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A Microarray Analysis of Rice Near-Isogenic Lines that Cover QTLs Associated with Callus Proliferation

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Abstract

Near-isogenic lines (NILs) were applied for the first time to microarray analysis of 8,987 randomly selected expressed sequence tags (ESTs) aiming at screening rice genes associated with seed callus proliferation. Callus proliferation of the variety Koshihikari is poor, since its callus tends to become brown with time. We developed ten NILs that each had a chromosomal segment of the variety Kasalath on chromosome 1 in a Koshihikari background, to cover a region that contains two QTLs for subcultured callus color, *qSc1-1* and *qSc1-2*. The existence of QTLs was verified through tissue culture of the seed calli of these NILs. mRNAs from the calli of three NILs that had the Kasalath allele in *qSc1-1* and/or *qSc1-2* were applied to microarray analysis. A comparison of expression profiles between Koshihikari and each NIL followed by Northern hybridization showed that 22 unique genes were induced and 15 unique genes were repressed in the calli of these Kasalath allele-containing NILs on chromosome 1. The results of a *cis*-element search using 500 bp of genomic sequence upstream of each gene suggested that the expression profiles of GA- and/or sugar-responsive genes were different between each NIL and Koshihikari, and that defense-related genes and genes that act negatively in metabolism were repressed in the NILs compared with Koshihikari.

Discipline: Biotechnology
Additional key words: cDNA microarray, NILs, *Oryza sativa* L., seed callus

Introduction

The rice seed callus derived from the scutellum of the mature embryo is most often used in the production of transgenic plants by the *Agrobacterium*-mediated transformation method. To produce transgenic plants efficiently, the seed callus must proliferate sufficiently and must be competent for somatic embryogenesis or regeneration. Koshihikari, a leading rice variety in Japan, has little callus proliferation ability, since its callus tends to become brown with time. To overcome this problem, media for Koshihikari cells have been improved by decreasing the nitrogen source[4,5,13].

Plant callus proliferation is a quantitative trait, and genes conferring callus maintenance and regeneration have been reported in several species[2,11,12]. In rice, quantitative trait loci (QTLs) that affect the proliferation of seed callus were identified[16]. Up to now, no QTL for callus proliferation has been cloned. Many genes expressed in regeneration have been reported, such as housekeeping genes, hormone-responsive genes, genes involved in signal transduction, homeotic genes, genes coding for extracellular proteins, and maturation genes[3]. However, it is

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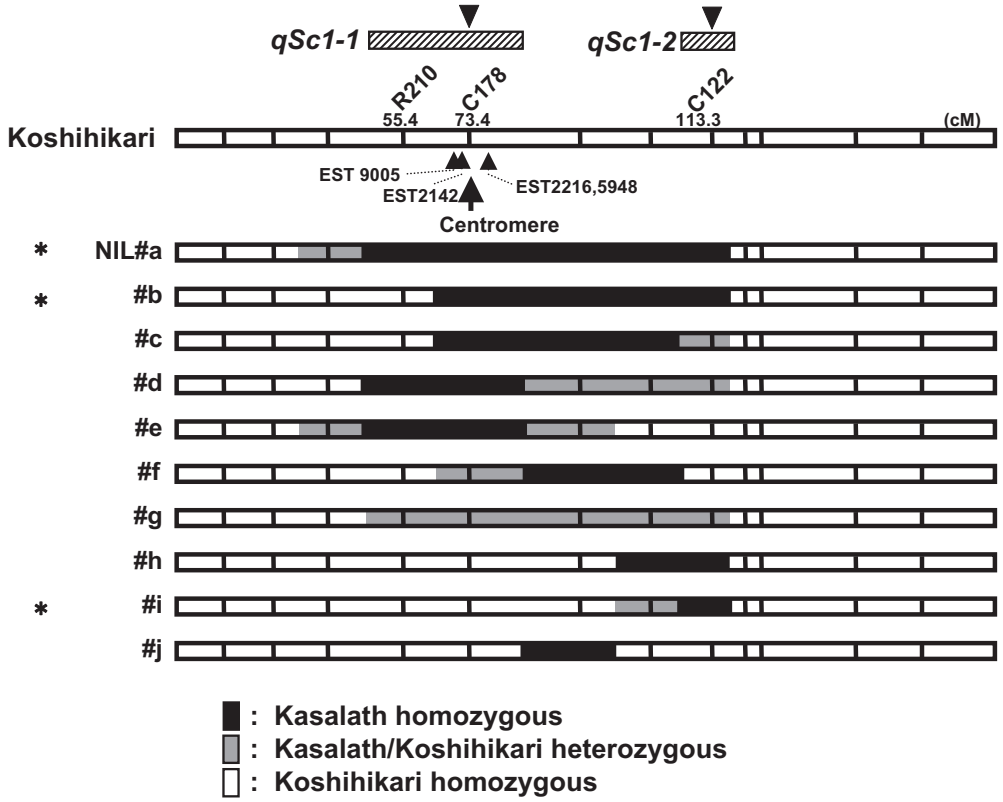

Fig. 1. Graphical genotypes of chromosome 1 of Koshihikari and ten near-isogenic lines (NILs) from #a to #j revealed by 14 RFLP markers

*: NILs applied to a microarray analysis. Locations of QTLs are shown on the upper side of Koshihikari. 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. Genetic distance is described according to 'The Latest High-Density Rice Genetic Map, Including 3267 Markers' (http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/index.html).

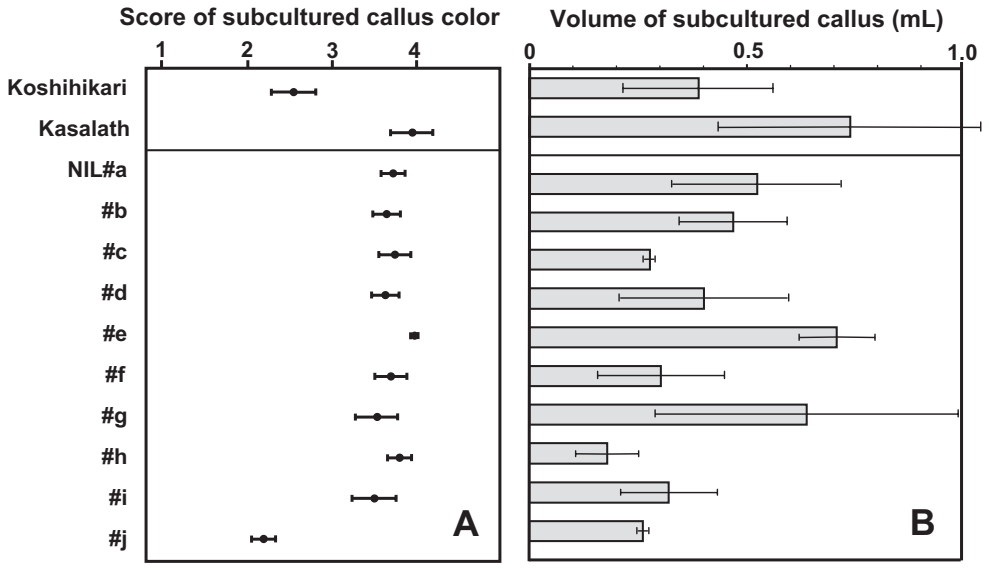

Fig. 2. Callus proliferation abilities of ten NILs and their parents, Koshihikari and Kasalath, after subculture for 1 week in a N6 medium supplemented with 30 g/L sucrose, 0.3 g/L casein acid hydrolysate, 1.15 g/L proline and 1 mg/L of 2, 4-D

About 80 mg of calli was used to initiate subculture. n = 7. The mean ± SE was used to represent Koshihikari, Kasalath, and each NIL. A: Colors of subcultured calli. Color scores: 1, brown; 2, brownish-yellow; 3, yellow; 4, yellowish-white. B: Volume of subcultured calli.

not clear what causes the differences in callus proliferation among different varieties.

To compare the expression profiles of rice lines with different callus proliferation abilities, we developed ten NILs in which the allele of an *indica* variety, Kasalath, was introduced in the region of two QTLs controlling subcultured callus color, *qSc1-1* and *qSc1-2*, in the Koshihikari background. The existence of two QTLs was demonstrated through the evaluation of callus proliferation of Koshihikari and these NILs. Koshihikari and three NILs that had greater callus proliferation than Koshihikari were applied to microarray analysis. To screen the genes induced or repressed by the Kasalath allele in the region of the QTLs, the expression profiles in subcultured calli were compared with Koshihikari and each NIL.

Materials and methods

1. Plant material and culture procedures

A BC_1F_3 population, derived from a cross between Koshihikari and Kasalath[17], was used to develop the NILs. Out of 187 BC_1F_3 plants, two plants were selected since they had Kasalath allele in *qSc1-1* and/or *qSc1-2,* and were backcrossed twice with Koshihikari to obtain 20 BC_3F_1 plants. Ten BC_3F_2 plants were screened using 52 RFLP markers dispersed throughout the genome. These plants were verified to have a Koshihikari allele in at most 2 markers in all chromosomes except for chromosome 1. Selfed seeds derived from ten BC_3F_2 NIL plants and mature seeds of Koshihikari and Kasalath were cultured. The procedures from callus induction to regeneration of the shoots have been described previously[16].

2. Microarray analysis, Northern hybridization, gene annotation, and *cis*-element search

Koshihikari and three NILs—NIL#a, NIL#b, and NIL#i—were used to prepare total RNAs. Calli after 1 week's subculture in liquid callus-inducing medium were used to isolate total RNA and were applied to the microarray analysis as described previously[1]. On glass microarray slides, 8,987 randomly selected ESTs were spotted in duplicate[18]. ArrayGauge (FujiFilm, Tokyo) was used for image analysis. When the fluorescence intensity ratio of each NIL to Koshihikari changed by more than threefold, the gene was judged to be induced or repressed. Northern hybridization procedures were performed as previously described[1], and 10 μg of total RNA was used.

To annotate genes, the GenBank database and the rice full-length cDNA database, KOME (http://cdna01.dna.affrc.go.jp/cDNA/)[9], were searched for the sequences of the ESTs, using the BLAST program to find mRNA sequences. Pfam (http://pfam.wustl.edu/) was used to predict protein domains. Using the PLACE *cis*-element database[6] (http://www.dna.affrc.go.jp/PLACE/), 500 bp of the genomic sequence upstream from the initiation codon of each mRNA or the 5' terminus of each full-length cDNA clone was searched for *cis*-elements.

Results and discussion

1. Development of NILs and tissue culture of their seed calli

Ten NILs were selected to have the Kasalath allele in the region containing two QTLs for callus color, *qSc1-1* and *qSc1-2*, in the Koshihikari background (Fig. 1).

Sometimes QTLs detected in primary QTL analysis are not detected in the NILs. In this study, the existence of QTLs having a sufficient effect was confirmed through tissue culture of ten NILs that had the Kasalath allele in the region containing *qSc1-1* and/or *qSc1-2* in the Koshihikari background. Nine NILs, from NIL#a to #i, showed better callus color in subculture (Fig. 2A). Seven NILs, from NIL#a to #g, increased the fresh weight of regenerated calli including the shoots, roots, and differentiated structures (Fig. 3). This suggests that a Kasalath allele in the region including *qSc1-1* would improve sub-

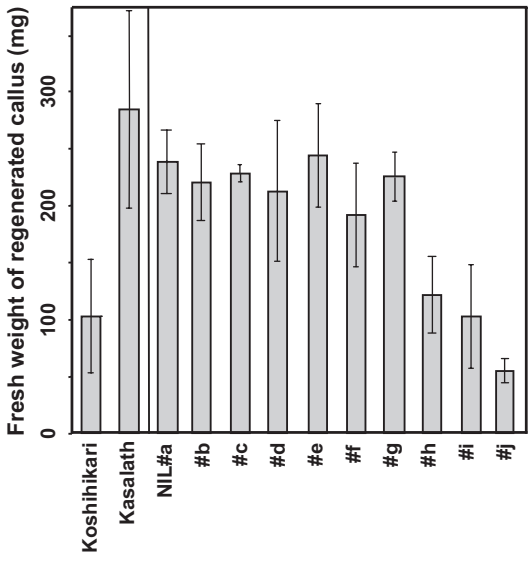

Fig. 3. Regenerated callus proliferation of ten NILs and their parents, Koshihikari and Kasalath, after regeneration for 4 weeks in medium containing kinetin and NAA

The average fresh weight of calli, including the shoots, roots, and differentiated structures, were calculated for each dish. The mean ± SE for each of the 5 dishes containing 10 calli was used to represent Koshihikari, Kasalath, and each NIL.

cultured callus color and increase the fresh weight of regenerated calli, regardless of whether the Kasalath allele is heterozygous or homozygous. This region contains ferredoxin-nitrite reductase gene and it was recently clarified to be a regeneration QTL[10]. In Koshihikari, nitrite reductase activity is lower than other varieties, and toxic nitrite ion (NO_2^-), which is not reduced, accumulates in cultured cells and causes browning of the callus followed by restrained cell growth[13]. Considering these facts, *qSc1-1* might be the ferredoxin-nitrite reductase gene.

On the other hand, a Kasalath allele in the region including *qSc1-2* improved subcultured callus color (Fig. 2A), however, it did not increase the fresh weight of regenerated calli (Fig. 3). Callus proliferation of NIL#j was the same as that of Koshihikari (Figs. 2 & 3), which implies the region that is covered by NIL#j does not include *qSc1-1* nor *qSc1-2* (Fig. 1).

2. Identification of genes induced or repressed in NILs

In microarray analysis, 44 unique genes were screened to show different profiles between Koshihikari and each NIL (Tables 1 & 2). Of these 44 genes, 37 were induced or repressed by a Kasalath allele in the region of QTL. To verify this result, 25 of the 44 genes were subjected to Northern hybridization, and 15 of them (15/25; 60%) were confirmed. Since the genotype of QTL did not explain the expression patterns of most genes (Tables 1 & 2), these genes are considered to act somewhere in the downstream of these QTLs in the gene regulation cas-

Table 1. List of 22 unique genes induced in at least one NIL after subculture in medium containing 2,4-D

EST clones	Chr	PAC or BAC clone	Gene identification	Full-length cDNA	Microarray analysis Induced ratio[a)]			Northern analysis Signal strength			
					NIL#a	NIL#b	NIL#i	Koshihikari	NIL#a	NIL#b	NIL#i
97, 378, 946, 1068	1	B1417F08	Cysteine endopeptidase	– [b)]	7.4 [c)]	5.8 [c)]	9.7 [c)]	+	+++	+++	+++
1539	5	P0554F08	Protease inhibitor/seed storage/LTP family, putative	AK119689	5.8	7.0	6.4	+	++	++	++
5350, 4191	5	P0016H04	Metallothionein-like protein	AK058529	3.4 [c)]	3.2 [c)]	3.4 [c)]	+	++	++	++
8705	1	P0504H10	Putative glycosyl hydrolase	AK110956	3.0	2.8	3.0	+	++	++	++
8850, 2214	9	P0515A04	Unknown	AK063844	4.3 [c)]	3.7 [c)]	3.0 [c)]	+	++	++	++
9005 [d)]	1	B1140D12	SnoRNA	AK069030	3.8	2.4	2.5	++	++	+	+++
9026	1	P0489A01	Unknown	–	3.5	2.5	2.4	+	++	+	+
7113, 7645	1	B1015E06	Metallothionein-like protein	AK120350	4.7 [c)]	5.2 [c)]	4.3 [c)]				
1492	12	OSJNBb0094E08	Unknown	AK068646	3.3	3.7	4.2				
164	11	OSJNBa0029D01	Nonphototropic hypocotyl 1 (protein kinase)	AK065447	2.2	3.5	3.9				
8872	6	P0416A11	Putative cell-wall protein	AK103715	4.8	3.9	2.4				
619	2	P0519E06	Protein kinase	AK060657	3.7	2.5	3.7				
871	8	P0013B04	α-Amylase	AK103413	2.3	3.8	3.0				
393	3	OSJNBa0017N12	Putative phosphatidylserine receptor long form	AK065339	2.9	2.2	3.2				
9046	4	OSJNBb0108J11	Unknown	AK102919	3.3	2.0	2.5				
120	5	OSJNBa0017N18	ADP glucose pyrophosphorylase	AK100910	2.5	2.0	3.0				
8956	3	OSJNBa0004L11	β-Glucosidase	AK103027	3.3	2.4	–				
3903	10	OSJNBa0062C05	Putative senescence-associated protein	AK103857	–	–	3.7				
749	6	P0505A04	Transmembrane amino acid transporter protein	AK073428	–	–	3.0				
4197	–	–	Unknown	–	–	–	2.9				
1723 [e)]	8	OJ1005_B05	ACT domain, putative	–	0.31	0.19	0.15	+	+	++	++
7050 [e)]	2	P0006C08	Unknown	AK069678	0.33	0.35	0.35	+	++	++	++

a): Fluorescence intensity ratios (each NIL/Koshihikari). Ratios between 0.50 and 2.0 are not shown.
b): B1417F08.21 was used as a substitute in *cis*-element search.
c): Average of induction ratio of 2 or 4 EST clones.
d): One EST clone, 9005, was located in the region of *qSc1-1*.
e): Two EST clones, 1723 and 7050, were repressed in microarray analysis, but their induction was clarified by Northern analysis.

Table 2. List of 15 unique genes repressed in at least one NIL after subculture in medium containing 2,4-D

EST clones	Chr	PAC or BAC clone	Gene identification	Full-length cDNA	Microarray analysis Induced ratio[a]			Northern analysis Signal strength			
					NIL#a	NIL#b	NIL#i	Koshi-hikari	NIL#a	NIL#b	NIL#i
1341	3	OSJNBa0023A13	Unknown	– [b]	0.26	0.18	0.25	++	+	+	+
1614	4	OSJNBb0049I21	Unknown	AK103013	0.22	0.22	0.20	++	+	+	+
2142 [c]	1	P0440D10	Jacalin-like lectin domain	AK062520	0.21	–	0.20	++	+	+	++
2216, 5948 [c]	1	P0043B10	Glutathione S-transferase	AK101436	0.30 [d]	– [d]	– [d]	++	+	+	++
3477	4	OSJNBa0004N05	Caleosin-related protein, putative	AK063625	0.30	–	0.30	++	+	+	++
6403	1	P0688A04	Cytochrome P450	AK063764	0.26	0.30	0.27	+++	+	++	++
8432	–	–	Unknown	–	0.23	0.17	0.14	+++	+++	++	++
439	4	OSJNBb0060M15	Unknown	–	0.30	0.21	0.28				
2382	4	OSJNBa0038O10	α-Amylase/subtilisin inhibitor	AK106723	0.38	0.43	0.31				
3389	5	OJ1004E02	VIP2 protein, putative	AK073521	–	0.47	0.32				
4860	1	P0485B12	Protease inhibitor/seed storage/ LTP family, putative	AK071598	0.19	0.26	0.40				
1916	9	P0501E09	Unknown	AK063582	0.22	–	0.22				
3660	8	OJ1300_E01	Ferredoxin I, chloroplast precursor	AK120393	–	–	0.34				
9016	6	P0564B04	ABC transporter-like protein	AK105826	0.32	–	–				
2964 [e]	2	OSJNBa0050G13	Unknown	AK121324	85.6	11.0	3.3	++	++	+	++

a): Fluorescence intensity ratios (each NIL/Koshihikari). Ratios between 0.50 and 2.0 are not shown.
b): OSJNBa0023A13.4 was used as a substitute in *cis*-element search.
c): EST clones 2142, 2216, and 5948 were located in the region of *qSc1-1*.
d): Average induction ratio of 2 EST clones.
e): An EST clone, 2964, was induced in microarray analysis, but was found to be repressed in Northern analysis.

cade. Three genes were located in the region of the *qSc1-1* (Fig. 1, Tables 1 & 2). It is not clear whether these genes were detected because of different transcript copy numbers or because of the different nucleotide sequences between Koshihikari and Kasalath.

Among the 22 induced genes (Table 1), six genes involved in seed germination were found, including cysteine endopeptidase (97, 378, 946, & 1068) and four genes contributing to starch metabolism: putative glycosyl hydrolase (8705), α-amylase (871), ADP glucose pyrophosphorylase (120), and β-glucosidase (8956). Genes associated with seed storage proteins were also found: two members of a protease inhibitor/seed storage/ LTP family (1539; 164), which might also be involved in the germination process. These results are consistent with the fact that calli of the NILs have more meristematic cells that proliferate well in comparison with Koshihikari callus.

Among the 15 repressed genes (Table 2), three genes were related to defense: Jacalin-like lectin (2142), glutathione S-transferase (2216 & 5948), and cytochrome P450 (6403). This is consistent with the fact that the calli of the NILs are sounder than that of Koshihikari. Three genes, caleosin-related putative protein (3477), α-amylase/subtilisin inhibitor (2382), and protease inhibitor/ LTP family (4860) were associated with seed storage proteins. VIP2 (3389) contained domains of the NOT2/ NOT3/NOT5 family; these domains form a nuclear complex that negatively regulates the basal and activated transcription of many genes (according to Pfam). This suggests that the NILs' calli cells were more activated in metabolism than those of the Koshihikari callus.

Ten genes had GA-response elements (GAREs), while ten had ABA-response elements (ABREs) (Table 3). Various GA-related *cis*-elements are known to act in concert with each other[15]. One of these is GARE as the main component, together with a pyrimidine box and an amylase element. Most genes having GARE also had other GA-related *cis*-elements in various combinations. This result implies that these GAREs are likely to act as *cis*-elements and that the genes are GA-responsive. The same goes for ABA-related *cis*-elements. Optimal ABA responsiveness usually requires the main component, ABRE, and other elements[7]. Ten genes had ABREs together with other ABA-related *cis*-elements, which implies these genes are likely to be ABA-responsive. In a microarray analysis in germinating Arabidopsis seeds, only 20% of promoters of GA-induced genes contained GARE-like sequences[14]. This suggests that factors other than GARE may regulate these genes. Amylase elements are known to be involved in the regulation of sugar level[8]. A total of 17 genes had sequences of GARE and/or amy-

Table 3. *Cis*-element search in the 500-bp region upstream from the initiation codon of each mRNA or the 5' terminus of each full-length cDNA clone

(A) Genes induced in NILs

EST clones	Gene identification	GA			ABA					Auxin	Ethylene	Nitrogen	
		GARE	Pyrimidine box	Amylase element	ABRE	DRE	AT-rich sequence	RY repeat	MYC	ARF	AGC box	Endosperm	Nia1 motif
97, 378, 946, 1068	Cysteine endopeptidase	4											
1539	Protease inhibitor/seed storage/LTP family, putative		1		1	1		1	2				
5350, 4191	Metallothionein-like protein	1		1	1				1				
8705	Putative glycosyl hydrolase				2								
8850, 2214	Unknown	1	3	1				1	2	1			
9005	SnoRNA	1	3		1						1		
7113, 7645	Metallothionein-like protein	3											
1492	Unknown		1	1	1	2		1			1		
164	Nonphototropic hypocotyl 1 (protein kinase)		2	1			1	1			1		
8872	Putative cell-wall protein		2					1	1				
619	Protein kinase		2						1		1		
871	α-Amylase			1		1							
393	Putative phosphatidylserine receptor long form		3										
9046	Unknown							1	2		1		
120	ADP glucose pyrophospho-rylase		2	1									
8956	β-Glucosidase					1	1	1	1		1		
3903	Putative senescence-associated protein					1				1			
749	Transmembrane amino acid transporter protein												
7050	Unknown		3		1		1		3				

(B) Genes repressed in NILs

EST clones	Gene identification	GA			ABA					Auxin	Ethylene	Nitrogen	
		GARE	Pyrimidine box	Amylase element	ABRE	DRE	AT-rich sequence	RY repeat	MYC	ARF	AGC box	Endosperm	Nia1 motif
1341	Unknown		1	1									
1614	Unknown												
2142	Jacalin-like lectin domain	1	1	1	1			2	1		1		
2216, 5948	Glutathione S-transferase	1	4										
3477	Caleosin-related protein, putative				3	1		1	1	1			
6403	Cytochrome P450		1										
2382	α-Amylase/subtilisin inhibitor			1	1	1							
3389	VIP2 protein, putative		1							1	1		
4860	Protease inhibitor/seed storage/LTP family, putative	1											
1916	Unknown		2	1		2						1	
3660	Ferredoxin I, chloroplast precursor		5		1			1	2				
9016	ABC transporter-like protein	2	1							2			1
2964	Unknown	1	3					1		1			

Sequence of *cis*-elements are as follows: GARE: TAAC(G/A)(A/T/G)A or CATGA(C/T)(G/A)TGG or CTACTC, pyrimidine box: (C/T)CTTTT or TTTTTTCC, amylase element: TATCCA(T/C) or TATCCA, ABRE: (T)ACGTG(G/T)C or ACGTSSSC (S = C/G), DRE: (G/A)CCGAC, AT-rich: CAAT(C/G)ATTG or CAATTATTA, RY repeat: CATGCA, MYC: CA(TG/CA)TG, ARF: TGTCTC, AGC box: AGCCGCC.

lase elements (Table 3), which indicates that more than half of the screened genes may be involved in germination.

Overall, NILs covering QTLs associated with callus proliferation were applied to a microarray analysis for the first time, and the results of the microarray analysis implied that the expression profiles of germination-related genes differed between each NIL and Koshihikari. More accurate microarray analysis using isogenic lines is needed to clarify characterization of the QTLs.

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