Polyamine Plays a Role in Subculture Growth of in Vitro Callus of Indica Rice

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In vitro embryogenic callus is a critical factor for genetic transformation of rice, especially for indica varieties. In this study, we investigated the relationship between polyamines, including putrescine (Put), spermidine (Spd) and spermine (Spm), and callus browning, and we studied the effect of exogenous Put on callus regeneration and on the content of endogenous polyamines. In addition, the expression levels of arginine decarboxylase gene (*Adc1*) and S-adenosylmethionine decarboxylase gene (*Samdc*) in embryogenic callus were studied by quantitative Real-time PCR analysis. The results showed that the contents of endogenous Put and Spd in the browning callus were significantly lower than those in normal callus. Exogenous Put could effectively improve the growing state of callus of indica rice and enhance the development of embryogenic callus. The content of endogenous polyamines in embryogenic callus, especially Spd and Spm, was increased after addition of exogenous Put. Additionally, exogenous Put also had an obvious impact on the expression levels of *Adc1* but partial effect on the expression levels of *Samdc* gene. This study could increase the knowledge of both embryogenic callus induction and polyamine catabolism in callus in indica rice.

Keywords: browning, callus, gene expression, indica rice, polyamine

INTRODUCTION

Plant tissue culture systems have been widely used in many areas of plant science and crop improvement. Successful application of plant tissue culture techniques for crop improvement and transformation needs suitable plant regeneration methods. Generally, it is not easy to culture and regenerate monocot plants, including agronomically important crops such as rice or maize. Up to now, there have been many reports of successful plant regeneration from callus of different rice varieties, nevertheless, information about utilizing tissue culture for rice indica variety is limited (Ge et al., 2006; Rachmawati and Anzai, 2006; Priya et al., 2011; Wani et al., 2011). One of the reasons for this phenomenon was that the embryonic callus of indica rice cultivars was easier to brown than that of japonica rice cultivars, whose genomic background differs from that of indica rice cultivars (Sang and Ge, 2007; Saika and Toki, 2010). As one of important rice cultivars, indica-type rice provides staple food for almost half of the world population (Datta

and Datta, 2006). To satisfy the growing demand of the ever-increasing population, more production improvement and deeper research on indica-type rice are needed. The applicable plant tissue culture methods should be carried out for producing embryonic callus cultures, which are genetically resistant to browning in indica rice.

Polyamine (Pa) is a universal kind of low molecular weight nitrogenous alkali in fat family in plant, including putrescine (Put), spermidine (Spd), spermine (Spm) and cadaverine (Cad). Polyamine controls some processes of development and growth, for example, stimulating cell growth, differentiation and proliferation, accelerating somatic cell embryogenesis, promoting root age and development of flower, boosting up plant resistance to environmental stress (Kusano et al., 2008; Cheng et al., 2009; Fuell et al., 2010). The degradation product of polyamine can also induce hypersensitivity feedback and accelerate the ability of disease resistance in plant (Yoda et al., 2003). In addition, polyamines could delay senescence and

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programmed cell death (PCD) in plants. Therefore, exogenous PA application or PA overexpression in plant can promote somatic cell embryogenesis in callus by changing the level of endogenous hormone, whereas the inhibitor of polyamine can postpone or restrain somatic cell embryogenesis in callus by inhibiting polyamine biosynthesis (Torné et al., 1994; Gray et al., 1995; Takeda et al., 2002). However, little is known about the content changes of endogenous polyamines and the relation between rice callus and endogenous polyamines during indica rice callus subculture.

In plants, arginine decarboxylase (ADC), ornithine decarboxylase (ODC), and S-adenosyl-methionine decarboxylase (SAMDC) are three key enzymes in the endogenous polyamine synthesis pathway (Pillai and Akiyama, 2004; Kusano et al., 2008; Hu et al., 2012). Putrescine could be synthesized directly from ornithine by the action of ODC (Hiatt et al., 1986) or synthesized indirectly from Arginine by ADC (Walden et al., 1997). The function of SAMDC is to provide decarboxylated AdoMet in spermidine biosynthesis for the aminopropyltransferases (Hanzawa et al., 2002). It was reported that ADC may regulate tobacco callus growth during early and late biosynthesis of Put while SAMDC mRNAs are translationally regulated by spermidine and spermine (Takeda et al., 2002; Capell et al., 2004; Pillai and Akiyama, 2004). Despite research efforts, the expression module of endogenous polyamine synthesis genes in indica rice embryonic callus during callus subculture was unclear.

In this study, we detected the content changes of the polyamine (mainly including Put, Spm, Spd) by a HPLC method, and analyzed the state of callus growth during callus subculture to study the relation between endogenous polyamine and callus growth. Then with exogenous Put treatment, the state of callus growth was valued by the sliced tissue and endogenous polyamine was also analyzed by a HPLC method. In addition, mRNA expression levels of *Adc1* and *Samdc* in embryogenic callus were investigated using real-time fluorescence quantitative PCR analysis. This research could be helpful to clarify the mechanism of polyamines regulating rice callus growth, and to further establish efficient callus from mature embryos of indica rice.

MATERIALS AND METHODS

PLANT MATERIAL

Yuetai B (YTB), Honglian-CMS maintainer, a typical *Oryza sativa* subsp. *indica*, was obtained from National Key Laboratory of Hybrid Rice, College of Life Science, Wuhan University, China.

CALLUS INDUCTION AND REGENERATION

Rice callus was induced and regenerated in solid following the method of Lin and Zhang (2005), the basic culture medium was N6 culture medium with 2.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 2.0 mg/l kinetin (Kin), 500 mg/l proline, 500 mg/l glutamine, 800 mg/l casein hydrolysate and 3% maltose, pH 5.9. YTB callus was cultured in the regeneration culture medium with addition of exogenous Put (30 mg/l) for 20 days. All of the calli were cultured in a growth chamber under light $(1800 \ \mu mol.m^{-2}.s^{-1})$ at $28 \pm 1°C$.

HISTOLOGICAL ANALYSIS OF RICE CALLUS

Rice calli were fixed in 4% formamide in PBS buffer overnight, before dehydration through a gradient series of ethanol. The samples were finally embedded in 100% Paraplast (Sigma, St. Louis, MO, USA) at 55–60°C and 7–9 μm thick sections were prepared using a rotary-microtome. Sections were stained using a 0.05% toluidine blue solution and observed through a Nikon ECLIPSE Ti (Nikon, Japan) microscope.

ISOLATION AND MEASUREMENT OF ENDOGENOUS POLYAMINES

The modification method followed the method of Flores and Galston (1982). 1.00 g of callus was homogenized in 5.0 ml 5% cool perchloric acid (V/V), extracted in ice water bath for 1 h, spun at 15,000 rpm for 30 min at 4°C, 500 μL of upper clear aqueous layer was carefully transferred to another 7 ml polypropylene tube, 1.0 ml of 2 mol/l NaOH and 10 μl α-chlorobenzaldehyde were added and the preparation was mixed in vortex for 20 s, incubated at 37°C in water bath for 30 min, 2.0 ml of NaCl saturation solution was added and the preparation was mixed in vortex, then extracted with 3.0 ml of aether, spun at 15,000 rpm for 5 min at 4°C; 1.0 ml of aether phase was transferred to another new polypropylene tube and dried, dissolved with 1.0 ml of methanol and filtrated with 0.22 μm membranes. 20 μl of filtrate was measured by HPLC.

The content of polyamine was measured with Agilent 1200 high performance liquid chromatography (HPLC). The parameters were as follows: Agilent ZORBAX Eclipse XDB-C18 liquid phase chromatogram columniation (4.6 × 150 mm, 5 um), methanol : H_2O (1:1, 7:13, 1:4 and 3:2) for gradient elution for mobile phase, 254 nm wavelength, 1.0 ml/min flow velocity, 25°C temperature of columniation, 30 min of the measurement time. An external standard method was used for chromatographic quantitive analysis.

RNA EXTRACTION AND CDNA SYNTHESIS

RNA was purified from callus at different stages with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions and we removed the genomic DNA using DNase I (TaKaRa, Japan). Total RNA was used as the template for cDNA synthesis with an M-MLV reverse transcriptase kit (TOYOBO, Japan) at 42°C for 1 h.

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS FOR *Adc1* AND *Samdc* GENE

The quantitative PCR was performed in a Rotor gene 2000 PCR system (Qiagen, German) using the following primer pairs: Adc1 gene, ADC1F (5'-AGAAGCTGTCCAAGAGGGTGA-3') and ADC1R (5'-CCGATCAGTTCAAGGACGGC-3'), Samdc gene, SamdcF (5'-GGTTTGAGGGCTATGAGA-3') and SamdcR (5'-AATAGATAAACAGGCTGGACT-3'), for Actin gene, ActinF (5'-TGCTATGTACGTC GCCATCCAG-3') and ActinR (5'-AATGAGTAACCAC GCTCCGTCA-3'). The Actin gene, a single copy gene in the rice nuclear genome, was used as a control for the analysis. The PCR conditions were as follows: 40 cycles of 94°C for 30 s, Tm (Actin 66°C, Adc1 62°C, Samdc 56°C) for 30 s, 72°C for 30 s. Fluorescence was quantified every 1°C from 57°C to 99°C after PCR cycles. In all PCR experiments, amplification of a single sample was confirmed by $2^{-\Delta\Delta CT}$ analysis of real-time PCR and gel electrophoresis of PCR products. All quantitative RT-PCR experiments were performed with biologically independent samples at least three times.

STATISTICAL ANALYSES

Statistical analyses were performed with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) and Means ± SE of three independent experiments with three replicates were shown in the Tables and Figures. Significant differences between the means were determined using one-way analyses of variance (ANOVAs) and the Duncan's test for multiple range comparison with significant level established at $P < 0.05$.

RESULTS

CONTENT OF ENDOGENOUS POLYAMINES

Endogenous polyamines of the callus were analyzed to investigate the relation between rice callus browning and endogenous polyamines. During YTB callus subculture growth, as shown in Fig. 1d, the callus gradually differentiated into two kinds on 15th day: the normal growing callus and the browning callus. Table 1 (N6 CK group) shows that, about 70% of callus appeared brown after being subcultured for 20 days. As shown in Table 2, the contents of endogenous Put and Spd
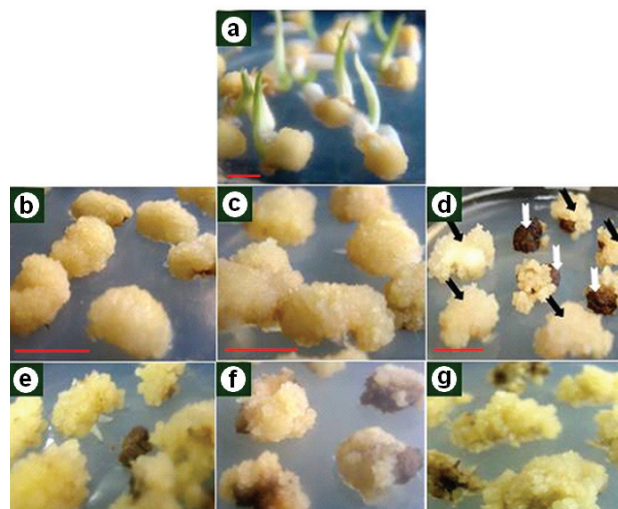

Fig. 1. Representative photographs of indica rice YTB callus in different conditions. (**a**) The indica rice YTB callus (induced after 8 days); (**b**) The indica rice YTB callus as a control (subcultured for 5 days); (**c**) The indica rice YTB callus treated with Put (subcultured for 5 days); (**d**) The indica rice YTB callus (subcultured for 15 days). The normally growing calli are marked with black arrows and the browning calli are marked with white arrows; (**e**) The indica rice YTB callus treated with Put (subcultured for 15 days); (**f**) The indica rice YTB callus as a control (subcultured for 20 days); (**g**) The indica rice YTB callus treated with Put (subcultured for 20 days). Bar = 5 mm.

TABLE 1. The browning status of indica rice callus after being subcultured for 20 days.

Variety	Medium	Callus number	Browning rate (%)		
			First degree	Second degree	Third degree
YTB	N6 (CK)	50	24.40	59.40	16.25
	N6 + Put	50	79.24	13.21	7.55

The first degree means that the callus browning rate is below 50%, the second degree means that the callus browning rate is between 50% and 100%, the third degree means complete browning. The browning rate (%) was the percentage of the browning callus accounted for the total subculture. (n=10)

TABLE 2. Changes in endogenous polyamines content (µg/g) during callus subculture from mature embryos.

Callus	Days	Put	Spd	Spm	Total Pa content
YTB-N	0	208.11 ± 1.73	52.66 ± 1.92	65.40 ± 1.85	319.24 ± 5.46
	5	305.57 ± 1.96	214.65 ± 2.35	77.83 ± 1.90	594.70 ± 5.35
	6	273.24 ± 1.62	220.27 ± 2.05	72.76 ± 1.85	565.14 ± 5.61
	10	242.36 ± 1.68	134.16 ± 1.73	63.53 ± 1.73	438.64 ± 5.32
YTB-N	15	344.96 ± 1.56**	143.48 ± 1.82**	65.84 ± 1.82	557.52 ± 5.21
YTB-B	15	314.75 ± 1.87	110.80 ± 1.76	67.92 ± 1.90	489.21 ± 5.63
YTB-N	20	360.21 ± 1.73**	145.35 ± 1.80**	65.31 ± 1.83	572.43 ± 5.42
YTB-B	20	315.89 ± 1.78	108.40 ± 1.85	67.96 ± 1.79	490.64 ± 5.67

YTB-N – normal YTB callus (not treated with Put); YTB-B – browned YTB callus (not treated with Put);
(* for $P < 0.05$ and ** for $P < 0.01$, based on one-way ANOVA analysis). (n=3)

reached 305.57 µg/g and 214.65 µg/g on 5th day of callus subculture, respectively. On 15th day of callus subculture, the contents of endogenous Put (314.75 µg/g) and Spd (110.80 µg/g) in the browning callus were obviously lower than those of normal callus. Also, endogenous Put (315.89 µg/g) and Spd (108.40 µg/g) in the browning callus were significantly lower than those of normal callus on 20th day. On the other hand, endogenous Spm in the browning callus did not differ from that in normal callus. Therefore, exogenous Put (30 mg/l) was added to rice callus culture to raise the polyamines concentrations and study the effort on rice callus.

EXOGENOUS PUT EFFECT ON THE GROWTH STATE OF CALLUS

In order to obtain better callus and define the effect of exogenous Put on callus during callus subculture, exogenous Put (30 mg/l) was added to the callus subculture medium. The growth state of the subcultured callus was observed to be different in the rice callus treated with exogenous Put from that in the control callus. As shown in Fig. 1, the rice callus treated with exogenous Put (Figs. 1c, 1e, 1g), was more compact, had a faster growth rate and a lower browning rate than that of the control callus (Fig. 1b, 1d, 1f). After 20 days of subculture, the calli treated with exogenous Put showed less browning and a lighter color than the control (Fig. 1g). As shown in Fig. 2, after 20 days of subculture, the embryogenic callus treated with Put had a smaller cell size, was more uniform in cell shape and denser in cell quality, and it was more deeply stained with toluidine blue than the control embryogenic callus. Moreover, according to the browning rate statistics of callus subcultured for 20 days, the callus treated with exogenous Put had a lower browning rate than the control callus (Table 2).

EXOGENOUS PUT EFFECT ON THE CONTENT OF ENDOGENOUS PUT

In order to define the effect of exogenous Put on endogenous Put in callus, the content of endogenous Put was analyzed by HPLC during callus
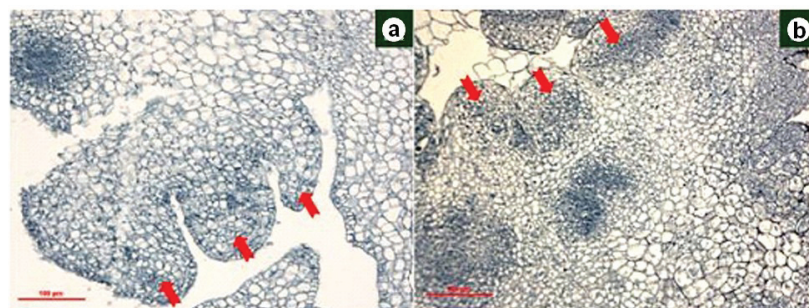

Fig. 2. Representative photographs of the embryogenic callus cells of indica rice. (**a**) The indica rice YTB callus as a control (subcultured for 20 days), the embryogenic cells are marked with red arrows; (**b**) The indica rice YTB callus treated with Put (subcultured for 20 days), the embryogenic cells are marked with red arrows. Bar = 100 µm.

subculture. On 5th day of callus subculture growth, the content of endogenous Put (440.21 μg/g) from the callus with exogenous Put treatment was much higher than that (305.57 μg/g) of the control callus (Table 3). On 15th day, the callus with exogenous Put treatment contained 473.13 μg/g Put and the control callus contained 346.49 μg/g Put. Finally, the content of endogenous Put (483.07 μg/g) from the callus with exogenous Put treatment was much higher than that (380.03 μg/g) of the control callus on 20th day.

EXOGENOUS PUT EFFECT ON THE CONTENT OF ENDOGENOUS SPD AND SPM

On 5th day, the contents of endogenous Spd in callus treated with exogenous Put increased to 263.43 μg/g while that in the control callus was 216.45 μg/g. (Table 3). Then on 15th day, the contents of endogenous Spd in callus treated with exogenous Put reached 287.68 μg/g while that in the control callus just reached 145.94 μg/g. On 20th day, the content of endogenous Spd (247.91 μg/g) in the callus treated with exogenous Put was significantly higher than that (147.67 μg/g) in the control callus. It can be concluded that exogenous Put could increase the content of endogenous Spd in the callus during callus subculture growth. Meanwhile, the content of endogenous Spm also increased in the callus treated with exogenous Put during callus subculture growth (Table 3) and reached the highest peak at 238.24 μg/g on 15th day.

EXOGENOUS PUT EFFECT ON THE EXPRESSION LEVEL OF *Adc1* AND *Samdc*

To further find out the exogenous Put effect on the expression of arginine decarboxylase gene *Adc1* and S-adenosylmethionine decarboxylase gene *Samdc* in callus subculture, the expression level of *Adc1* and *Samdc* gene was analyzed in the subculture at different stages (Figs. 3 and 4). Under exogenous Put (30 mg/l) treatment, the expression level of *Adc1* was significantly raised in callus after 10 days, while the expression of *Adc1* of the control callus was significantly raised after 20 days (Fig. 3).
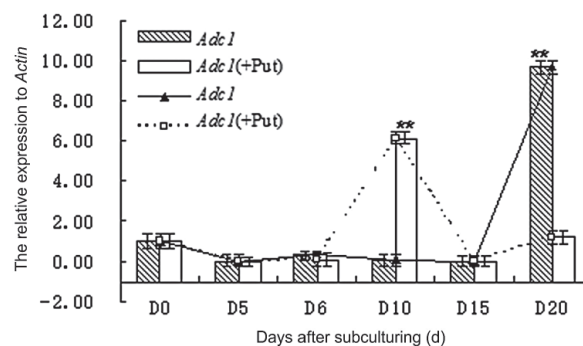

Fig. 3. Quantitative expression analysis of *Adc1* in subcultured callus of indica rice at different stages (n=3). Asterisks indicate that the difference in the value of relative expression between Put-treated callus cultures and the corresponding control is significant (* for $P < 0.05$ and ** for $P < 0.01$, based on one-way ANOVA analysis).

Table 3. Changes in endogenous polyamines contents (μg/g) during callus subculture with exogenous Put treatment.

Callus	Days	Put	Spd	Spm	Total Pa content
YTB-N	0	211.11 ± 1.65	55.66 ± 1.86	64.30 ± 1.83	331.08 ± 5.34
	5	305.57 ± 1.96	216.45 ± 2.60	78.23 ± 1.95	834.90 ± 6.25
	6	265.81 ± 1.54	226.19 ± 1.95	72.76 ± 1.85	654.14 ± 5.61
	10	222.66 ± 1.59	136.27 ± 1.75	61.71 ± 1.84	420.64 ± 5.17
	15	346.49 ± 1.53	145.94 ± 1.71	66.33 ± 1.80	558.77 ± 5.05
	20	380.03 ± 1.66	147.67 ± 1.74	64.96 ± 1.81	592.66 ± 5.21
YTB-Put	0	213.98 ± 1.79	160.61 ± 1.90	134.84 ± 2.02	509.43 ± 5.71
	5	440.21 ± 1.70	263.43 ± 2.24	179.96 ± 2.40	878.96 ± 6.60
	6	355.18 ± 1.82	184.86 ± 1.87	187.48 ± 2.27	638.15 ± 5.68
	10	313.38 ± 1.50	278.95 ± 1.76	211.92 ± 1.99	804.25 ± 5.24
	15	473.13 ± 2.18	287.68 ± 2.07	238.24 ± 1.91	999.05 ± 6.15
	20	483.07 ± 1.68	247.91 ± 1.90	217.56 ± 2.18	948.54 ± 5.76

YTB-N – normal YTB callus (not treated with Put); YTB-Put – YTB callus treated with Put. (n=3)

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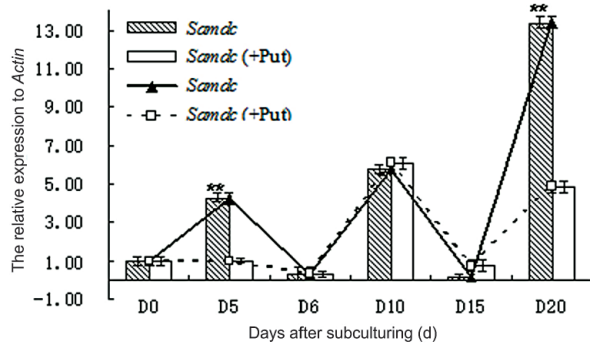PAN
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Fig. 4. Quantitative expression analysis of *Samdc* in subcultured callus of indica rice at different stages (n=3). Asterisks indicate that the difference in the value of relative expression between Put-treated callus cultures and the corresponding control is significant (*for $P < 0.05$ and **for $P < 0.01$, based on one-way ANOVA analysis).

During YTB control callus subculture, the expression of *Samdc* gene of the control callus was significantly raised on 5th, 10th, and 20th day. Meanwhile, the expression of *Samdc* gene was also raised on 10th day in the callus subculture with exogenous Put, while the content of endogenous Put, Spd and Spm also increased on 10th day (Table 3). However, there was no perceptible change of *Samdc* gene expression in rice callus treated with exogenous Put.

DISCUSSION

Rice callus induction culture is one of the key steps in rice callus subculture, and the browning of rice callus could be related to the quantities of endogenous hormones (Priya et al., 2011; Wani et al., 2011). Putrescine (Put), as a kind of polyamines in plants, could protect plants from abiotic and biotic stresses (Bais and Ravishankar, 2002). In this study, the content of endogenous Put in the browning callus was obviously lower than that of normal callus during YTB callus subculture growth. In this study we also tried to add different concentrations of exogenous Put to improve the rice callus growth (data not shown). The results showed that exogenous Put caused a marked improvement in rice callus growth and reduction of callus browning. From all these data, it was concluded that exogenous Put was helpful in the callus subculture and embryonic callus could show a lower browning rate than the control callus. It was also suggested that Put could be considered as a growth regulator of YTB callus. Therefore, it could increase the regeneration capacity of rice callus and reduce the callus browning

effect. However, there were also reports that the different genotypes or physiological status of plants or explants in the process of somatic embryogenesis did not have the same demand for the polyamines (Kakkar et al., 2000), and this kind of phenomenon did not appear in this research.

As for plant polyamine biosynthesis, Put is the diamine precursor and promotes the synthesis of endogenous polyamines Spd and Spm (Tiburcio et al., 1997; Bouchereau et al., 1999). In this study, the content of endogenous Spd in the browning callus was also lower than that of normal callus during YTB callus subculture growth, while the content of endogenous Spm did not significantly differ in the browning callus and the normal callus. After exogenous Put was added to rice callus culture, the content of endogenous Spd and Spm was correspondingly increased. This showed that exogenous Put treatment led to increase of the content of endogenous Put in callus during rice callus subculture growth. Thus, it was suggested that Put in YTB callus could be used as the precursor to promote the synthesis of endogenous polyamines Spd and Spm. On the other hand, it is reported that spermidine (Spd) could also protect plants from abiotic and biotic stresses (Shi and Chan, 2014). Exogenous Put and Spd have been reported to be helpful to recover browning tissues into normal callus cultures and improving plant regeneration by acting as plant growth substances (Tang et al., 2004). In this study, the content of endogenous Spd was increased by exogenous Put treatment and this implied that the increasing Spd could also act as a growth regulator of YTB callus and promote the regeneration capacity of rice callus. Therefore, the function of Spd in YTB callus needs to be analyzed in a further study.

In plants, *Adc* and *Samdc* genes had different expression patterns under different abiotic stresses, while rice *Odc* gene has not been confirmed yet (Alcazar et al., 2006; Kusano et al., 2008). In this study, the mRNA levels of rice *Adc1* gene were in agreement with the alterations of endogenous Put content in callus growth, implying that *Adc1* gene was induced by exogenous Put, which agrees with other reports (Liu and Moriguchi, 2007; Liu et al., 2009). As for *Samdc* gene, it was reported that higher levels of putrescine could induce *Samdc* gene expression and promote spermidine and spermine synthesis, accordingly (Capell et al., 2004). In YTB control callus culture, *Samdc* gene expression was induced at the beginning and the end of callus culture (5th, 10th, 20th day). This could be like a response to starvation stress caused by callus culture, since this kind of induction has been mentioned for a variety of plants such as peach (Liu and Moriguchi, 2007), soybean (Tian et al., 2004), and *Arabidopsis* (Urano

et al., 2003). Due to inadequate endogenous Put in YTB control callus, the content of endogenous spermidine and spermine did not increase with *Samdc* gene expression induced on 10th or 20th day. By contrast, in callus treated with exogenous Put, which had higher levels of putrescine, the content of endogenous spermidine and spermine was remarkably increased with *Samdc* gene expression induced on 10th day. On the basis of the above data, it was suggested that exogenous Put could induce *Adc1* gene expression and partly affect the expression levels of *Samdc* gene, and then accelerate the synthesis of endogenous polyamines in YTB callus.

CONCLUSIONS

During the callus subculture growth, the content of endogenous Put and Spd in the browning callus was much lower than that of normal callus. It was suggested that endogenous Put and Spd could be related to browning of rice callus. Exogenous Put (30 mg/l) was added to indica rice callus subculture medium to improve the quality of the embryo callus and investigate the effect of exogenous putrescine during rice callus growth. The results showed that exogenous putrescine could effectively improve the growing state of indica rice callus and enhance the callus embryogenic traits. The content of endogenous polyamines in embryo callus, especially for Spm and Spd, was increased by exogenous putrescine during the callus subculturing. As for two key polyamine biosynthetic enzyme genes, *Adc1* and *Samdc*, exogenous putrescine had an obvious impact on the expression levels of *Adc1* gene and a partial effect on the expression levels of *Samdc* gene. Exogenous Put treatment could be a feasible method of improving the quality of the embryo callus in indica rice. These results could increase the knowledge of both indica rice embryogenic callus induction and polyamine catabolism in rice callus growth.

AUTHORS' CONTRIBUTIONS

YP Tan and G Cheng designed the research experiments. YP Tan, W Hu, X Xu, J zhou, CT Wang, XQ Liu and G Cheng performed the experimental research. YP Tan, X Xu, and G Cheng analyzed the data. YP Tan and G Cheng prepared and wrote the manuscript. The authors declare that there are no conflicts of interest.

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