



Quantitative trait loci for mature embryo culturability traits from Yuanjiang common wild rice (*Oryza rufipogon* Griff.)

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Abstract

Low callus subculture and regeneration ability are the main factors restricting the genetic transformation potential of *indica* rice varieties. In the present study, an *Oryza rufipogon* introgression line population in the background of *indica* cultivar 93-11 was used to evaluate mature embryo culturability after callus induction, two-round of subculture, and differentiation of culture. A total of 25 quantitative trait loci related to tissue culturability were detected that included QTLs for callus browning index after one- and two-round subculture and callus regeneration rate. Among them, QTLs detected at the RM335 locus on chromosome 4 were found to control callus browning index after one- and two-round subculture and accounted for 11 and 15 % of the phenotypic variation. QTLs detected at the RM341 locus on chromosome 2 and at the RM8206 on chromosome 9 were found to control regeneration rate with 10 % contributions to the observed variation, respectively. The alleles of *O. rufipogon* were found to reduce callus browning and were possibly involved in the improvement of callus differentiation ability. Six introgression lines with excellent culturability characteristics were identified. It is expected that these lines will be useful materials for genetic transformation and fine mapping of culturability-trait.

Keywords: Common wild rice, callus browning, callus regeneration, QTL, genomic region

Introduction

Compared with *japonica* subspecies, callus of *indica* rice is typically susceptible to browning during subculture, leading to a reduction in its differentiation ability. Genetic transformation of *indica* rice has been confined to a limited number of varieties (Rashid et al. 1996; Lin and Zhang 2005; Hiei and Komari 2006; Sahoo et al. 2011; Shri et al. 2013). A highly efficient

tissue culture system suitable for genetic transformation has not yet been established for most of the *indica* varieties. Since rice mature embryos are easy to use and have no seasonal restriction, they are widely used in tissue culture. Reports on analyses of quantitative trait loci (QTLs) using rice mature embryos include studies on callus induction (Kwon et al. 2001; Taguchi-Shiobara et al. 2006; Zhao et al. 2009; Li et al. 2013), callus proliferation (Taguchi-Shiobara et al. 2006; Li et al. 2007; Zhao et al. 2009; Li et al. 2013), and callus regeneration (Taguchi-Shiobara et al. 1997; Takeuchi et al. 2000; Kwon et al. 2001; Nishimura et al. 2005; Ozawa and Kawahigashi 2006; Zhao et al. 2009; Li et al. 2013). Using a mature embryo culture system and map-based cloning, Nishimura et al. (2005) mapped a main effect QTL on the short arm of chromosome 1; this QTL, which controlled rice regeneration ability, was associated with a gene encoding a ferredoxin-nitrite reductase.

Several researchers have developed introgression lines from *Oryza rufipogon* and *O. sativa* hybridization for the purpose of detecting and mapping of quantitative traits loci by different methods (Tan et al. 2007). Previous genetic analyses of rice culturability have mostly involved cultivated rice, with limited studies using common wild rice (*Oryza rufipogon* Griff.). Common wild rice, the wild progenitor of cultivated rice, possesses an important gene pool that includes several genes no longer present in cultivated rice (Sun et al. 2001). QTLs identified in common wild rice that are beneficial for improving rice varieties

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include QTLs for yield improvement (Li et al. 2002; He et al. 2006), disease resistance (Huang et al. 2001), drought tolerance (Zhang et al. 2006), and cold tolerance (Liu et al. 2003).

Mature embryos of an *O. rufipogon* introgression line population in the background of *indica* cultivar 93-11 as explants were used for the mapping of QTLs for genetic transformation efficiency from common wild rice. The present study focused to measure and analyse the QTLs for callus browning index (CBI) after two-round subculture and plant regeneration rate (RR) and to find out molecular markers for the screening and improvement of rice tissue culturability traits, which may be useful for the cloning of related genes. In addition, introgression lines identified as having high tissue culturability in this study represent excellent plant material for genetic transformation.

Materials and methods

Plant materials

To assess culturability, 127 *O. rufipogon* introgression lines in the background of *indica* cultivar, 93-11 (BC₄F₄, henceforth called "9YJ") were selected from 354 lines on the basis of chromosomal genotype distributions. The introgression line was constructed using an *O. rufipogon* accession collected from Yuanjiang County and Yunnan Province, China as the donor and the elite *O. sativa indica* cultivar 93-11 as the recipient (Fu et al. 2010).

Callus subculture and regeneration

After hulling, 90 completely healthy mature seeds for each line were sterilized with 70 % ethanol for 1-2 min and then 25 % sodium hypochlorite for approximately 30 min. The seeds were rinsed three times with sterilized water, placed on induction medium in three dishes (30 seeds per dish), and cultured. After incubation in darkness at 27°C for 12 days, the calli were separated from the seeds, transferred to subculture medium, and incubated in darkness at 27°C for 25 days. Calli of similar size were selected for a second subculture round. At 25 days post-subculture, calli of uniform size were chosen and transferred to regeneration medium; these were maintained under 16-h light/8-h darkness at 27°C for 30 days. The traits of the calli in each dish were investigated and recorded.

Media and Investigation of callus culturability

Callus culture (CC) basic medium, prepared according to Portrykus et al. (1979), consisted of CC

macroelements, CC microelements, and CC organic components supplemented with mannitol (36.43 g/L), maltose (20.0 g/L), and Phytigel (sigma) (3.0 g/L) adjusted to pH 5.8. To obtain induction and subculture medium, CC basic medium was supplemented with 2 mg/L 2, 4-dichlorophenoxyacetic acid. Regeneration medium was composed of CC basic medium supplemented with 2 mg/L 6-benzylaminopurine and 1 mg/L naphthylacetic acid. The degree of callus browning was categorized using four levels as follows: 0) browning of less than 1/10 of the callus tissue area (recorded as no browning); 1) callus light browning; 2) callus medium browning; and 3) callus deep browning. Callus culturability was assessed based on two parameters i.e., CBI and RR. The calculation of CBI and RR were according to Gu et al. (1992).

$$\text{CBI} = (\sum \text{number of calli at each browning level} \times \text{browning level} / \text{number of transferred calli} \times \text{highest browning level}) \times 100 \%$$

$$\text{RR} = (\text{number of calli producing small green buds} / \text{number of transferred calli}) \times 100 \%$$

Statistical analysis

The data acquired in this study were expressed as percentages, with the phenotypic values transformed using the arcsine function in Microsoft Excel. Analyses of variance were performed using SPSS 13.0 software for Windows. QTLs were detected by single-point analysis using the software program Map-Manager QTXb17 (Manly et al. 2001) based on the simple sequence repeat marker data reported by Fu et al. (2010). The statistical thresholds for each trait were calculated based on permutation tests at an experiment-wise level of $P < 0.05$.

Results and discussion

Phenotypic analysis of 9YJ introgression lines

Values of the three measured traits related to mature embryo culturability are shown in Table 1 for the

Table 1. Mature embryo culturability traits of *Oryza rufipogon* 9YJ introgression lines and recurrent parent 93-11

	CBI1t ^a %	CBI2t ^a %	RR ^a %
93-11	38.6±1.5	37.5±1.17	18.9±1.9
9YJ	1.2±0.2-	8.3±1.2-	0.0±0.0-
	91.6±2.0	95.2±1.6	65.0±3.1

^aAbbreviations of culturability-related traits are as follows: CBI, callus browning index; RR, regeneration rate; 1t, one round of subculturing; 2t, two rounds of subculturing

recurrent parent 93-11 and the 9YJ population. The recurrent parent 93-11 showed a moderate or low level of culturability on the three traits as compared to the 9YJ population, which showed a wide distribution (Fig 1). The ANOVA revealed that genotypes differed

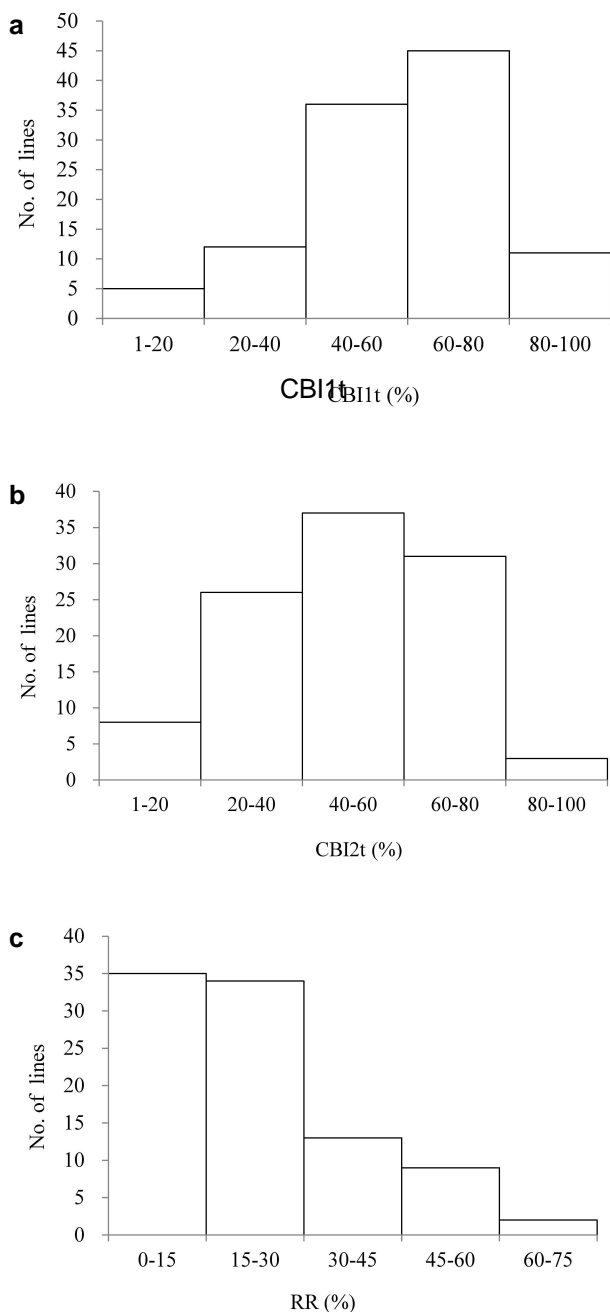


Fig. 1. Distributions of culturability traits of *Oryza rufipogon* 9YJ introgression lines. (a) Callus browning index after one round of subculturing (CBI1t); (b) Callus browning index after two round of subculturing (CBI2t); (c) Regeneration rate(RR)

significantly for the culturability traits (the mean sum of square for CBI1t, CBI2t and RR were 0.58, 0.37 and 0.12, Significant at $p < 0.05$, the P value were 0.00, 0.00 and 0.04).

Several lines showing overall superior characteristics were identified, including 9YJ127, 9YJ125, 9YJ120, 9YJ100, 9YJ45 and 9YJ22. For example, line 9YJ100 exhibited more obvious reduction in callus browning compared with 93-11 (Fig. 2a), while line 9YJ127 displayed a higher differentiation ability than that of 93-11 (Fig. 2b). These lines can be used

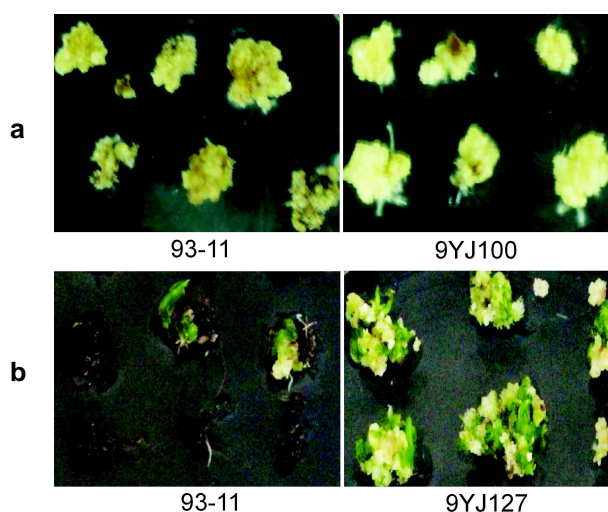


Fig. 2. Callus subcultures and differentiation of 93-11 and 9YJ. (a) Calli after two rounds of subculturing; (b) Callus differentiation

as *indica* rice genetic transformation receptors and should also be useful as parent materials for fine mapping of culturability traits.

Analysis of QTLs for callus subculture traits

Nine QTLs for CBI 1t (CBI after first sub-culturing) detected on chromosomes 1, 2, 3, 4, 7 and 8 accounted for 4-11 % of observed phenotypic variation, while 11 QTLs for CBI 2t (CBI after second sub-culturing) on chromosomes 1, 2, 3, 4, 5, 6, 8, 10 and 11 explained 5-15 % of the phenotypic variation (Table 2 and Fig. 3). Previous studies investigating callus status and proliferation ability have used calli that were freshly induced or subcultured for only a short time (Taguchi-Shiobara et al. 2006; Li et al. 2007; Zhao et al. 2009; Li et al. 2013). However, to obtain resistant clones in rice transformation experiments, calli should be screened for 40-60 days. In this study, we evaluated calli that were subcultured twice (25 days each)

Table 2. QTLs for mature embryo callus browning index (CBI) following subculturing in the 9YJ population

Chr ^a	QTL	Locus	One-round subculture			Two-round subculture		
			PV (%) ^b	P ^c	Add (%) ^d	PV (%)	P	Add (%)
1	<i>qBI1-1</i>	RM243	9	0.0014	-21.1			
1	<i>qBI1-2</i>	RM212				9	0.0021	-21.3
1	<i>qBI1-3</i>	RM6489				5	0.0255	10.0
2	<i>qBI2-1</i>	RM1358				6	0.0357	-15.1
2	<i>qBI2-2</i>	RM341	5	0.0250	-14.2	6	0.0168	-16.3
3	<i>qBI3-1</i>	RM251	6	0.0105	-23.0			
3	<i>qBI3-2</i>	RM16	6	0.0113	9.0			
3	<i>qBI3-3</i>	RM55	4	0.0348	-8.0	6	0.0099	-11.1
4	<i>qBI4-1</i>	RM335	11	0.0005	-14.3	15	0.0001	-17.3
4	<i>qBI4-2</i>	RM255	4	0.0360	-8.0			
5	<i>qBI5-1</i>	RM334				5	0.0288	7.2
6	<i>qBI6-1</i>	RM217				7	0.0064	17.4
7	<i>qBI7-1</i>	RM505	7	0.0052	-17.2			
8	<i>qBI8-1</i>	RM223				5	0.0231	7.0
8	<i>qBI8-2</i>	RM447	5	0.0209	-14.2			
10	<i>qBI10-1</i>	RM333				8	0.0041	6.0
11	<i>qBI11-1</i>	RM202				5	0.0306	-8.0

^aChromosome; ^bPhenotypic variance explained by the QTL; ^cThe probability that the marker genotype had no effect on the trait;

^dAdditive effect of the allele from *Oryza rufipogon*

following 12 days of induction a total culture time of 62 days. Most QTLs were detected in either one-round or the two-round subculture but the QTLs *viz.*, *qBI2-2*, *qBI3-3*, and *qBI4-1* were detected only in two-round subcultures. The QTL *qBI4-1*, located at the RM335 locus on chromosome 4, accounted for 11 and 15 % of phenotypic variation. Its effect was negative (Table 2), indicating that the alleles from *O. rufipogon* reduced callus browning. Genes that control callus browning with various expression patterns may, therefore, be present. The larger effect QTLs at the RM335 locus was detected in both subcultures and may thus be the most suitable for improvement of the efficiency of genetic transformation.

So far, only a few of the detected QTLs have been mapped to chromosomal regions as previously reported. A series of QTLs underlying important agronomic traits have been identified from introgression lines by using single marker analysis (Tian et al. 2006; Fu et al. 2010). The *qBI1-2* at locus RM212 on chromosome 1 was located near a QTL for callus subculture capability reported by Zhao et al. (2009). The *qBI4-1* at the RM335 locus on

chromosome 4 was in close vicinity of the two QTLs reported by Taguchi-Shiobara et al. (2006) at R2373 locus *i.e.*, *qlc4* related to induction callus color and *qSc4* contributing to subculture callus color. Most of QTLs detected in the present study are not in agreement with those of previously identified, perhaps because of the differences in materials, populations, callus growth stages, media and other factors. Nevertheless, QTLs for culturability were repeatedly detected on chromosome 4 at locus RM335.

Table 3. QTLs for mature embryo callus regeneration rate in the 9YJ population

Chr ^a	QTL	Locus	PV (%) ^b	P ^c	Add (%) ^d
2	<i>qR2-1</i>	RM341	10	0.0004	16.4
3	<i>qR3-1</i>	RM6676	5	0.0141	9.0
4	<i>qR4-1</i>	RM335	5	0.0301	11.0
9	<i>qR9-1</i>	RM8206	10	0.0004	17.1
12	<i>qR12-1</i>	RM277	6	0.0075	12.2

^aChromosome; ^bPhenotypic variance explained by the QTL; ^cThe probability that the marker genotype had no effect on the trait;

^dAdditive effect of the allele from *Oryza rufipogon*

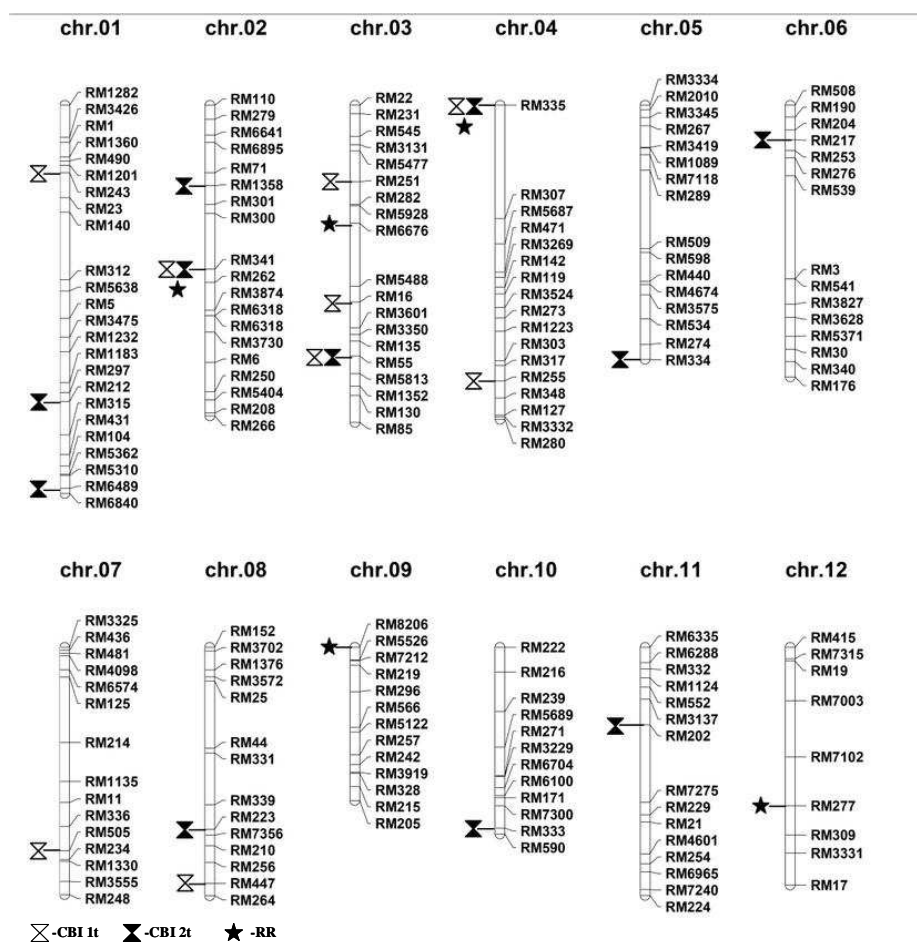


Fig. 3. Location of QTLs for mature embryo tissue culture traits on the 12 rice chromosomes

Analysis of QTLs for callus regeneration traits

Five QTLs for callus RR detected on chromosomes 2, 3, 4, 9 and 12 explained 5-10 % of the phenotypic variation (Table 3 and Fig. 3). QTL *qR2-1* located at locus RM341 on chromosome 2 and *qR9-1* located at RM8206 on chromosome 9 accounted for 10 % of the phenotypic variation, while *qR4-1* at locus RM335 on chromosome 4 explained 5 % of the variation. QTLs for callus differentiation traits have positive effect, indicating that common wild rice probably possess genes, which can enhance rice tissue culturability and can be fully expressed in the 93-11 genetic background.

Previous investigations on culturability have mostly used populations derived from crosses within or between subspecies, whereas in present study, introgression lines having *O. rufipogon* as the donor parent were used. The important QTLs for callus differentiation and callus browning near the RM341 locus (*qR2-1* and *qBI2-2*) did not map to previously

reported locations, suggesting that these genes may be unique to common wild rice.

Acknowledgments

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