 medium were organized nodular-like structures (Fig. 1B) and these structures had bigger size than those cultured on the NBMI2 medium (Fig. 1C). Moreover, this result indicated that callus induction frequency from seed cultured on NBMI1 medium was significantly higher (96%) than that on NBMI2 medium (80%, $p \leq 0.05$). In NBMI1 treatment, the average mass of calli was larger than that of calli in NBMI2 treatment (size, FW and DW; 7.55 mm, 65 mg and 14 mg, respectively) (Fig. 2). 2,4-D, a mimic auxin, at high concentration in media led to develop embryogenic calli from culturing mature seed (Noor *et al.,* 2005; Saqlan Naqvi *et al.,* 2005; Hussain *et al.,* 2010; Noor *et al.,* 2011). The addition of small amount of kinetin (0.5-1.0 mg/l) has been reported to improve embryogenic calli and shoot formation efficiency (Nhut *et al.,* 2000; Afrasiab & Jafar, 2011) including in *indica* rice (Revathy *et al.,* 2000; Bano *et al.,* 2005; Pravin et al., 2011). Contradictory to other *indica* rice, KDML105 callus induced from NBMI2 medium could not regenerate into planlets after culture on regeneration medium (data not show). Genetic variation among *indica* rice may respond differently to plant growth regulators in culture medium.

Effect of partial desiccation on the plant regeneration: Dehydration of callus before transferring to the regeneration medium has been reported to promote plant regeneration frequency, especially *indica* rice callus (Saharan *et al.,* 2004; Ikram-ul-Haq *et al.,* 2009). After 2 weeks incubation of non desiccated and partial desiccated calli on regeneration medium, green spots appeared on the callus (Fig. 1D). Shoot and root formations appeared within 3 weeks after transferring callus to the regeneration medium (Fig. 1E, 1F and 1G). Shoots were clearly identified within 4 weeks (Fig. 1H). The regenerated plants were transferred to soil where they grew well and attained maturity (Fig. 1I). After 4 weeks, the green spots and shoot bud frequencies of desiccated calli (23.33 and 13.33%, respectively) were significantly more than those of non-desiccated callus (18.33 and 6.67%, respectively, $p \leq 0.05$). Therefore, partial desiccation of callus before transferring onto regeneration medium should affect the enhancing regeneration frequency in KDML105 varieties. Partial desiccated callus could be reabsorbed water and nutrients when it was transferred to the regeneration medium (Chand & Sahrawat, 2001; Saharan *et al.,* 2004). Callus dehydration had been reported to induce the biosynthesis of abscisic acid (ABA) which involved in progressing of tissue adaptation under stresses (Srinivas *et al.,* 2006). The proper concentration of ABA can control the embryo formation and maturation (Gawronska *et al.,* 2000).

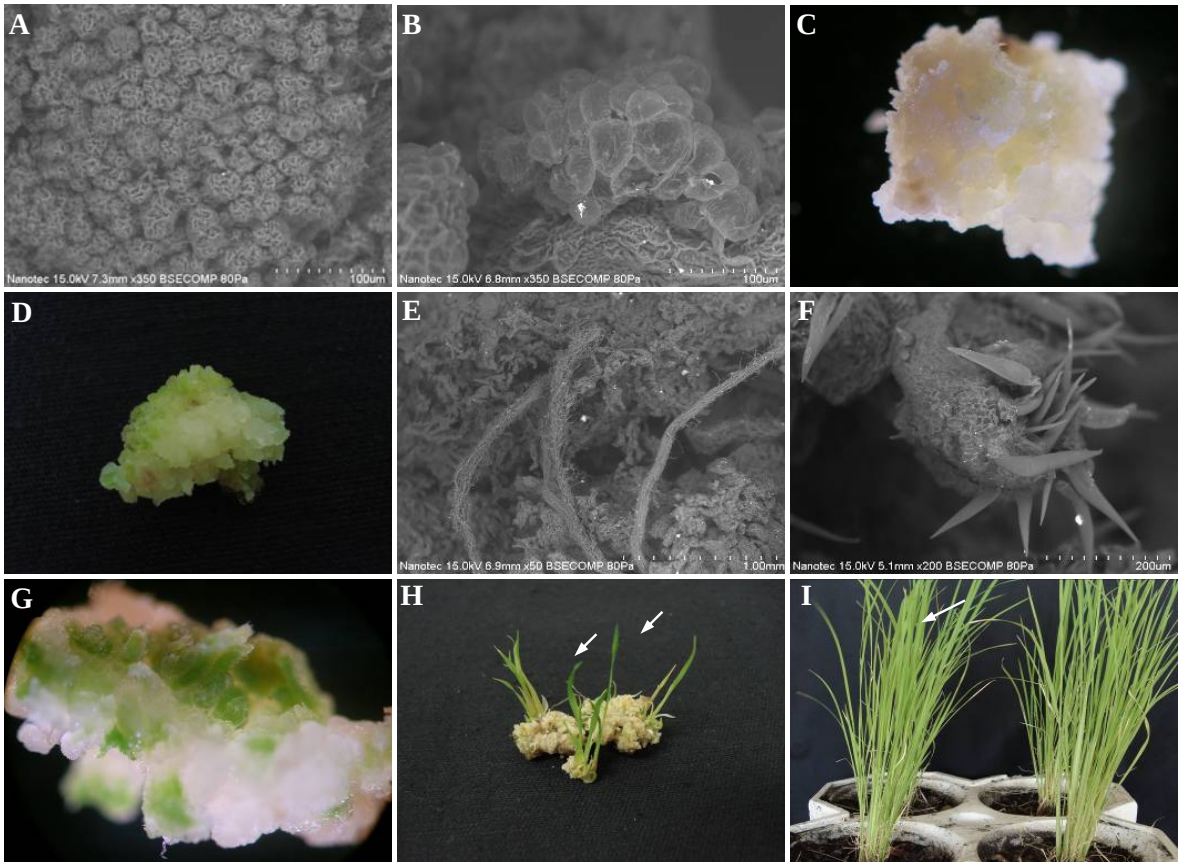

Fig. 1. Callus induction and plant regeneration of Thai *indica* rice (*Oryza sativa* L. cv. KDML105). Callus induction from scutellum of mature seed in NBMI medium (A). Scanning electron microscopic of compact callus structures derived from NBMI1 (B) and NBM2 (C) media after 4 weeks. Characteristic of green spots on the surface of callus after transferring to regeneration medium (NBMR2 medium) for 14 days (D). Roots (E; white point) and shoot buds formation (F; white point) were subjected to scanning electron microscopic after 21 days of transfer to NBMR2 medium. Shoot regeneration of callus on NBMR2 medium for 21 days (G) and 28 days (H). Plantlets of rice from callus were transferred to soil (I).

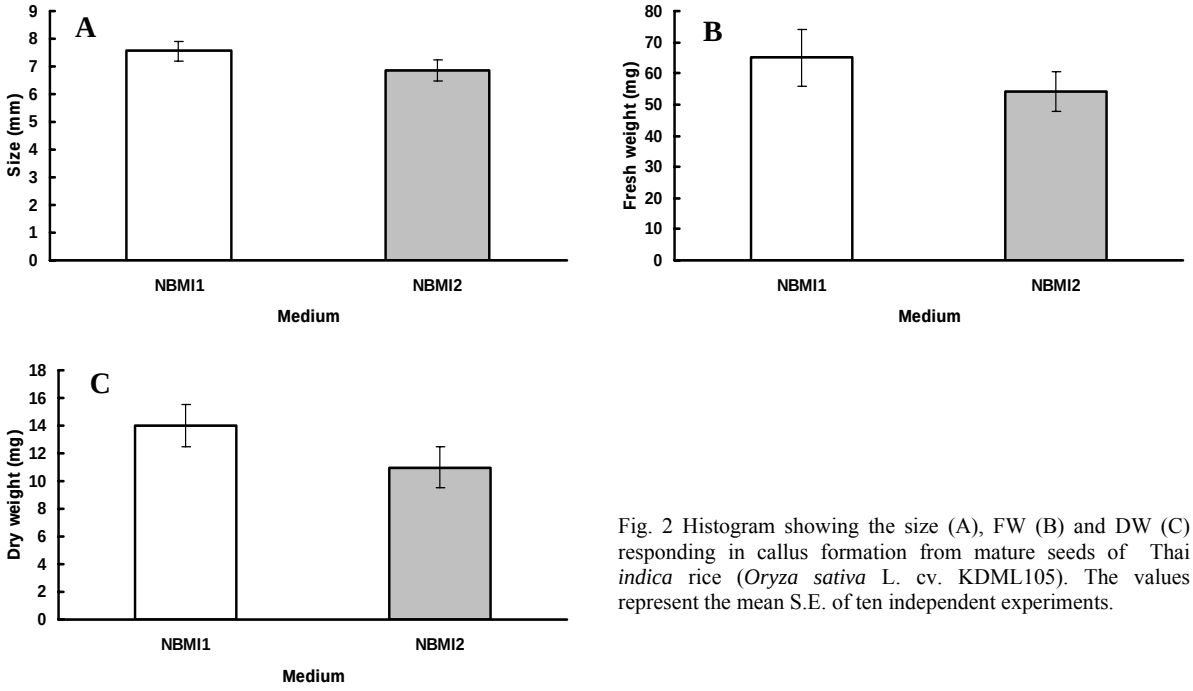

Fig. 2 Histogram showing the size (A), FW (B) and DW (C) responding in callus formation from mature seeds of Thai *indica* rice (*Oryza sativa* L. cv. KDML105). The values represent the mean S.E. of ten independent experiments.

Effect of plant growth regulators on the regeneration medium: We tested the combinations of various BA concentrations with 1 mg L^{-1} IAA in medium on the callus regeneration of rice cultivar KDML105. The result of plant regeneration form callus was presented in Table 1. The average regeneration frequencies of callus derived from media containing 3, 4, 5 and 6 mg L^{-1} BA combination with 1 mg L^{-1} IAA were 7.35%, 13.64%, 20.29% and 10.45%, respectively. The result showed the highest frequency of plant regeneration after transferring callus to regeneration medium containing 5 mg L^{-1} BA and 1 mg L^{-1} IAA (NBMR3 medium). The appropriate concentrations of plant growth regulators used for shoot buds formation from rice were varied from different genotype (Saqlan Naqvi *et al.,* 2006; Rachmawati & Anzai, 2006; Javed *et al.,* 2007).

Table 1. Effect of different concentrations of BA in combination with IAA on regeneration from desiccated Thai *indica* rice (*Oryza sativa* L. cv. KDML105) callus.

Media	No. of seeds	No. of green spots callus	% Green spots callus	No. of shoot buds callus	% Shoot buds callus (the plant regeneration frequency)
NBMR1	68	11	16.18[a]	5	7.35[a]
NBMR2	66	16	24.24[b]	9	13.64[c]
NBMR3	69	32	46.38[c]	14	20.29[d]
NBMR4	67	15	22.39[b]	7	10.45[b]

Data were expressed as the average of four replicates. Values followed by different letters indicating significant differences according to Duncans's Multiple Range Test ($p \leq 0.05$)

Effect of supporting materials on the regeneration medium: The result in Table 2 showed that type and concentration of supporting materials have also effective in promoting green spots and shoot buds of KDML105. Calli cultured on NBMR9 (5 g L^{-1} Phytagel® containing) showed the highest number of green spots and shoot buds per cultured seeds (73.33 and 30.00% respectively). The next orders were the treatment of calli cultured on NBMR2 or NBMR4 media (12 g L^{-1} agar or 4 g L^{-1} Phytagel® containing) which were presented 56.67% and 23.33% green spots and shoot buds per cultured seeds. With a suitable Phytagel® concentration, embryogenic callus were obtained high-frequency green spots and plant regeneration (Lee & Lee, 2003; Ramesh & Gupta, 2005). Phytagel® is one of supporting materials that has been reported to be effective for the transfer of nutrients and maintenance of embryogenic callus in many plant species including *indica* rice (Pons *et al.,* 2000; Garg *et al.,* 2002; Ma & Pulli, 2004).

Table 2. Effect of different types and concentrations supporting materials on regeneration from desiccated Thai *indica* rice (*Oryza sativa* L. cv. KDML105) callus.

Media	No. of seeds	No. of green spots callus	% Green spots callus	No. of shoot buds callus	% Shoot buds callus (the plant regeneration frequency)
NBMR3	60	28	46.67[c]	12	20.00[d]
NBMR5	60	34	56.67[d]	14	23.33[e]
NBMR6	60	20	33.33[b]	10	16.67[c]
NBMR7	60	14	23.33[a]	6	10.00[a]
NBMR8	60	36	63.33[e]	14	23.33[e]
NBMR9	60	54	73.33[f]	18	30.00[f]
NBMR10	60	34	56.67[d]	10	16.67[c]
NBMR11	60	32	53.33[d]	8	13.33[b]

Data were expressed as the average of four replicates. Values followed by different letters indicating significant differences according to Duncans's Multiple Range Test ($p \leq 0.05$)

Effect on light irradiation on the culture condition: The effects of light intensify and light sources on the regeneration of embryogenic callus rice were investigated. As shown in Table 3 and 4, the number of regenerated callus (shoot bud formations) enhanced when light intensity in callus incubation were increased within the range from 500-1000 lux (30.00%, 35.19% and 40.74%, under cultured on 500, 800 and 1000 lux light intensity, respectively). In addition, rice regeneration cultured under the different ratio of Gro-lux and fluorescent lights at 1000 lux light intensity were determined (Table 4). Calli cultured at 2:6 and 4:4 ratios of Gro-lux tubes to fluorescent light tubes, significantly increased green spots and shoot buds (92.31% and 47.69% when used 2:6, 94.03% and 56.72% when used 4:4). The result indicated the ratio of Gro-lux tubes to fluorescent light tubes at 4:4 were suitable for callus regeneration enhancement in KDML105.

The previous researches are reports on the high intensity light-irradiation culture of callus that were beneficial for the formation of compact granular structure cell and the higher regeneration frequency (Liu *et al.,* 2001; Meneses *et al.,* 2005). Not only light intensity is influenced the plant regeneration frequency but also light

source or spectral quality as well. Gro-lux light emits strong light from the deep red and blue regions of the light spectrum which are common light spectrum in plant biosynthesis while fluorescent light provide blue, yellow and green but very little red region (Torne *et al.,* 2001). Thus, Gro-lux light is a one of light source using for plant tissue culture and many research reported the enhancement of shoot and root formation in response to various plant species (Torne *et al.,* 2001; Ascencio-Cabral *et al.,* 2008). In this study, plant regeneration cultured under fluorescent light sources combining Gro-lux light had highly significant effects on plant regeneration frequency when compared to those cultured under fluorescent light source alone.

Table 3. Effect of lights intensity on regeneration from desiccated Thai *indica* rice (*Oryza sativa* L. cv. KDML105) callus.

Media	No. of seeds	No. of green spots callus	% Green spots callus	No. of shoot buds callus	% Shoot buds callus (the plant regeneration frequency)
500 lux	50	37	74.00[a]	15	30.00[a]
800 lux	54	43	79.63[b]	19	35.19[b]
1000 lux	54	49	90.74[c]	22	40.74[c]

Data were expressed as the average of four replicates. Values followed by different letters indicating significant differences according to Duncans's Multiple Range Test ($p \leq 0.05$)

Table 4. Effect of different lights sources on regeneration from desiccated Thai *indica* rice (*Oryza sativa* L. cv. KDML105) callus.

Treatments (the ratio of Gro-lux tubes to fluorescent light tubes)	No. of seeds	No. of green spots callus	% Green spots callus	No. of shoot buds callus	% Shoot buds callus (the plant regeneration frequency)
0:8 (Fluorescent)	65	59	90.76[a]	24	36.92[a]
2:6	65	60	92.31[ab]	31	47.69[b]
4:4	67	63	94.03[b]	38	56.72[c]
6:2	65	61	93.85[b]	35	53.85[c]

Data were expressed as the average of four replicates. Values followed by different letters indicating significant differences according to Duncans's multiple range test ($p \leq 0.05$)

Conclusions

In conclusion, high frequency of green spots and plant regeneration of KDML105 mature rice seeds were achieved by using desiccated callus from NBMI1 medium (2 mg L^{-1} 2,4-D, 8 g L^{-1} agar) and transferred to NBMR9 medium (5 mg L^{-1} BA, 1 mg L^{-1} IAA, 5 g L^{-1} Phytagel®). The culture was incubated at 1000 lux light intensity with 4:4 ratios of Gro-lux tubes to fluorescent light tubes. The regenerated plants had normal growth in green house. Application of this knowledge base should facilitate the generation of transgenic and other biotechnological objectives.

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