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ORIGINAL PAPER

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# Mapping QTLs that control the performance of rice tissue culture and evaluation of derived near-isogenic lines

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**Abstract** Quantitative trait loci (QTLs) that control the performance of tissue culture in rice were detected by using 116 RFLP markers and 183 $BC_1F_3$ lines derived from two varieties, Koshihikari and Kasalath. With time, the seed callus of Koshihikari tends to turn brown and stop growing, while that of Kasalath remains yellowish-white and proliferates continuously. The performance of tissue culture in the induction of calli from seed, the subculture of induced calli, and shoot regeneration were evaluated by five indices: induced-callus weight, induced-callus color, subcultured-callus volume, subcultured-callus color, and regeneration rate. Through callus induction and subculture, eight putative QTLs ($P < 0.001$) were located on chromosomes 1, 4, and 9. Among these QTLs, five Kasalath alleles and three Koshihikari alleles improved tissue culture performance. No QTL for regeneration was found. Among all the QTLs, *qSv1* explained the largest phenotypic variance, 33%, in subcultured-callus volume. In induced-callus color, two detected QTLs accounted for 36.4% of the total phenotypic variance; this was the highest score among the five indices used to evaluate the performance of tissue culture. Three near-isogenic lines for QTLs, located in two regions on chromosome 1, were developed to evaluate their tissue culture performance. The Kasalath alleles in *qSv1* and *qSc1-1* improved callus color through callus induction and subculture, and increased the subcultured-callus volume and the fresh weight of regenerated calli, including shoots, roots, and differentiated structures. In *qSc1-2*, the Kasalath allele improved callus color through induction and subculture. These results verified the presence of QTLs for the volume and color of subcultured callus on chromosome 1, *qSv1*, *qSc1-1*, and *qSc1-2*.

**Keywords** Tissue culture · Seed callus · QTL · Rice · *Oryza sativa* L

**Abbreviations** ICW: Induced-callus weight · ICC: Induced-callus color · SCV: Subcultured-callus volume · SCC: Subcultured-callus color

## Introduction

Koshihikari has been a leading rice variety in Japan for more than 20 years because of its appeal to the tastes of Japanese consumers. Many varieties that appeal to the Japanese palate have been produced by crosses of Koshihikari. In the production of transgenic plants and in anther cultures to produce doubled haploid lines, calli induced from Koshihikari tend to turn brown and stop growing, as is the case in many related varieties. Changing the nitrogen source in the medium often improves callus growth in Koshihikari. Tissue culture media for Koshihikari have been developed (Daigen and Abe 1993; Ogawa et al. 1999; Hashizume et al. 1999; Daigen et al. 2000), all of which contain less ammonium ion ($NH_4^+$) and/or nitrate ion ($NO_3^-$) than N6 or R2 medium, which is often used in rice tissue culture. However, even when these new media are used, tissue cultures of Koshihikari cells are still unstable because of the calli's tendency to turn brown. If we were to modify the genes that cause this browning and thus improve the performance of tissue culture, culture work with Koshihikari cells would be easier, and N6 or MS, the usual media in plant tissue cultures, would be applicable

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to the cultured cells of many varieties, including Koshihikari and its relatives. Moreover, genes modified to improve the performance of tissue culture could be used as selection marker genes in the production of transgenics when transformed Koshihikari cells are cultured in media in which the Koshihikari callus becomes brown.

The rice seed callus, which is derived from the scutellum of the mature embryo, is an excellent material for making transgenics because it regenerates efficiently, and more transgenics can be obtained than other tissues using the Agrobacterium-mediated transformation method (Shimamoto et al. 1989; Hiei et al. 1994). In several species, tissue culture performance is quantitatively controlled. Also in several species, genes associated with regeneration from mature or immature embryos, in the process of somatic embryogenesis, have been reported. Many QTLs have been identified for somatic embryogenesis in maize (Armstrong et al. 1992), alfalfa (Yu and Pauls 1993), rice (Taguchi-Shiobara et al. 1997b), barley (Mano et al. 1996; Mano and Komatsuda 2002), and wheat (Ben Amer et al. 1997). In rice, QTLs that affect the regeneration ability of seed callus were identified using $BC_1F_5$ lines derived from a cross between a *japonica* variety, Nipponbare, and an *indica* variety, Kasalath (Taguchi-Shiobara et al. 1997b). Since that study focused on regeneration from the callus, both parental varieties were chosen because of their performance in callus growth and regeneration on N6 and MS media. In those media, cultured cells of Nipponbare and Kasalath proliferate well and severe browning seldom occurs, but regeneration of Kasalath is more efficient than that of Nipponbare.

Here we tried to detect QTLs that affect tissue culture performance using $BC_1F_3$ lines derived from a cross between Koshihikari and Kasalath. The tissue culture system, including the media, was the same as that used to identify QTLs involved in regeneration ability using a population derived from a cross between Nipponbare and Kasalath (Taguchi-Shiobara et al. 1997b). In the media used, cultured Koshihikari cells turn brown. To evaluate tissue culture performance more precisely, the procedures were divided into three steps: callus induction from seed, subculture of induced calli, and regeneration from subcultured calli. At each step, the detection of QTLs was attempted. Near-isogenic lines for QTLs on chromosome 1—*qSv1*, *qSc1-1*, and *qSc1-2*—were developed to demonstrate the existence of the QTLs. The QTLs detected here, using a Koshihikari/Kasalath population, were compared with those identified using a Nipponbare/Kasalath population.

## Materials and methods

### Plant materials and linkage map construction

The $BC_1F_3$ population used in this study was developed from a cross between Koshihikari and Kasalath as described in Yamamoto et al. (2001). One $BC_1F_3$ individual from each line was used to construct the linkage map using 116 RFLP markers distributed over all 12 rice chromosomes. The RFLP markers were selected from a high-density linkage map (Kurata et al. 1994).

### Culture procedures

To construct the linkage map, mature Koshihikari, Kasalath, and selfed seeds derived from 183 of 187 $BC_1F_3$ individuals were cultured. The procedures from callus induction to regeneration of the shoots have been described previously in detail (Taguchi-Shiobara et al. 1997a, b). About 30 $BC_1F_4$ seeds per experimental group were incubated on callus-inducing solid medium for 4 weeks. The calli derived from each seed were weighed, and the mean fresh weight was used as 'induced-callus weight (mg)' to represent each $BC_1F_3$ individual and its parents. The color of the induced calli, or 'induced-callus color', was also observed and categorized into four levels: brown, brownish-yellow, yellow, and yellowish-white. About 80 mg of calli per seed from five independent seeds were selected to initiate suspension cultures. After subculture for 1 week, the volume of calli derived from each seed was separately measured using a volumetric pipette, the digital balance of which was accurately adjusted, and the mean volume was used as 'subcultured-callus volume (ml)'. The color of the callus, or the 'subcultured-callus color', was observed in the same way as was done previously. Ten calli, each 1 mm in diameter and originating from one seed, were incubated on a shoot-inducing medium. Five dishes, each with ten calli, were cultured for each experimental group. After incubation for 4 weeks, the percentage of calli that had more than one regenerated shoot was calculated for each dish, and the mean for each of the five dishes was used as the 'regeneration rate (%)' to represent each of the 183 $BC_1F_3$ individuals and its parents.

### Statistical analysis

Prior to analysis, the regeneration rate was transformed to normalize variances: $y = \arcsin \sqrt{x}$. Scores were assigned to the four levels of callus color: four for yellowish-white, three for yellow, two for brownish-yellow, and one for brown. Analysis of variance (ANOVA) was used to detect markers linked to loci for the performance of tissue culture. We employed a 0.001 probability level as the threshold for the detection of putative QTLs for the five indices: induced-callus weight, induced-callus color, subcultured-callus volume, subcultured-callus color, and regeneration rate. To determine associations between markers and regeneration ability, the general linear model (GLM) procedure in the SAS program (SAS Institute 1989) was used to detect significant differences between the mean values of Koshihikari and

Kasalath homozygotes at each marker locus. The marker locus showing the highest *F*-value around the region of significance was considered to be the nearest marker. The MAPMAKER/QTL program (Lincoln et al. 1992) was also used to estimate each detected QTL gene effects and to calculate the multigenic variance of all detected QTLs.

### Development and tissue culture of near-isogenic lines for QTLs on chromosome 1

Of the 183 $BC_1F_3$ individuals used for QTL analysis, two were selected to develop near-isogenic lines for QTLs on chromosome 1. These two $BC_1F_3$ individuals were backcrossed to Koshihikari twice to obtain 20 $BC_3F_1$ individuals. After they were selfed, these $BC_3F_2$ individuals were screened using 38 RFLP markers, so that the genome of each contained a Kasalath fragment in the region of the QTL in the Koshihikari background. The culture procedure was the same as that described earlier. After the 4-week regeneration step, the fresh weight of calli, including the shoots, roots, and differentiated structures, was calculated for each dish, and the mean for each of the five dishes was used to represent each near-isogenic line and its parents.

## Results

### Phenotypic variation among the two parents and 183 $BC_1F_3$ lines

The tissue culture performance of Koshihikari was much lower than that of Kasalath. Figure 1 shows shoot regeneration from seed calli derived from Koshihikari and Kasalath. Most Koshihikari calli turned brown, while the Kasalath calli grew well. The frequency distribution for each trait in both parents and in the 183 $BC_1F_3$ lines is shown in Fig. 2. The Koshihikari callus started to turn brown (score = 2.6 ± 0.5) after the callus induction step (4 weeks). During the following subculture (1 week), the color of the Koshihikari callus did not become worse (score = 2.7 ± 0.5), but the callus turned brown and stopped growing by the end of regeneration step (4 week) (Fig. 1). On the other hand, the Kasalath callus did not turn brown after the callus induction and subculture steps (scores = 4.0 ± 0.0). The subcultured-callus color of the $BC_1F_3$ lines became worse than their induced-callus color (Fig. 2a, b). Transgressive segregation was observed in induced-callus weight and in subcultured-callus volume (Fig. 2a, b).

### Correlations between indices used to evaluate performance of tissue culture

Table 1 shows the correlation coefficients (r) among the five indices used to detect the QTLs' tissue culture performance. The induced-callus color and subcultured-callus color were highly correlated with each other, and these two indices in the subculture step were also highly correlated with the index in the following regeneration step. The induced-callus color was correlated with regeneration rate but not with subcultured-callus volume, even though the subculture step followed the induction step. Induced-callus weight was not correlated with the indices in subculture or in regeneration.

### QTLs for performance of tissue culture

In total, eight QTLs were detected for four indices. Each QTL's gene effect was estimated (Table 2), and each QTL was located on the linkage map (Fig. 3).

#### *Callus induction*

Two QTLs on chromosomes 4 and 9, *qIw4* and *qIw9*, were detected for induced-callus weight. In *qIw4*, the Koshihikari allele increased induced-callus weight, as did the Kasalath allele in *qIw9*. A multilocus model involving *qIw4* and *qIw9* accounted for 13.3% of the

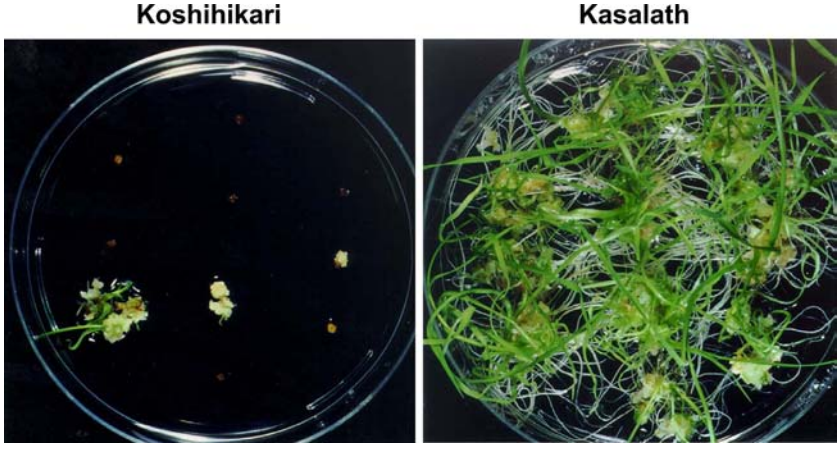


**Fig. 1** Regeneration from seed calli derived from two parents, Koshihikari and Kasalath

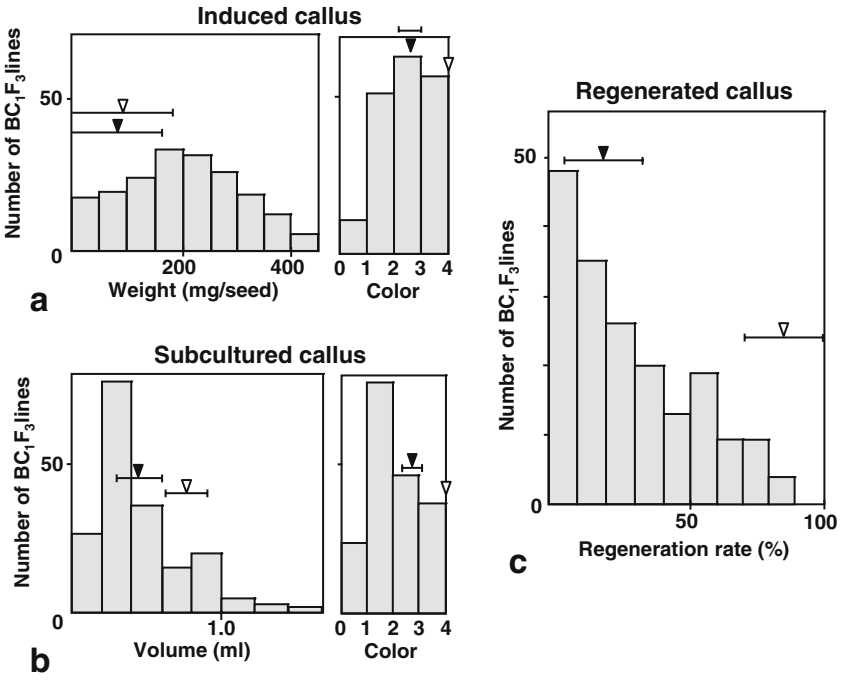


**Fig. 2** Frequency distributions for $BC_1F_3$ lines of; (**a**) fresh weight and color of induced-callus, (**b**) volume and color of subcultured-callus, and (**c**) regeneration rate. Scores for colors in (**a**) and (**b**) are 1: brown, 2: brownish-yellow, 3: yellow, and 4: yellowish-white. Phenotypic values with standard deviations of Koshihikari and Kasalath are denoted by *black* and *white triangles*, respectively

total phenotypic variance in the $BC_1F_3$ lines. As for callus color, two QTLs on chromosomes 4 and 9, *qIc4* and *qIc9*, were detected, and a multilocus model involving them accounted for 36.4% of the total phenotypic variance. In the callus induction step, *qIc4* had the highest score of total phenotypic variance explained: 29% by single-point analysis.

*Subculture*

Only one QTL on chromosome 1, *qSv1*, was mapped for subcultured-callus volume, which the Kasalath allele increased. This *qSv1* explained 33% of the total phenotypic variance, which was the highest score in the single-point analysis. Three significant QTLs on chromosomes 1 and 4, *qSc1-1*, *qSc1-2*, and *qSc4*, were detected for subcultured-callus color. A multilocus model involving these three QTLs accounted for 30.5% of the total phenotypic variance. The Kasalath alleles in *qSc1-1* and *qSc1-2*, and the Koshihikari allele in *qSc4*, improved subcultured-callus color.

*Regeneration*

No QTL was detected for regeneration rate.

Near-isogenic lines for QTLs on chromosome 1

Of the $BC_3F_2$ individuals, three near-isogenic lines, #1, #2, and #3, were selected to have the Kasalath alleles in the regions of QTLs on chromosome 1 (Fig. 4). Line #1, which had a Kasalath fragment in two RFLP markers, R210 and C178, was selected as a near-isogenic line for *qSv1* and *qSc1-1*. Line #2, with a Kasalath fragment on C122, was also selected as a near-isogenic line for *qSc1-2*. To understand the effects of the Kasalath alleles in both these regions, one $BC_3F_2$ line that had a Kasalath fragment covering both regions was chosen and named #3. Most of the genomes of these three near-isogenic lines were derived from Koshihikari and did not contain Kasalath fragments in other QTL regions.

**Table 1** Correlation coefficients ($r$) between indices in the 183 $BC_1F_3$ lines derived from the cross between Koshihikari and Kasalath

| Index used to detect | QTL | ICW | ICC | SCV | SCC |
|---|---|---|---|---|---|
| Induced-callus weight | ICW | | | | |
| Induced-callus color | ICC | 0.198** | | | |
| Subcultured-callus volume | SCV | −0.124 | 0.007 | | |
| Subcultured-callus color | SCC | 0.116 | 0.410*** | 0.305*** | |
| Regeneration rate | RS | 0.034 | 0.254*** | 0.272*** | 0.365*** |

**, *** Indicate significant differences at 0.01 and 0.001 probability levels, respectively

**Table 2** QTLs for tissue culture traits detected in a Koshihikari/Kasalath $BC_1F_3$ population, based on a single-point analysis of variance and MAPMAKER/QTL

| QTL | Chromosome | Nearest marker | SAS/GLM | | MAPMAKER/QTL | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Probability | $R^2$ [a] | LOD | Percent variation | AE[b] | DPE[c] |
| Induced callus | | | | | | | | |
| Weight | | | | | | | | |
| *qIw4* | 4 | C513 | 0.0007 | 0.08 | 3.0 | 7.2 | −20.2 | Koshihikari |
| *qIw9* | 9 | R2638 | 0.0009 | 0.07 | 3.0 | 7.4 | 16.3 | Kasalath |
| | | | | | | Total 13.3 | | |
| Color | | | | | | | | |
| *qIc4* | 4 | R2373 | < 0.0001 | 0.29 | 8.0 | 18.5 | −0.272 | Koshihikari |
| *qIc9* | 9 | R2638 | < 0.0001 | 0.14 | 5.4 | 13.2 | 0.184 | Kasalath |
| | | | | | | Total 36.4 | | |
| Subcultured callus | | | | | | | | |
| Volume | | | | | | | | |
| *qSv1* | 1 | C178 | < 0.0001 | 0.33 | 16.0 | 33.2 | 0.099 | Kasalath |
| Color | | | | | | | | |
| *qSc1-1* | 1 | C178 | < 0.0001 | 0.25 | 9.3 | 21.7 | 0.280 | Kasalath |
| *qSc1-2* | 1 | C122 | 0.0006 | 0.07 | 2.8 | 6.9 | 0.149 | Kasalath |
| *qSc4* | 4 | R2373 | 0.0006 | 0.07 | 2.2 | 5.5 | −0.128 | Koshihikari |
| | | | | | | Total 30.5 | | |
| Regenerated callus | | | | | | | | |
| Regeneration rate | | | | | | | | |
| – | – | – | – | – | – | – | – | – |

[a]Phenotypic variance explained by each QTL
[b]Additive effects (1/2 weight) of Kasalath allele
[c]Direction of phenotypic effect

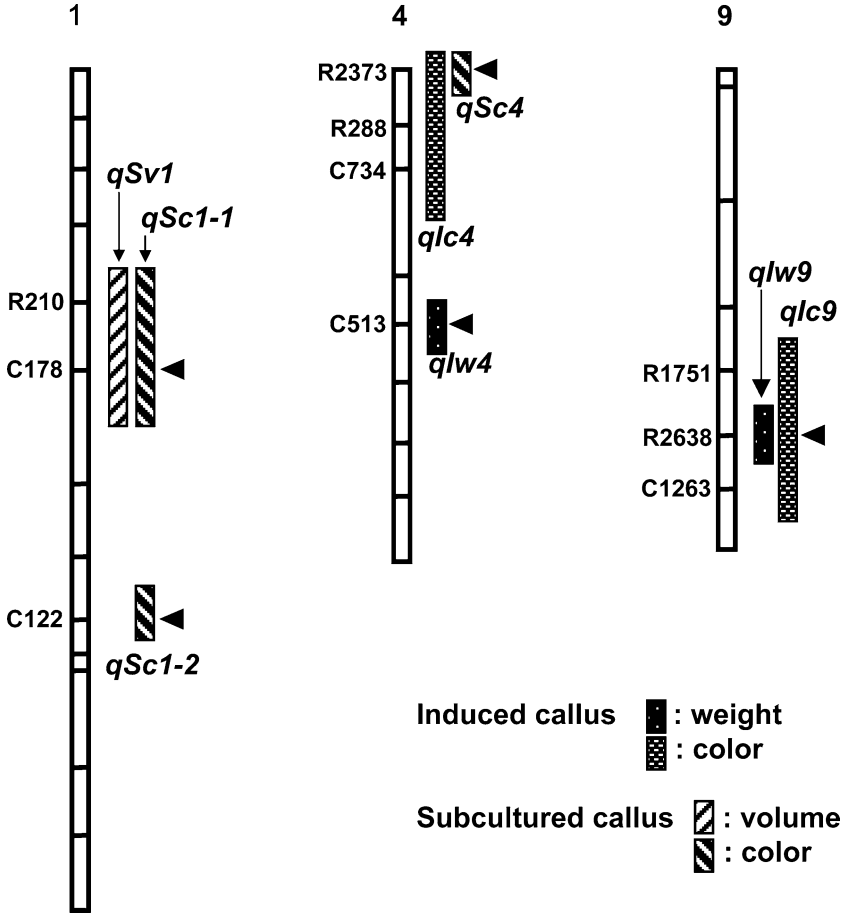


**Fig. 3** Locations of the QTLs for tissue culture performance. The RFLP markers significant at the 0.1% level are named. *Arrows* indicate the most significant marker in each QTL detected in single-point analysis of variance

Tissue culture performance of the three near-isogenic lines for QTLs on chromosome 1

All three of the near-isogenic lines showed improved callus color during callus induction and subculture (Fig. 5a). The induced-callus weight of line #1 was lower than that of Koshihikari, while those in lines #2 and #3 were the same as in Koshihikari (Fig. 5b). In subculture, lines #1 and #3 had a higher subcultured-callus volume than Koshihikari, while #2 produced the same volume as Koshihikari (Fig. 5c). In regeneration, the regeneration rates of the three near-isogenic lines were higher than that of Koshihikari but much lower than Kasalath (Fig. 5d). As for the fresh weight of the regenerated callus including shoots, roots, and other differentiated structures, lines #1 and #3 were as high as Kasalath, while #2 was as low as Koshihikari (Fig. 5e). Lines #1 and #3 also produced many shoot primordia on the surface of the regenerated callus, although these primordia did not contribute to the regeneration rate. Koshihikari and line #2 did not produce shoot primordia.

## Discussion

We detected eight QTLs for four indices: induced-callus weight, induced-callus color, subcultured-callus volume, and subcultured-callus color; we detected no QTL for the fifth index, regeneration rate (Fig. 3, Table 2). When two QTLs share the same location on a linkage map and their positive alleles have the same parents, and when there is at least one significant correlation between indices used, those two QTLs might actually be one QTL with pleiotropic effects. For example, *qIc4* and *qSc4* were located on the same region on chromosome 4 (Fig. 3), and both of them shared the same direction of phenotypic variance; thus, the Koshihikari allele had a positive effect (Table 2). Also, the indices used to detect these QTLs, induced-callus color and subcultured-callus color, were correlated with each other (Table 1). So *qIc4* and *qSc4* might be the same QTL. The same goes for *qSv1* and *qSc1-1* and also for *qIw9* and *qIc9*.

We mapped the QTLs that control the performance of tissue culture at callus induction from mature seeds and in the subculture of induced calli. Because of the low total phenotypic variance (13.3%) explained by the QTLs for induced-callus weight (Table 2), there likely exist QTLs that went undetected here. Since the quality of mature seeds tends to affect callus induction from them, the variance tends to be larger in the measurement of tissue culture performance. Some $BC_1F_3$ lines produced more than 300 mg of calli per seed (Fig. 2a), but often such calli were not embryogenic. That is, they contained more water and less cytoplasm than embryogenic calli, and seldom produced regenerated shoots. This is consistent with the fact that induced-callus weight did not correlate with the indices in subculture and regeneration (Table 1). These results showed that induced-callus weight is not a proper index of callus growth, particularly when the calli are extremely watery.

Among the four indices by which at least one QTL was detected in this study, all but induced-callus weight explained 30.5–36.4% of total phenotypic variance

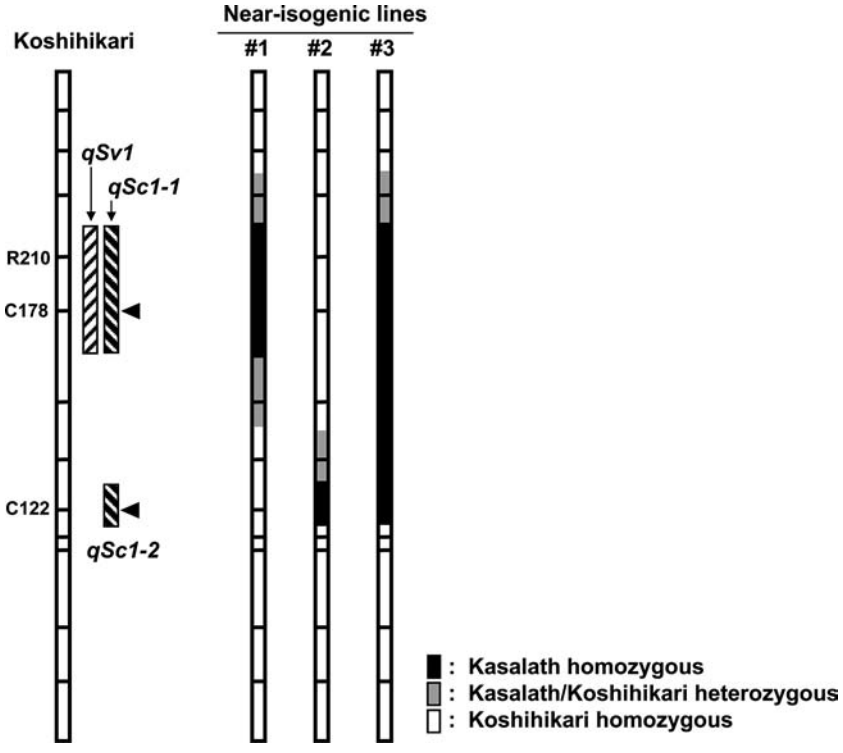


**Fig. 4** Graphical genotypes of chromosome 1 of Koshihikari and three near-isogenic lines (NILs): #1, #2, and #3. Locations of QTLs are shown on the right side of Koshihikari

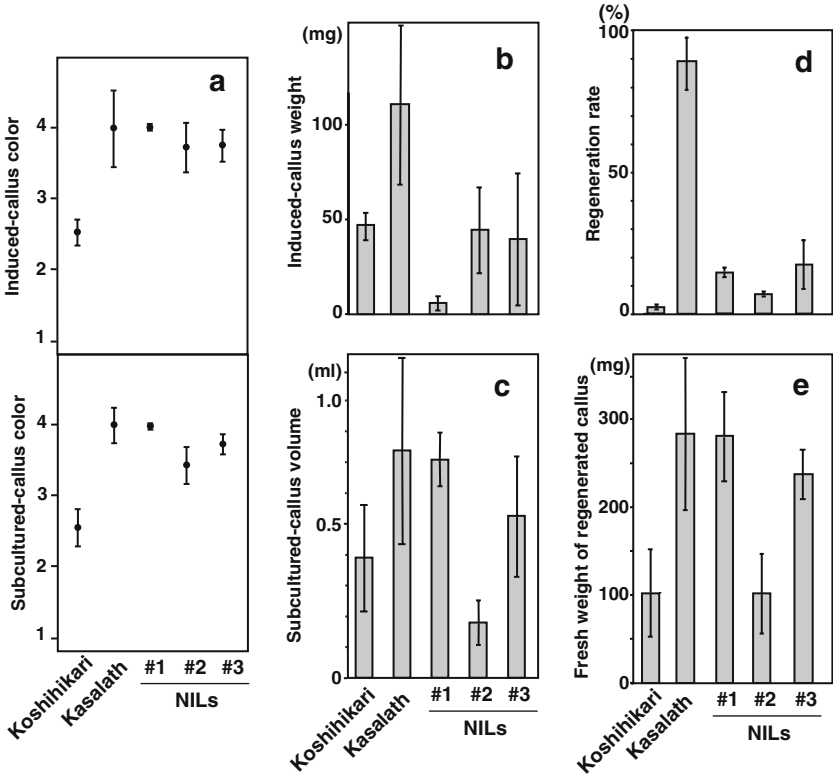


**Fig. 5** Tissue culture performance of NILs and their parents, Koshihikari and Kasalath. (**a**) Colors of induced-and subcultured-calli. Color scores are described in Fig. 2. (**b**) Fresh weight of induced-callus. (**c**) Volume of subcultured-callus. (**d**) Regeneration rate. (**e**) Fresh weight of callus including shoots, roots, and other differentiated structures after regeneration

(Table 2), indicating that those three indices—induced-callus color, subcultured-callus volume, and subcultured-callus color—are suitable for evaluating the performance of tissue culture. However, the performance of subcultured cells was more stable than that in primary culture, as with callus induction from seed. We considered that a smaller experimental error can be expected when subcultured cells are used to evaluate the performance of tissue culture.

A callus's color reflects its nature. A yellowish-white callus, like that of Kasalath, tends to be embryogenic and produces more regenerated shoots, while a browner callus, like that of Koshihikari, is less embryogenic and stops growing when the culture period becomes longer. In our experiment, *qIc4* and *qSc4*, in both of which the Koshihikari allele has a positive effect, were also detected (Table 2). However, there were few lines in which the callus color was better than that of Kasalath (Fig. 2a, b). This is because the highest score in callus color was limited to 4, and the Kasalath score was also $4.0 \pm 0.0$ in callus induction and subculture.

To isolate QTLs by map-based cloning, it is best to choose the QTLs that have the largest effect on an index for certain traits. In our results, these QTLs were *qSv1*, *qSc1-1*, and *qIc4* (Table 2). *qSv1* explained the largest phenotypic variance in subcultured-callus volume: 33% in single-point analysis and 33.2% in MAPMAKER/QTL. As for the subcultured-callus color, *qSc1-1* accounted for 25% of the phenotypic variance in single-point analysis and 21.7% for color in MAPMAKER/QTL. *qSv1* and *qSc1-1* might be the same QTL, since they are at the same location on the linkage map, specifically the region that includes RFLP markers R210 and C178 (Fig. 3). Near-isogenic line #1, which covered this region of about 9.7 Mb (Fig. 4), showed improved performance of tissue culture in both the color and volume of subcultured callus (Fig. 5a, c), thus confirming the existence of *qSv1* and *qSc1-1*. In regeneration, line #1 had a higher fresh weight of regenerated callus including shoots, roots, and differentiated structures, and its regenerated callus produced many shoot primordia, which were not observed in the callus of Koshihikari. Line #1 also improved the induced-callus color (Fig. 5a), although there was no QTL in callus induction. This region may contain a gene that causes browning of the Koshihikari callus. *qIc4* had the largest phenotypic variance of induced-callus color: 29% in single-point analysis and 18.5% in MAPMAKER/QTL (Table 2). In the same region, the QTL for subcultured-callus color, *qSc4*, was also detected (Fig. 3). Since the Koshihikari allele had positive effects on both *qIc4* and *qSc4* (Table 2), they also might be the same gene related to callus browning in this region.

The existence of QTLs with smaller effects, such as *qSc1-2*, was also confirmed, since near-isogenic line #2 improved the subcultured-callus color (Fig. 4 and Fig. 5a). It is considered to be more difficult to isolate a QTL that has a smaller effect than that having a larger effect, such as *qSv1* and *qSc1-1*. No QTL for induced-callus color was detected here, but line #2 showed improved induced-callus color. The *qSc1-2* region also may have a gene associated with the browning of the callus.

To figure out what happens if both of the regions covered by lines #1 and #2 contain the Kasalath allele (Fig. 4), line #3 was evaluated for its tissue culture performance. That ability was better than line #2's and almost the same as line #1's in subcultured-callus volume and also in the fresh weight of regenerated callus (Fig. 5c, e). No additive effect was observed.

Taguchi-Shiobara et al. (1997b) reported five QTLs—*qRg1*, *qRg2*, *qRg4a*, *qRg4b*, and *qRg4c*—associated with the rice seed calli's regeneration ability, using 98 $BC_1F_5$ lines derived from another *japonica* variety, Nipponbare, and an *indica* variety, Kasalath, which was also used in this study. They considered neither callus color nor callus volume. In our experiment, no QTL for regeneration rate was found (Table 2). However, three near-isogenic lines, which covered the *qSv1* and *qSc1-1* region and/or the *qSc1-2* region, showed higher regeneration rates than Koshihikari (Fig. 5d). Lines #1 and #3, which covered the *qSv1* and *qSc1-1* region, produced many shoot primordia in regeneration even if no QTL for regeneration rate was detected in the region. And three indices—induced-callus color, subcultured-callus volume, and subcultured-callus color—were highly correlated with regeneration rate (Table 1). Considering these findings, the QTLs for these three indices could be genes affecting regeneration. They were not detected since QTLs for regeneration rate may be because the recurrent parent was Koshihikari the callus of which tended to turn brown and stop growing. *qRg4c* might be the same gene as the two QTLs, *qIc4* and *qSc4*, that we detected here (Fig. 3; Table 2), since they were located on the same region in the linkage map and the *japonica* parent had the positive allele. *qRg1* also might be the same gene as *qSc1-1*.

The browning of the Koshihikari callus is reportedly related to nitrogen metabolism in the cells. To avoid browning of the Koshihikari callus, the use of media containing smaller amounts of ammonium and/or nitrate ions was found to be effective. Ogawa et al. (1999) reported that the Koshihikari seed callus showed improved growth when alanine replaced ammonium sulfate [$(NH_4)_2SO_4$] as the source of reduced nitrogen and when sucrose concentration was decreased. Hashizume et al. (1999) used modified N6 basal media containing one-fourth of potassium nitrate ($KNO_3$), aspartic acid, and no ammonium nitrate. Daigen et al. (2000) developed DKN (a designation for diluted $KNO_3$) medium, a modified R2 medium containing one-fifth of potassium nitrate and ammonium sulfate. Ogawa et al. (1999) clarified that Koshihikari and related varieties having low tissue culture performance had low levels of nitrite reductase activity, and that toxic nitrite ion ($NO_2^-$), which was not reduced, accumulated in cultured cells and caused browning of the callus followed by restrained cell growth. They also showed that decreased nitrite reductase activity also prevented the accumulation of nitrite ion in cultured cells, which improved cell growth.

Genes involved in nitrate metabolism might be QTLs associated with callus color—*qSc1-1*, *qSc1-2*, *qSc4*, *qIc4*, and *qIc9*. These QTLs are likely to be related to genes involved in nitrate metabolism and thus cause browning of the Koshihikari callus. Since nitrite reductase genes and nitrate reductase genes might be candidate genes for these QTLs, we tried to search for them in the rice genome using DDBJ. It is worth mentioning that a putative ferredoxin-nitrite reductase gene (P0025H06.19) is predicted near C178 in the 9.7 Mb region of *qSc1-1*. No nitrate reductase genes were found in the regions where QTLs for callus color were located.

Isolation and modification of the QTLs that control the performance of tissue culture will make it easier to produce Koshihikari transgenics. Such QTLs can be used to enhance the tissue culture performance of varieties with lower performance, and thus can be used as selection markers in the production of transgenics. When such a QTL is transformed to plants of a specific variety, the transformants can be selected using the media on which the callus of that variety cannot grow. It is not clear whether there are any common genes that control the performance of tissue culture between different cereal species, such as maize and wheat, whose transgenics are still difficult to obtain. The development of comparative mapping will clarify whether or not there are homologous genes that cause differences in tissue culture performance between varieties.

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## References

Armstrong CL, Romero-Severson J, Hodges TK (1992) Improved tissue culture response of an elite maize inbred through backcross breeding, and identification of chromosomal regions important for regeneration by RFLP analysis. Theor Appl Genet 84:755–762

Ben Amer IM, Korzun V, Worland AJ, Borner A (1997) Genetic mapping of QTLs controlling tissue-culture response on chromosome 2B of wheat (*Triticum aestivum* L.) in relation to major genes and RFLP markers. Theor Appl Genet 94:1047–1052

Daigen M, Abe S (1993) Callus formation from seed explant, growth in suspension culture and plant regeneration in rice (*Oryza sativa* L. cv. Koshihikari). Plant Tissue Cult Lett 10:176–179

Daigen M, Kawakami O, Nagasawa Y (2000) Efficient anther culture method of the Japonica rice cultivar Koshihikari. Breed Sci 50:197–202

Kurata N, Nagamura Y, Yamamoto K, Harushima Y, Sue N, Wu J, Antonio BA, Shomura A, Shimizu T, Lin SY, Inoue T, Fukuda A, Shimano T, Kuboki Y, Toyama T, Miyamoto Y, Kirihara T, Hayasaka K, Miyao A, Monna L, Zhong HS, Tamura Y, Wang ZX, Momma T, Umehara Y, Yano M, Sasaki T, Minobe Y (1994) A 300 kilobase interval genetic map of rice including 883 expressed sequences. Nature Genet 8:365–372

Hashizume F, Tsuchiya T, Ugaki M, Niwa Y, Tachibana N, Kowyama Y (1999) Efficient Agrobacterium-mediated transformation and the usefulness of a synthetic GFP reporter gene in leading varieties of Japonica rice. Plant Biotech 16:397–401

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa*, L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282

Lincoln S, Daly M, Lander E (1992) Tutorial for MAPMAKER/QTL. In: Mapping genes controlling quantitative traits with MAPMAKER/QTL 1.1. Whitehead Institute Technical Report, 2nd edn. Cambridge, pp 7–42

Mano Y, Takahashi H, Sato K, Takeda K (1996) Mapping genes for callus growth and shoot regeneration in barley. Breed Sci 46:137–142

Mano Y, Komatsuda T (2002) Identification of QTLs controlling tissue-culture traits in barley (*Hordeum vulgare* L.). Theor Appl Genet 105:708–715

Shimamoto K, Terada R, Izawa T, Fujimoto H (1989) Fertile transgenic rice plants regenerated from transformed protoplasts. Nature 338:274–276

Ogawa T, Fukuoka H, Yano H, Ohkawa Y (1999) Relationships between nitrite reductase activity and genotype-dependent callus growth in rice cell cultures. Plant Cell Rep 18:576–581

SAS Institute (1989) The GLM procedure. In: SAS/STAT users guide, version 6, 4th edn. SAS Institute, Cary, pp 891–996

Taguchi-Shiobara F, Komatsuda T, Oka S (1997a) Comparison of two indices for evaluating regeneration ability in rice (*Oryza sativa* L.) through a diallel analysis. Theor Appl Genet 94:378–382

Taguchi-Shiobara F, Lin SY, Tanno K, Komatsuda T, Yano M, Sasaki T, Oka S (1997b) Mapping quantitative trait loci associated with regeneration ability of seed callus in rice, *Oryza sativa* L. Theor Appl Genet 95:828–833

Yamamoto T, Taguchi-Shiobara F, Ukai Y, Sasaki T, Yano M (2001) Mapping quantitative trait loci for days-to-heading, and culm, panicle and internode lengths in a $BC_1F_3$ population using an elite rice variety, Koshihikari, as the recurrent parent. Breed Sci 51:63–71

Yu K, Pauls K (1993) Identification of a RAPD marker associated with somatic embryogenesis in alfalfa. Plant Mol Biol 22:269–277