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Original Article

Improvement of drought tolerance in Thai rice cultivar RD6 through somaclonal variation

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Abstract

Thai rice cultivar RD6 was induced for drought tolerance through somaclonal variation. Nitsch and Nitsch (NN) medium supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 700 mg/l casein hydrolysate were employed for callus induction and proliferation, whilst the same medium containing 0.5 mg/l naphthaleneacetic acid (NAA) combined with 4 mg/l 6-benzylaminopurine (6-BA) was suitable for plant regeneration. Drought stress was stimulated by treatment with polyethylene glycol (PEG, 0, 0.5, 1, 1.5 and 2% in v/v, MW6000) and/or mannitol (0,150, 300, 450 and 600 mM, MW182.17). The results showed the decreased survival percentage of calli exposed to the increasing PEG or mannitol concentration. Following drought treatment, surviving calli could develop into whole plants when grown on regeneration mediums. RAPD analysis employed to determine the genetic relationship between the control plants and the drought-tolerant plants showed that the control plants and the drought-tolerant plants were genetically different.

Keywords: drought tolerance, RD6, somaclonal variation, polyethylene glycol, mannitol

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important food crops, providing a staple diet for almost half of the world's population (Song *et al.*, 2003). More than 90% of the world's rice is grown and consumed in Asia, where rice is cultivated in 135 million ha with a production of 516 million tons annually (Roy & Misra, 2002). In Thailand, a total of 11.116 million ha are dedicated to growing rice (Office of Agricultural Economics [OAE], 2013) and the major areas are in the north and northeast of the country. Unfortunately, rice yield in these regions is low and fluctuates due to drought (Jongdee, 2003). Drought is regarded as the most important limiting factor that negatively affects rice production, particularly in many rainfed lowland areas all over the world (Duan *et al.*, 2012; Jeanneau *et al.*, 2002). Rice is a paddy field crop that is particularly susceptible to water stress due in part to its small root system, rapid stomatal closure and poor production during mild water stress (Hirasawa, 1999; Tao *et al.*, 2006; Yang *et al.*, 2008). Drought may develop at any time during the growing season. Early season drought occurs in most areas, affecting timely transplanting of seedlings and the growth of direct seeded rice. Late season drought develops in most years at the end of the rainy season before crop maturation, particularly in paddy rice in a high topo-sequence position (Jongdee, 2001). Drought stress suppresses leaf expansion, tillering and midday photosynthesis (Kramer & Boyer, 1995) and reduces photosynthetic rate and leaf area due to early senescence (Nooden, 1988). In addition, water deficit increases the formation of reactive oxygen species

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(ROS) resulting in lipid peroxidation, protein denaturation and nucleic acid damage with severe consequences on overall metabolism (Hansen *et al.*, 2006), leading to a reduction in grain yield. Improvement of rice for drought tolerance by selection under actual field conditions is tedious owing to low heritability and time required. On the other hand, selection for drought tolerance in vitro is recognized as one way to confer better selection efficiency. However, standardized protocols are required for this method. Several substances are used as selection agents for *in vitro* selection, and polyethylene glycol (PEG) and mannitol are amongst the most commonly used ones. PEG and mannitol are a non-penetrable and non-toxic osmotic that are used to lower the water potential of the medium, thus stimulating drought stress in plant (Abdel-Raheem *et al.*, 2007).

This study was carried out to improve drought tolerance in Thai rice cultivar RD6 through somaclonal variation induced by treatment with PEG and mannitol. Random amplified polymorphic DNA (RAPD) analysis was employed to determine the genetic relationship between the control plants and the drought-tolerant plants.

2. Materials and Methods

2.1 Plant materials, callus induction and proliferation, and plant regeneration

Dehusked mature seeds of rice cultivar RD6 were washed with mild detergent and surface-sterilized by soaking in 70% ethanol for 2 min and in 30% Clorox containing the wetting agent "Tween 20" for 30 min. Following three times of washing in sterile water, these seeds were cultured on Nitsch and Nitsch (NN) mediums (Nitsch & Nitsch, 1969) supplemented with 20 g/l sucrose, or Murashige and Skoog (MS) mediums (Murashige & Skoog, 1962) containing 30 g/l sucrose, both of which contained different concentrations (0, 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 mg/l) of 2,4-dichlorophenoxyacetic acid (2,4-D). Effects of proline and casein hydrolysate (0, 100, 300, 500 and 700 mg/l) on callus proliferation were included in the study. The cultures were kept for 3 weeks at 25±2°C under a long photoperiod (16 h light: 8 h dark) with a photon dose of 40 μmol m^{-2} s^{-1}. For plant regeneration, calli were transferred to NN or MS medium supplemented with 0.5 mg/l naphthaleneacetic acid (NAA) combined with different concentrations of benzylaminopurine (6-BA) (0, 1, 2, 3, 4 and 5 mg/l). The cultures were kept for 8 weeks at 25±2°C under a long photoperiod (16 hrs light:8 hrs dark) with a photon dose of 40 μmol m^{-2} s^{-1}.

2.2 Drought stress treatments and measurements

Rice calli were subjected to drought stress on NN or MS medium amended with polyethylene glycol (PEG; 0, 0.5, 1, 1.5 and 2% in v/v; MW6000) or mannitol (0, 150, 300, 450 and 600 mM; MW182.17). Each treatment was performed in triplicate, with five calli per treatment. The cultures were kept for 4 weeks at 25±2°C under a long photoperiod (16 hrs light: 8 hrs dark) with a photon dose of 40 μmol m^{-2} s^{-1}. At the end of the experiment, survival percentage of calli exposed to different concentrations of PEG or mannitol was recorded.

2.3 DNA extraction and RAPD genotyping

Genomic DNA was extracted from young leaf tissues of unstressed tissue culture grown rice plants, field grown rice plants and drought-tolerant rice plants (somaclones) produced through PEG and mannitol treatments, according to the method described by Saghai-Maroof (1984) with minor modifications. The DNA quantity as well as quality was checked by a microplate spectrophotometer. Isolated high quality DNA was diluted to required concentrations for further use. Initially, 36 RAPD primers were screened by amplifying randomly selected rice plants, and then eight decamer primers were used in this study because they could generate clear and reproducible DNA fingerprints. PCR amplifications were carried out in a final volume of 20 μL containing 0.7 μL of template DNA, 2 μL of 10X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM primer, 1.25 U Taq DNA polymerase in a 96 well Thermo Hybaid Thermal Cycler. Each amplification profile consisted of one initial DNA denaturation step at 94°C for 5 min followed by one step at 94°C for 1 min, 36°C for 1 min, and 30 cycles at 72°C for 2 min followed by one step at 72°C for 5 min, and finally held at 25°C. PCR products were separated by electrophoresis on a 1.5% agarose gel and viewed under UV illumination after staining with ethidium bromide. For data analysis, PCR fragments of RAPD with the same molecular weight were scored as identical, and the presence (1) or absence (0) of amplified DNA fragments was determined for each isolate. Only clear, unambiguous and reproducible RAPD markers were used for analysis. A matrix of the different RAPD phenotypes was assembled for analysis. The UPGMA approach was used to construct a dendrogram.

2.4 Statistical analysis

The data were assessed and quantified using one-way ANOVA. Statistical analyses were performed using SPSS (Statistical package for social sciences, version 17.0) computer software (SPSS, Inc., Chicago, IL, U.S.A.).

3. Results and Discussion

3.1 Callus induction and proliferation, and plant regeneration

The results showed that RD6 seed explants produced yellowish compact calli on both mediums. As presented in Table 1 which shows differences in callus size, and fresh and dry weights, NN medium supplemented with 1 mg/l 2,4-D was found suitable for callus induction, giving rise to the maximum callus size of 8.07 mm (Figure 1) with fresh and dry weights

Table 1. Differences in callus size, and fresh and dry weights observed on NN and MS mediums supplemented with different concentrations of 2,4-D.

2,4-D (mg/l)	NN medium			MS medium		
	Callus size (mm)	Fresh weigh (g)	Dry weight (g)	Callus size (mm)	Fresh weight (g)	Dry weight (g)
0.0	0.00±0.00a	0.00±0.00a	0.000±0.000a	0.00±0.00a	0.00±0.00a	0.000±0.000a
0.5	7.87±0.54e	0.13±0.00c	0.019±0.004d	6.80±0.65de	0.09±0.01c	0.010±0.001c
1.0	8.07±0.64e	0.18±0.03d	0.025±0.004e	7.73±0.51fg	0.12±0.02d	0.013±0.002cd
1.5	6.33±0.55d	0.09±0.01b	0.015±0.001c	8.00±0.59g	0.15±0.00e	0.011±0.001c
2.0	6.80±0.40d	0.10±0.01b	0.014±0.001bc	5.73±0.51c	0.10±0.01cd	0.014±0.001d
2.5	3.67±0.36b	0.72±0.01e	0.010±0.001b	7.13±0.46ef	0.10±0.02cd	0.012±0.002cd
3.0	4.47±0.39c	0.08±0.01b	0.012±0.001bc	6.20±0.44cd	0.09±0.00c	0.010±0.001c
3.5	5.13±0.56c	0.08±0.01b	0.012±0.001bc	4.27±0.18b	0.06±0.01b	0.007±0.001b

Values are the mean of three replicates (±SD) of three replications of five seed explants. Values with the same letter in the same column are not significantly different at $p<0.05$ according to LSD test. Results after three weeks of culture.

observed to be 0.18 and 0.025 g, respectively. Similarly, MS medium containing 1.5 mg/l 2,4-D was also effective for callus induction, resulting in the callus size of 8 mm, fresh weight of 0.15 g and dry weight of 0.011 g (Figure 2). Even though these two treatments were both effective, only NN medium amended with 1 mg/l 2,4-D was selected for callus production because it was more cost-effective. Effects of proline and casein hydrolysate on callus proliferation were also investigated. The results demonstrated that both proline and casein hydrolysate enhanced callus proliferation (Table 2). However, casein hydrolysate showed better performance on the proliferation. The maximum callus size of 8.5 mm was observed on NN mediums supplemented with 1 mg/l 2,4-D and 300 mg/l casein hydrolysate, whilst the highest fresh weight of 0.89 g was achieved on the same mediums containing 700 mg/l casein hydrolysate. 2,4-D has been extensively used for callus induction. NN medium supplemented with 0.5 mg/l NAA combined with 4 mg/l 6-BA was most effective in inducing plant regeneration, giving the maximum number of regenerated calli of 24% with total regenerated plants of 35

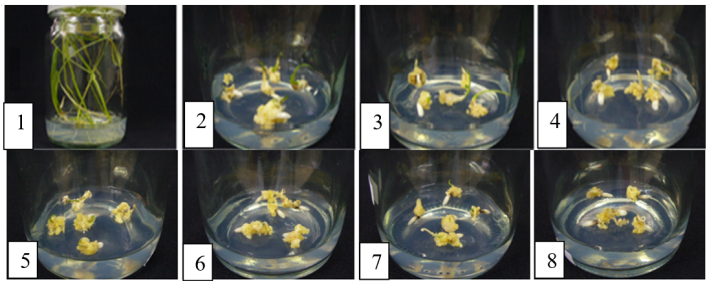

Figure 1. Callus induction of the rice cultivar RD6 seed explants on NN mediums. 1, NN; 2, NN + 0.5 mg/l 2,4-D; 3, NN+ 1 mg/l 2,4-D; 4, NN + 1.5 mg/l 2,4-D; 5, NN + 2 mg/l 2,4-D; 6, NN + 2.5 mg/l 2,4-D; 7, NN + 3 mg/l 2,4-D; 8, NN + 3.5 mg/l 2,4-D.

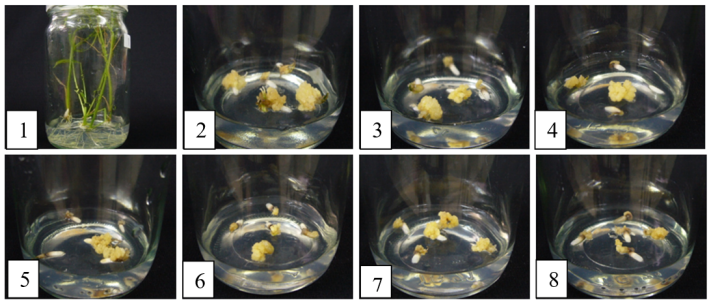

Figure 2. Callus induction of the rice cultivar RD6 seed explants on MS mediums. 1, MS; 2, MS + 0.5 mg/l 2,4-D; 3, MS+ 1 mg/l 2,4-D; 4, MS + 1.5 mg/l 2,4-D; 5, MS + 2 mg/l 2,4-D; 6, MS + 2.5 mg/l 2,4-D; 7, MS + 3 mg/l 2,4-D; 8, MS + 3.5 mg/l 2,4-D.

Table 2. Differences in callus size and fresh weight observed on NN supplemented with 1 mg/l 2,4-D and different concentrations of proline or casein hydrolysate.

Proline (mg/l)	Casein hydrolysate (mg/l)	Callus size (mm)	Fresh weight (g)
-	-	0.49±0.20a	0.83±0.00b
100	-	0.53±0.02ab	0.73±0.00a
300	-	0.67±0.02cd	1.11±0.01e
500	-	0.65±0.02bcd	1.11±0.00e
700	-	0.58±0.01abcd	0.89±0.06c
-	100	0.54±0.01abc	1.28±0.00g
-	300	0.70±0.01d	1.19±0.00f
-	500	0.67±0.02cd	1.20±0.00f
-	700	0.65±0.02bcd	1.07±0.00d

Values are the mean of three replicates (±SD) of three replications of five seed explants. Values with the same letter in the same column are not significantly different at $p < 0.05$ according to LSD test. Results after three weeks of culture.

Table 3. Effects of NAA and BA on plant regeneration efficiency.

NAA (mg/l)	BA (mg/l)	Regenerated Explants (RE %)[a]		Total regenerated plants	
		NN medium	MS medium	NN medium	MS medium
0.5	-	0±0a	0±0a	0±0a	0±0a
0.5	1	12.17±1.53c	16.34±0.99d	7.00±1.00b	19.34±1.18f
0.5	2	12.17±0.55c	16.26±1.01d	8.21±0.92b	14.01±1.76c
0.5	3	12.17±1.48c	16.43±1.80d	12.38±1.15c	33.23±1.74g
0.5	4	24.33±1.53d	12.24±1.52c	35.12±1.67d	11.19±0.80b
0.5	5	4.19±0.85b	8.13±0.85b	1.19±0.35a	16.17±0.66d

Values are the mean of three replicates of three replications of five callus clump explants. Values with the same letter in the same column are not significantly different at $p<0.05$ according to LSD test. Results after 8 weeks of culture.

[a] Regenerated explants RE %: number of callus clump explants producing at least one plant/total number of callus clump explants × 100.

(Table 3). Rueb *et al.* (1994) reported that mediums supplemented with 2 mg/l 2,4-D resulted in high callus proliferation efficiency in the Japonica rice line Taipei 309. Oinam and Kothari (1993) found that 2.5 mg/l 2,4-D was suitable for callus induction in 15 indica rice cultivars using embryos as explants. Darshanie *et al.* (1991) reported that 3 mg/l 2,4-D was optimum for callus induction from seeds of the rice cultivar Bg94-1. Proline and casein hydrolysate have been reported to enhance callus proliferation. Shahsavari (2010) reported that the addition of proline and casein hydrolysate in mediums supported the development of the rice cultivar Selasi tissue. Khaleda and Al-Forkan (2006) tested the efficiency of proline and casein hydrolysate on callus proliferation in five rice cultivars and found that callus proliferation of all rice cultivars was greatly enhanced. Ali *et al.* (2004) reported that casein hydrolysate concentrations of 300-500 mg/l not only inhibited necrosis in the rice cultivar XC95 calli, but also enhanced callus proliferation.

3.2 Callus viability and acclimation to drought stress

The results demonstrated a decrease in the number of surviving calli against the increasing PEG or mannitol concentration. At the end of Week 4, as observed for PEG treatment the lowest survival percentage was found to be 34.26 and 58% on NN and MS medium amended with 2% PEG, respectively, as presented in Table 4. Meanwhile, calli cultured on NN and MS medium supplemented with 600 mM mannitol exhibited the lowest survival percentage of 51.75 and 45%, respectively. It was obvious that callus growth and proliferation were profoundly inhibited when the levels of PEG and mannitol were above 1.0% and 300 mM, respectively.

Table 4. Survival percentage of calli cultured on NN or MS mediums supplemented with different concentrations of PEG and mannitol.

PEG (%)	Mannitol (mM)	NN medium			MS medium		
		Minimum	Maximum	Mean ± SD	Minimum	Maximum	Mean ± SD
-	-	100.00	100.00	100.00±0.00a	100.00	100.00	100.00±0.00a
0.5	-	83.34	92.69	88.00±4.68bc	87.16	96.87	92.00±4.86ab
1.0	-	73.41	85.65	80.00±6.23b	82.99	91.48	88.00±4.45b
1.5	-	62.46	71.59	68.00±4.91d	59.15	66.01	62.00±3.55f
2.0	-	34.26	46.79	40.00±6.33e	58.00	72.55	64.00±7.59ef
-	150	91.58	99.39	96.00±4.02ab	76.00	91.00	84.00±7.55bc
-	300	80.51	96.52	88.00±8.03bc	68.59	83.45	76.00±7.43cd
-	450	60.01	76.00	68.00±8.00d	67.21	77.00	72.00±4.90de
-	600	51.75	74.00	64.00±11.30d	45.00	68.00	56.00±11.52f

Values are the mean of three replicates (±SD) of three replications of five seed explants. Values with the same letter in the same column are not significantly different at $p<0.05$ according to LSD test.
Results after 4 weeks of culture.

Calli that failed to grow were found to be contused and brown in color. Following drought treatment, surviving calli could grow and develop into whole plants (Figure 3). The current study was well-supported by one previous study reporting that development of wheat calli into whole plantlet was hindered when high concentrations of PEG were applied (Barakat & Abdel-Latif, 1995). Another study reported that mannitol concentrations above 300 mM reduced the development of spearmint and peppermint explants into whole plantlets (Faure *et al.*, 1998). Lutts *et al.* (1999) reported that plantlet regeneration percentages in the rice cultivars I Kong, Pao Aiwn, IR2153 and Nona Bokra were reduced when treated with 5% PEG.

Figure 3. RD6 plants obtained from surviving calli, following drought treatment with PEG and mannitol.

3.3 RAPD analysis

Both control plants and drought-tolerant plants were subjected to RAPD using 36 arbitrary primers. Out of the 36 primers used, eight primers revealed polymorphism showing distinctly different banding patterns in the drought-tolerant plants, which were equally prominent in their differences from the control (Table 5, Figure 4). The number of polymorphic fragments between the control plants and the drought-tolerant plants was calculated. A maximum number of six

Table 5. List of RAPD primers tested for their efficiency in generating polymorphism in the control rice plants and the drought-tolerant rice plants.

Primer designation	Primer sequence	Fragment length (bp)	Total no. of bands	Polymorphic bands	% Polymorphism
OPA01	CAGGCCCTTC	300-2,000	7	6	85.71
OPA02	TGCCGAGCTG	700-1,500	4	3	75.00
OPA11	CAATCGCCGT	300-1,200	6	4	66.67
OPA14	TCTGTGCTGG	300-1,700	5	4	80.00
OPA18	AGGTGACCGT	500-1,800	7	4	57.14
OPBH05	GTAGGTCGCA	400-2,000	8	6	60.00
OPBH19	GTCGTGCGGA	300-1,300	7	6	75.00
OPB10	CTGCTGGGAC	300-1,000	5	2	40.00

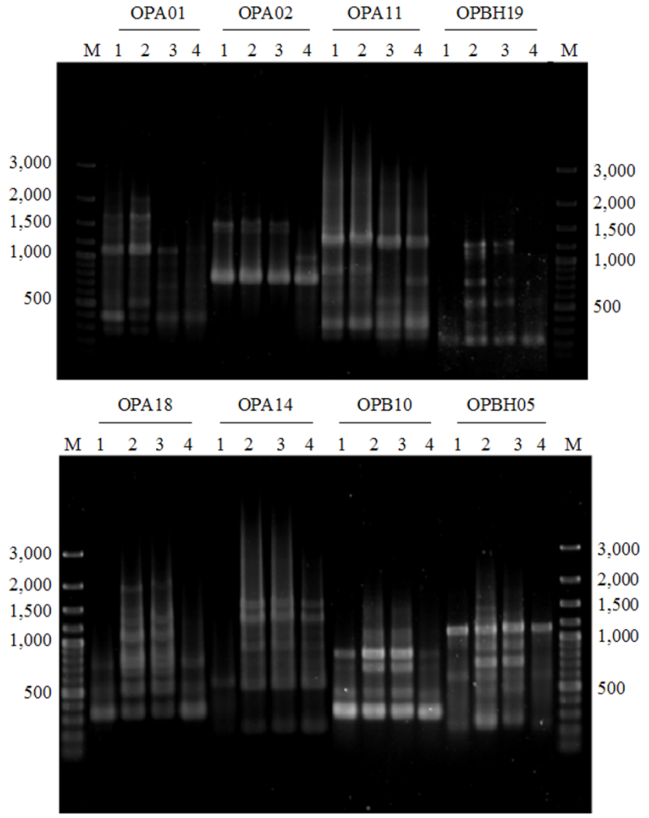

Figure 4. RAPD profiles of 8 selected primers of genetic DNA extracted from rice plants subjected to drought stress. Tracks 1-4 denote profiles of unstressed tissue culture grown rice plant, PEG selected somaclone, mannitol selected somaclone and field grown rice plant, respectively. DNA molecular size marker is represented by M.

polymorphic fragments were obtained between the control plants and the drought-tolerant plants with a minimum number of two polymorphic fragments.

In this experiment, eight of out 36 primers gave 4-8 distinct bands per primer, ranging in molecular size from 300 to 2000 base pairs. A maximum of 6 loci were amplified with the primers OPA01, OPBH05 and OPBH19 while a minimum of 2 loci were recorded with the primer OPB10 with an average of 6.13 bands per RAPD primer (Table 5). UPGMA cluster analysis was employed to assess the genetic relationships between the control plants and the drought-tolerant plants and it was evident that the control plants and the drought-tolerant plants showed genetic differences (Figure 5).

4. Conclusions

In summary, an efficient and reproducible procedure for plant regeneration and selection for drought tolerance in Thai rice cultivar RD6 was developed. Seeds used as explants in this study were suitable for micropropagation.

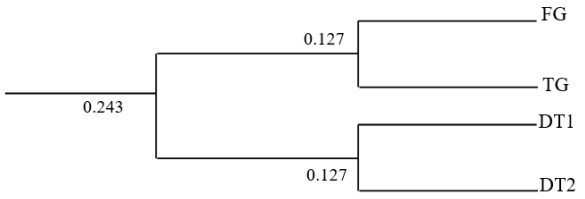

Figure 5. UPGMA-Dendrogram of genetic similarities using RAPD data among tested rice plants. FG: Field grown plants; TG: Tissue culture grown plants; DT: Drought-tolerant plants.

Induction of drought tolerance in the cultivar RD6 through somaclonal variation using PEG or mannitol is simple, cost-effective and easy to handle.

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