J. Plant Biol. (2011) 54:321–328
DOI 10.1007/s12374-011-9170-y

ORIGINAL RESEARCH

Dynamic QTL Analysis of Rice Protein Content and Protein Index Using Recombinant Inbred Lines

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Received: 23 April 2011 / Revised: 13 June 2011 / Accepted: 17 June 2011 / Published online: 7 July 2011

Abstract Protein content (PC) and protein index (PI) play important roles in determining nutritional quality in rice (*Oryza sativa* L.). We used 71 lines derived from "Asominori/IR24" to analyze the developmental behavior of PC and PI through unconditional and conditional QTL mapping methods. In all, 10 unconditional QTLs and 6 conditional QTLs for PC, and 11 unconditional QTLs and 9 conditional QTLs for PI, were identified at four stages of grain filling. More were identified in the first three stages than at the final stage. Temporal patterns of gene expression for PC and PI differed over time, with several QTLs being expressed across two or three stages but many being expressed at only one stage. Some of these QTLs were closely linked with maturity QTLs reported previously. Many QTLs for PC and PI were co-localized, supporting the significant correlation found between PC and PI. Our results suggest that dynamic QTL mapping might be a valid means for revealing more genetic information about protein accumulations during seed development.

Keywords Protein content · Protein index · QTL mapping · Rice (*Oryza sativa* L.)

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Abbreviations

DAF	Days after flowering
PC	Protein content
PI	Protein index
RFLP	Restriction fragment length polymorphism
RIL	Recombinant inbred line

As a food staple, rice (*Oryza sativa* L.) is a valuable source of energy, protein, and nutrients for half of the world's population. Its proteins are more nutritive than those from any other cereal crop. For example, the storage protein in rice is superior in lysine content to wheat, corn, and sorghum (Hegsted 1969), and it has a more balanced amino-acid profile. Nevertheless, enhancing its protein content (PC) has increasingly become one of the main breeding objectives for improving the nutritional quality of rice.

During grain filling, amino acids and other metabolites are transported into the developing seeds for the production of storage reserves. Thus, it is this biosynthesis of storage proteins and the final deposition of this major reserve that largely determines seed quality. To improve the efficiency of breeding efforts for that trait, we must understand the variability in expression of genes for controlling protein content.

Several QTLs for PC have been found in rice (Shi et al. 1999; Tan et al. 2001; Yoshida et al. 2002; Hu et al. 2004; Li et al. 2004; Zhang et al. 2008; Yu et al. 2009). For example, Hu et al. (2004) have detected five QTLs that collectively explain 74% of the total phenotypic variation, while Tan et al. (2001) have reported the identification of two QTLs for PC. However, most of these studies have utilized the final phenotypic value of traits at maturity but have been unable to provide information on genes at any

particular developmental time/stage. The process of protein accumulation in the seed is not a single event but, rather, it happens through a series of steps that are well orchestrated by the temporal and spatial expression of genes that begins soon after fertilization (Variath et al. 2009). Hence, variations might exist in expression and the genetic effects that are associated with different stages of grain development. Therefore, understanding the dynamic behavior of gene expression for PC during these processes is an important prerequisite for further revealing the genetic mechanism of rice protein.

The link between the developmental behavior of quantitative traits and molecular markers has been extensively examined in rice and cotton (Yan et al. 1998; Wu et al. 1999; Ye et al. 2003). Our study objectives here were to investigate the developmental genetics of PC and protein index (PI), another important indicator of quality in rice grains. We examined the dynamic QTLs for PC and PI developmental behavior by monitoring time-dependent changes. This involved identifying conditional QTLs for PC and PI that accounted for gene expression at a specific stage, as well as describing the temporal expression of those related genes.

Materials and Methods

Mapping Population

We examined 71 recombinant inbred lines (RILs) of rice (*O. sativa*), derived from a cross between "Asominori" and "IR24" that were developed by Tsunematsu et al. (1996) through the single-seed descent method.

Field Experiments

To synchronize their grain filling, we grouped the parents and 71 RILs into three bulks based on flowering time, as determined from data collected prior to this experiment. The RILs and their parents, "Asominori" and "IR24," were grown on the experimental farm of Nanjing Agricultural University, Nanjing, China, in a randomized complete design with two replications. Germinated seeds were placed in a seedling bed during the growing season of 2008, and the seedlings were transferred to a paddy field 30 days later, with a single plant per hill spaced at 15×20 cm. Each plot included two lines of 10 plants each. Field management essentially followed normal agricultural practices. Flowering time was recorded for each panicle. We divided the course of grain filling into four stages: (1) early, 1 to 7 days after flowering (DAF); (2) middle, 8 to 14 DAF; (3) late, 15 to 21 DAF; and (4) mature, 22 to 28 DAF. Panicles were then sampled at 7, 14, 21, and 28 DAF.

Trait Evaluation

The panicle samples were fixed and de-hulled as described by Wang et al. (2008). In two replications, 100 grains of sound brown rice were weighed to 0.001 g on a Hangping FA1004 balance (Shanghai Balance Instrument Factory, China). The average value from those replicates was referred to as the 100-kernel weight. The grains were then ground into power with a sample mill (JFS-13A; Qianjiang Machinery Co., Hangzhou, China) and passed through an 80-mesh sieve.

PC in this rice flour was determined by the semi-micro-Kjeldahl method (Chinese Bureau of Standardization 1982) and a Kjeltec 2300 Autoanalyzer (Foss AB, Sweden). A nitrogen conversion factor of 6.25 was used to compute the protein value. The protein index was described in terms of milligrams of protein per rice grain, calculated as PI=100-kernel weight×1,000×protein content/100. Each sample was analyzed in duplicate.

Statistical Analysis

7Following the method described by Tsunematsu et al. (1996), we created a linkage map of a RIL that comprised 375 RFLP markers and spanned a total of 1,275.4 cM on all 12 chromosomes, with an average interval of 4.4 cM between adjacent markers.

The unconditional phenotypic mean was assessed based on the phenotypic value at measuring time t [$y_{(t)}$] (Zeng 1993). The genetic effect of the unconditional QTL was the accumulation of individual gene effects from the initial time of grain development to a measuring time t:

$$y_{j(t)} = \beta_{0(t)} + \beta^{*}_{(t)}X^{*}_{j} + \sum_{i}\beta_{i(t)}X_{ij} + \varepsilon_{j(t)},$$

where $y_{j(t)}$ is the phenotypic value of the jth individual measured at time t; $\beta_{0(t)}$ is the population mean at time t; $\beta^{*}_{(t)}$ is the accumulated QTL effect at time t; X^{*}_{j} is the coefficient for the QTL effect; $\beta_{i(t)}$ is the accumulated effect for the ith marker at time t; X_{ij} is the coefficient for the ith marker effect; and $\varepsilon_{j(t)}$ is the residual error of the jth individual at time t.

We used a mixed model approach (Zhu 1995) to obtain the conditional phenotypic mean. The genetic effect of a conditional QTL was defined as the net genetic effect contributed by a specific developmental stage between time $t-1$ and time t ($t|t-1$):

$$y_{j(t|t-1)} = \beta_{0(t|t-1)} + \beta^{*}_{(t|t-1)}X^{*}_{j} + \sum_{i}\beta_{i(t|t-1)}X_{ij} + \varepsilon_{j(t|t-1)},$$

where $y_{j(t|t-1)}$ is the conditional phenotypic value of the jth individual; $\beta_{0(t|t-1)}$ is the conditional population mean; $\beta^{*}_{(t|t-1)}$ is the conditional QTL effect; X^{*}_{j} is the coefficient for the conditional QTL effect; $\beta_{i(t|t-1)}$ is the conditional effect for the ith marker; X_{ij} is the coefficient for the ith

marker effect; and $\varepsilon_{j(t|t-1)}$ is the conditional residual error of the *j*th individual.

Analyses of both conditional and unconditional QTLs were conducted using QTL Cartographer 2.5 (Wang et al. 2007) and a composite interval mapping module. QTL nomenclature followed that of McCouch et al. (1997).

Results

Phenotypic Analyses

Phenotypic values were obtained for PC and PI of the RIL population and its parents at four developmental stages (Table 1). The difference in PC and PI between the two parents was highly significant at all stages ($P<0.01$). Values for PC and PI were greater from "IR24" than from "Asominori" at most stages. During early filling, PC showed a high accumulation, then decreased at 14 DAF, before peaking at 28 DAF. Meanwhile, across all stages, PI displayed a consistent increment in "Asominori" but not in "IR24." We observed transgressive segregation of PC and PI in the RIL population at all stages (Table 1, Fig. 1).

Unconditional QTLs for PC

Ten unconditional QTLs that significantly influenced PC were identified at our four stages. These resided on nine of 12 chromosomes (Table 2, Fig. 2). No QTLs were present on Chromosomes 2, 5, or 11. Three QTLs were detected at the final stage between markers R886 and R1485 on Chromosome 1 (*qPC-1*) and XNpb212-G1318 on Chromosome 3 (*qPC-3.1*), as well as between markers C483 and C259 on Chromosome 8 (*qPC-8*). Of these, *qPC-8* was also detected at 21 DAF (i.e., Stage 3). At these loci, the alleles with increasing PC were from "Asominori" for *qPC-1* and *qPC-3.1*, and from "IR24" for *qPC-8*.

In addition to the significant QTLs that were detected in these three chromosomal regions at the final stage, another seven regions with significant QTLs were found at one or two other stages, with the phenotypic variation explained (PVE) ranging from 8.53% to 19.59%. Of these, *qPC-6* was detected at two consecutive stages—7 and 14 DAF. The others were recorded for only one stage.

At each stage, three QTLs significantly affected PC. None was detected during more than two stages probably due to the temporal expression of different genes.

Conditional QTLs for PC

A total of six genomic regions with conditional QTLs significantly affected PC values at different stages. They were mapped to five out of the 12 chromosomes (Table 2, Fig. 2). Four chromosomes (3, 6, 9, and 12) carried only one while Chromosome 7 had two significant regions. In five out of six genomic locations, QTLs were significantly detected at just one specific stage. Only *qPC-9* was expressed during two consecutive stages—7 and 14 DAF. This indicated that the relevant genes were being expressed continuously rather than there being a cumulative result of genetic effects for two specific growth stages in unconditional QTLs.

In all, three conditional QTLs affecting PC were detected at 7 DAF, thereby demonstrating that they were being expressed from the very beginning. Conditional QTLs numbered two for 14 DAF | 7 DAF, then decreased to zero for 21 DAF | 14 DAF before rising again to two for 28 DAF | 21 DAF. These results indicated that new genes for PC were expressed as the endosperm developed, a trend that was temporarily halted at 21 DAF but then continued at 28 DAF. QTL *qPC-7.2* accounted for 23.7% of the total variation at the final stage, having the larger effect.

Unconditional QTLs for PI

At all stages, we detected 11 unconditional QTLs for PI. These were distributed on 10 of 12 chromosomes (Table 3, Fig. 2). None was present on Chromosome 5 or 6. Three QTLs were detected at the final stage between markers

Table 1 Phenotypic values of protein content and protein index for recombinant inbred lines and its parents at four grain-filling stages

	Filling stage (day)	Parents		RIL	
		Asominori	IR24	Mean	Range
PC	7	9.38±0.01	10.76±0.01**	10.78±1.44	4.62–13.2
	14	9.11±0.01	10.44±0.01**	10.40±1.45	6.07–12.82
	21	8.53±0.01	9.19±0.01**	10.64±1.65	5.97–13.95
	28	9.42±0.01	11.45±0.01**	11.44±1.42	8.47–14.13
PI	7	0.50±0.00	1.04±0.00**	0.95±0.29	0.40–1.68
	14	1.05±0.00	1.56±0.01**	1.81±0.33	1.11–2.62
	21	1.69±0.00	1.52±0.00**	1.87±0.41	0.90–2.921
	28	2.16±0.00	2.03±0.00**	2.15±0.44	1.26–3.29

PC protein content, *PI* protein index, *RILs* recombinant inbred lines

**$P<0.01$

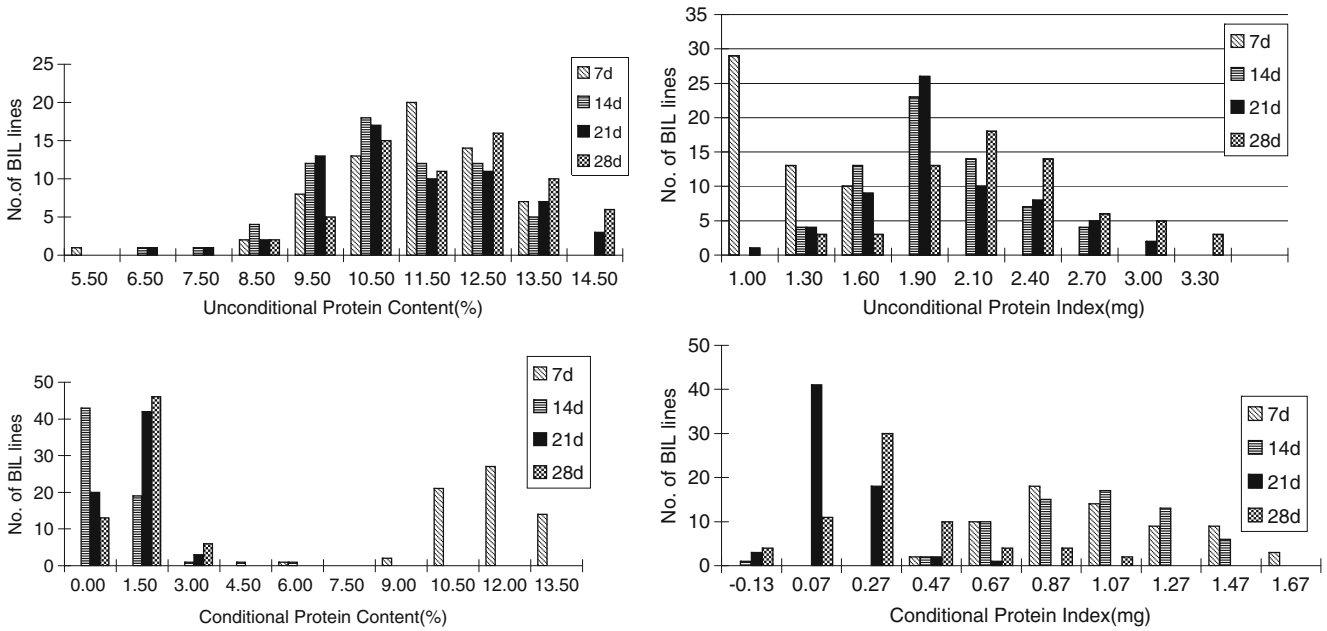

Fig. 1 Phenotypic distribution from unconditional and conditional analyses of PC and PI in RIL population of "Asominori/IR24" at four grain-filling stages

XNpb45G1314 on Chromosome 2 (*qPI-2*) and C609-XNpb108 on Chromosome 9 (*qPI-9.2*), and between markers XNpb148 and XNpb258 on Chromosome 12 (*qPI-12*). QTL *qPI-9.2* was also detected at both 7 and 21 DAF, indicating that the accumulated effects of this QTL were too small to be detected at 14 DAF, even though it was stably expressed afterward.

The numbers of unconditional QTLs for PI varied over time. Five were detected at the early stage, four at 14 DAF, and three at each of the last two stages. Moreover, both *qPI-8* and *qPI-10* were found during two separate stages.

Conditional QTLs for PI

Nine conditional QTLs for PI (Table 3, Fig. 2) were mapped to eight out of 12 chromosomes. For example, Chromosome 9 carried two significant regions while the rest had only one. In six of nine genomic locations, QTLs were significantly detected at only a single stage. Using a conditional method, we also detected unconditional QTL *qPI-8* at two stages (Table 3). Although QTL *qPI-11* was detected consecutively at 7 and 14 DAF, it manifested opposite effects between those times.

Five conditional QTLs affecting PI were detected for 7 DAF | initial. Another one was found for 14 DAF | 7 DAF, two for 21 DAF | 14 DAF, and four for 28 DAF | 21 DAF. QTL *qPI-3* accounted for 18.88% of the total variation at the final stage. The PVE for other QTLs was relatively small.

Discussion

Previous research on QTLs for rice protein has focused on a single developmental stage, usually maturity, although most genetic information is not revealed then. Here, we found only three QTLs for PC and three for PI at the final stage. However, by analyzing unconditional and conditional QTLs at other grain-filling stages, we were able to identify 12 QTLs for PC and 13 for PI. This demonstrated that more QTLs could be detected if monitoring was conducted at times other than seed maturity. Because a greater number of QTLs was detected in the three earlier stages, we can infer that more genes participate in protein synthesis at those times.

By comparing our results from conditional and unconditional mapping, we discovered that most of the conditional QTLs were identical to their unconditional QTLs. Four out of six QTLs for PC detected by conditional mapping were also found by unconditional mapping, while seven for PI were identical in both unconditional and conditional cases. Another four conditional QTLs for PC and PI went undetected by the unconditional method. Conditional mapping can detect QTLs acting at a specific growth period but not affected by the genes expressed in

Table 2 Unconditional and conditional quantitative trait loci (QTL) for protein content (PC) of recombinant inbred lines (RIsL) at four grain-filling stages

QTL	Marker interval	Type of QTL	7 DAF			14 DAF			21 DAF			28 DAF		
			LOD	A	PVE(%)	LOD	A	PVE(%)	LOD	A	PVE(%)	LOD	A	PVE(%)
qPC-1	R886-R1485	t										2.35	−0.48	9.91
		t | t−1												
qPC-3.1	XNpb212-G1318	t										3.15	−0.56	13.86
		t | t−1												
qPC-3.2	R758-XNpb15	t												
		t | t−1										3.59	−0.59	19.74
qPC-3.3	C606-XNpb238	t				3.24	−0.62	14.63						
		t | t−1												
qPC-4	R1854-R2373	t							3.55	0.66	15.65			
		t | t−1												
qPC-6	C1003-C688	t	2.09	−0.45	8.76	2.87	−0.54	12.67						
		t | t−1	2.09	−0.45	8.76									
qPC-7.1	XNpb338-C796	t	4.30	−0.67	19.59									
		t | t−1	4.30	−0.67	19.59									
qPC-7.2	XNpb268-R411	t												
		t | t−1										4.19	−0.72	23.70
qPC-8	C483-C259G	t							2.69	0.60	12.94	2.04	0.44	8.53
		t | t−1												
qPC-9	R265B-XNpb36	t	2.47	0.50	10.50									
		t | t−1	2.47	0.50	10.50	4.07	−0.40	13.97						
qPC-10	C16-C809	t							2.03	−0.50	8.54			
		t | t−1												
qPC-12	XNpb24-C562	t				3.84	−0.74	17.60						
		t | t−1				3.53	−0.46	16.15						

DAF days after flowering, *LOD* likelihood of odds, *PVE%* phenotypic variance explained, *A* additive effect, *QTL* quantitative trait loci, *PC* protein content, *RILs* recombinant inbred lines

the previous stages (Yan et al. 1998), which may explain why some conditional QTLs were not detected by unconditional mapping. Because unconditional QTLs explain the cumulative actions of a gene from the initial time to time *t*, the variation in its cumulative effects might be diminished if genes with opposite genetic effects are expressed at the same or nearby locations. This may have been the case with QTL *qPI-11* here.

Our study revealed multiple patterns of gene expression. Many QTLs for PC and PI were expressed at only one stage, while several, e.g., *qPC-6*, were expressed in two consecutive stages. In addition, some QTLs appeared at non-sequential intervals, such as *qPI-8*, which was found at 7 DAF and again at 21 DAF. This phenomenon indicates that some genes repeat their expression within different zones of time (Sun et al. 2006). On the other hand, an individual gene or genes in the same genomic region can have opposite genetic effects at various growth stages (Yan et al., 1998). At the map location of *qPC-9*, an unconditional QTL was detected at 7 DAF, but not afterwards (Table 2). This observation can be well explained by the conditional QTL mapping result. A conditional QTL with a negative genetic effect was detected for (14 DAF | 7 DAF), which might counteract the early gene effects at this locus. Similar observations were made at the *qPI-11* locus.

We noted that a few QTLs for PC and PI were closely linked with published maturity QTLs. For example, *qPC-3.2* and *qPC-3.3* were very near a QTL for PC in the interval RM251 and RM282 previously reported by Yu et al. (2009). Similarly, *qPC-6* was located in the *Wx* gene region, the same as Tan et al. (2001) has described. Other examples of this include *qPC-7.1* and *qPI-7.1* in the vicinity of a QTL for PC observed by Hu et al. (2004), i.e., between ZG34B and G20; and *qPC-10*, in the same region as a QTL for prolamin

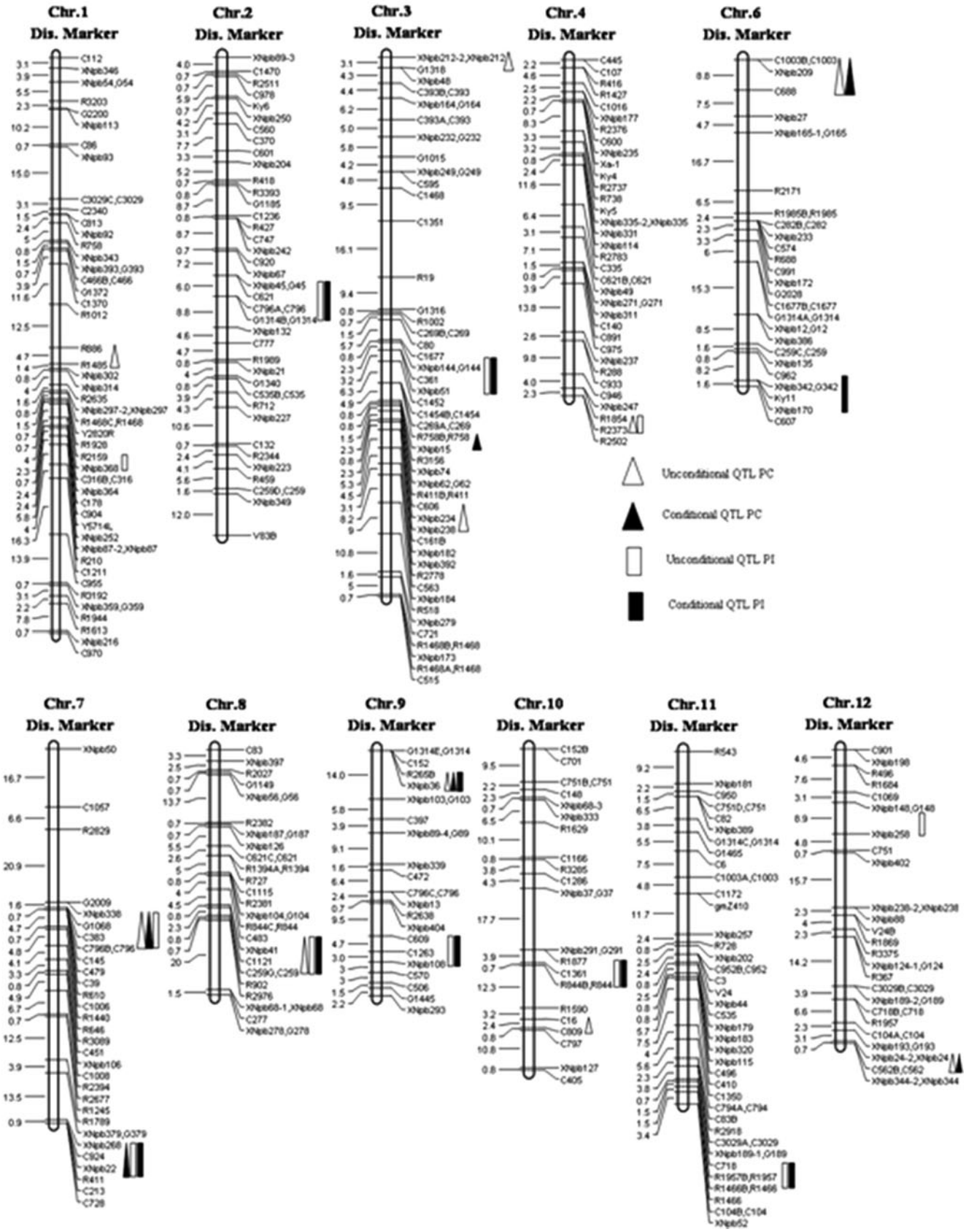

Fig. 2 Molecular linkage map showing locations of unconditional and conditional QTLs for PC and PI in RIL population of "Asominori/IR24"

Table 3 Unconditional and conditional quantitative trait loci (QTL) for protein index (PI) of recombinant inbred lines (RILs) at four grain-filling stages

QTL	Marker interval	Type of QTL	7 DAF			14 DAF			21 DAF			28 DAF		
			LOD	A	PVE(%)	LOD	A	PVE(%)	LOD	A	PVE(%)	LOD	A	PVE(%)
qPI-1	R2159-XNpb368	t				3.31	−0.13	13.84						
		t|t−1												
qPI-2	XNpb45-G1314B	t										2.38	−0.15	11.57
		t|t−1							4.28	−0.17	22.25			
qPI-3	C1677-XNpb51	t				2.29	−0.13	10.09						
		t|t−1										3.83	−0.12	18.88
qPI-4	R1854-R2373	t							2.72	0.14	11.34			
		t|t−1												
qPI-6	C962-XNpb170	t												
		t|t−1										3.17	0.11	14.54
qPI-7.1	XNpb338-C796	t				3.33	−0.13	14.54						
		t|t−1												
qPI-7.2	XNpb268-R411	t	2.07	−0.08	7.46									
		t|t−1	2.07	−0.08	7.46							2.17	−0.08	9.57
qPI-8	C483-C259G	t	4.25	0.13	16.97				3.32	0.16	15.48			
		t|t−1	4.25	0.13	16.97				2.16	0.08	9.80			
qPI-9.1	R265B-XNpb36	t												
		t|t−1										2.58	−0.09	11.46
qPI-9.2	C609-XNpb108	t	3.14	0.11	11.63				2.01	0.12	8.18	3.56	0.18	15.14
		t|t−1	3.14	0.11	11.63									
qPI-10	R1877-R844B	t	3.36	−0.12	15.33	3.56	−0.16	15.53						
		t|t−1	3.36	−0.12	15.33									
qPI-11	C718-R1466	t	2.60	0.10	9.55									
		t|t−1	2.60	0.10	9.55	3.03	−0.13	16.20						
qPI-12	XNpb148-XNpb258	t										2.41	0.14	10.15
		t|t−1												

DAF days after flowering, *PVE%* phenotypic variance explained, *A* additive effect, *LOD* likelihood of odds, *QTL* quantitative trait loci, *PC* protein content, *RILs* recombinant inbred lines

and glutelin contents (Zhang et al., 2008). Finally, our *qPC-12* coincided with a QTL for glutelin and crude protein contents described as well by Zhang et al. (2008). Therefore, the similarity in locations between our results and previous findings confirms a genetic component for protein content in rice grains. However, *qPC-3.2* was the only QTL to be expressed just at the mature stage. The fact that PC can be highly affected by environmental conditions may account for this. In addition, many factors, including the stage at which seed is harvested, the means by which samples are prepared, the procedure used for evaluating PC and PI, as well as random errors, all can influence the values obtained for PC and PI and the outcome of subsequent QTL-mapping.

PC was significantly correlated with PI ($P<0.05$) at all four stages. Moreover, many QTLs affecting PC and PI were mapped to the same genome regions. A total of five QTL clusters for PI and PC were found on Chromosomes 4, 7, 8, and 9. Co-localization of these QTLs, as a result of either pleiotropic effects or close linkage, may explain the genetic basis for correlations among those two quality traits.

Conclusions

Our conditional and unconditional QTL analyses for PC and PI at four filling stages showed that the accumulation of protein in rice grains is governed by time-dependent gene expression. More QTLs for PC and PI were detected when the entire process of filling was examined rather than concentrating only on the final stage. Some QTLs existed across two or three stages while others were detected only once. Therefore, our approach to obtaining genetic information demonstrated that a thorough and dynamic QTL analysis is more

productive when evaluating grain development. Several QTLs for PC and PI were closely linked with published maturity QTLs while others were co-localized and expressed at different stages.

Acknowledgements This research was supported by grants from the National Key Transformation Program (2008ZX01001-006), the National Natural Science Foundation of China (30971759), the Jiangsu Cultivar Development Program (BE2008354 and BE2009301-3), the National Science and Technology Supporting Program, and the ear-marked fund for a Modern Agro-industry Technology Research System.

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