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ORIGINAL PAPER

**Mitsuhiro Obara · Tadashi Sato · Shohei Sasaki
Kenji Kashiba · Atsushi Nagano · Ikuo Nakamura
Takeshi Ebitani · Masahiro Yano · Tomoyuki Yamaya**

Identification and characterization of a QTL on chromosome 2 for cytosolic glutamine synthetase content and panicle number in rice

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Abstract A quantitative trait locus (QTL) associated with the protein content of cytosolic glutamine synthetase (GS1; EC 6.3.1.2) in senescing leaves, panicle number, and panicle weight was characterized in rice (*Oryza sativa* L.). A near-isogenic line (NIL), C-22, developed by marker-assisted selection was grown under different nitrogen levels in the greenhouse and in a paddy field. Chromosome 2 of C-22 had an approximately 50-cM segment substituted from the Kasalath (*indica*) chromosome in a Koshihikari (*japonica*) genetic background. C-22 showed a 12–37% lower content of GS1 protein in leaf blades than Koshihikari, which was in good agreement with a QTL region positively affected by the *japonica* chromosome. At an early vegetative stage, C-22 had more active tillers than Koshihikari in the greenhouse. At the reproductive stage, both panicle number and total panicle weight of C-22 were significantly higher than those of Koshihikari, particularly when the plants were grown under a low-nitrogen condition. These traits of C-22 were further confirmed in a paddy field. Thus, tiller development was positively affected by the Kasalath chromosome at an early vegetative stage, which resulted in an increased panicle number and panicle weight at the mature stage in C-22. These data indicate that the target QTL (*Pnn1*; *panicle number 1*) is important in the development of tillers and panicles in rice. Linkage analyses for panicle number and ratio of developing tiller formation in the second axil (RDT) revealed that *Pnn1* was delimited at the 6.7-cM region.

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M. Obara · S. Sasaki · K. Kashiba · A. Nagano · T. Yamaya
Graduate School of Agricultural Science,
Tohoku University, 1-1 Tsutsumidoori-Amamiyamachi,
Aoba-ku, Sendai 981-8555, Japan

M. Obara
Research Fellow of the Japan Society for the Promotion of Science,
6 Ichibancho, Chiyoda 102-8471, Japan

T. Sato (✉)
Graduate School of Life Sciences, Tohoku University,
2-1-1 Katahira, Aoba-ku, Sendai 981-8577, Japan
E-mail: tadashi@ige.tohoku.ac.jp
Fax: +81-22-2175689

I. Nakamura
Graduate School of Science and Technology,
Chiba University, 648 Matsudo, Chiba 271-8510, Japan

T. Ebitani
Toyama Agricultural Research Center,
1124-1 Yoshioka, Toyama 939-8153, Japan

M. Yano
Department of Molecular Genetics,
National Institute of Agrobiological Sciences,
2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan

T. Yamaya
Plant Science Center, RIKEN, 1-7-22 Suehiro-cho,
Tsurumi-ku, Yokohama 230-0045, Japan

Introduction

Agronomic traits, such as nitrogen use efficiency, the size of seeds, and time for heading, are determined by the interaction of multiple gene functions. This is also true for biochemical and/or physiological traits such as the levels and activities of enzymes involved in plant metabolism. The analysis of quantitative trait loci (QTLs) using DNA markers is an efficient method for estimating the number of genes involved with these traits and the loci on chromosomes (Tanksley 1993). QTL analysis using interspecific crosses has been conducted to identify loci controlling such traits; for example, fruit size of tomato (Frary et al. 2000), elongation factor content in maize endosperm (Wang et al. 2001), and time to heading of rice (Yano et al. 2000; Takahashi et al. 2001). Hirel et al. (2001) demonstrated the mapping of QTLs for nitrogen use efficiency and various enzyme

activities related to nitrogen assimilation and metabolism in maize. Our group has also mapped QTLs associated with cytosolic glutamine synthetase (GS1; EC 6.3.1.2) levels in senescing leaves and those associated with NADH-glutamate synthase (NADH-GOGAT; EC 1.4.1.14) levels in non-green, unexpanded immature leaves, together with QTLs for various agronomic traits in rice (Obara et al. 2001; Yamaya et al. 2002).

In Sasanishiki, which is a popular cultivar of *japonica* rice (*Oryza sativa* L.) in northern Japan, approximately 80% of the total nitrogen found in the panicle has been remobilized through the phloem from senescing organs (Mae and Ohira 1981). Consequently, an understanding of the process of nitrogen recycling is very important in determining both the productivity and the quality of the rice grain. Glutamine and asparagine are the major forms of nitrogen in the phloem sap of rice plants (Hayashi and Chino 1990). As the synthesis of asparagine from glutamine is mediated by asparagine synthetase (Lea et al. 1990; Sechley et al. 1992; Ireland and Lea 1999), the first step in nitrogen recycling in rice plants is the synthesis of glutamine in senescing organs. In plants, glutamine synthetase (GS) is the only enzyme capable of synthesizing glutamine by catalyzing the ATP-dependent condensation of NH_3 with glutamate (Ireland and Lea 1999). In rice, there are two or possibly three cytosolic GS isoenzymes (Tabuchi et al. 2004) and one chloroplastic/plastidic GS (Sakurai et al. 1996). GS1 may be an important mediator in the export of nitrogen from senescing leaves as GS1 protein in rice has been located in companion cells, which are important for the phloem loading of solutes, and vascular parenchyma cells (Sakurai et al. 1996; Obara et al. 2000). This particular function of GS1 has also been supported by Dubois et al. (1996) in tobacco and Avila et al. (2001) in Scots pine. Some *indica* cultivars, including Kasalath, have been found to contain twice as much GS1 protein in their senescing leaf blades as *japonica* cultivars (Obara et al. 2000). In general, the total biomass production of *indica* cultivars is greater than that of *japonica* cultivars (Takahashi 1984), and this smaller capacity of source strength, including a less efficient export of glutamine via the GS1 reaction, may be the cause of this lower biomass production in the *japonicas*. However, the molecular mechanism involved in the regulation of GS1 levels is not known.

To identify the regulatory gene(s), we mapped seven QTLs associated with GS1 protein content using 98 backcross inbred lines (BILs) developed between Nipponbare, a *japonica* cultivar, and Kasalath, an *indica* cultivar (Obara et al. 2001; Yamaya et al. 2002). Some of these QTLs were co-located in QTL regions for physiological and agronomical traits affected by nitrogen recycling (Obara et al. 2001; Yamaya et al. 2002). A putative QTL for GS1 protein content linked to the DNA marker C777 on the long arm of chromosome 2 was located at the QTL regions for soluble protein content, panicle weight, and spikelet number. In this case, the alleles from Nipponbare contributed to an increase in both GS1 and soluble protein content, while the alleles negatively contributed to an increase in panicle weight and spikelet number on the main stem. Although the structural gene encoding GS1 was detected on the short arm of chromosome 2, it was not co-localized with any QTL for GS1 protein content. This suggests that the structural gene itself does not contribute to the determination of GS1 protein content in rice.

An efficient way of characterizing the target QTLs would be to identify phenotypic expression using nearly isogenic lines (NILs) (Lin et al. 2000; Nesbitt and Tanksley 2001). Agronomical and physiological traits such as yield and its components fluctuate greatly with the level of fertilizer supply. Nitrogen availability is one of the most important factors in the soluble protein content in a flag leaf, spikelet number per panicle and panicle weight in rice (Makino et al. 1988; Mae 1997).

In the study reported here, we characterized QTLs for GS1 protein content and panicle weight on the long arm of chromosome 2 using NILs grown under greenhouse and paddy field conditions and supplied with various levels of nitrogen. A QTL for panicle number was also delimited using substituted lines at the early vegetative stage.

Materials and methods

Plant materials and growth conditions

A rice (*Oryza sativa* L.) cv. Koshihikari (*japonica*) was crossed with *indica* cv. Kasalath, and the plant materials used in this study were selected from advanced backcross progenies between Koshihikari as the recurrent parent and Kasalath as the donor parent by marker-assisted selection (MAS; T. Ebitani et al., unpublished data). The seeds were germinated and sown on a synthetic culture soil (Mitsui-Toatsu No.3, Tokyo, Japan) in a cell [1.5 (dia.) × 3 cm] tray (60×30 cm). Both Koshihikari and the line selected as the NIL were planted in a greenhouse in pots or in a paddy field, respectively. Three seedlings were planted in 4-l pots containing 3 kg of soil supplement in a greenhouse in 2001 and 2002, and each was provided with one of three different levels of nitrogen fertilizer as described previously (Yamaya et al. 1997). The amount of nitrogen fertilizer supplied per pot was 0.32 g, 0.64 g, and 1.28 g of active ingredient in the form of fertilizer (N, 16%; P, 16%; K, 16%: Coop Chemical Co., Tokyo, Japan), designated 1/2N (low level), 1N (normal level) and 2N (high level), respectively. Also supplied to each pot was 1.28 g each of phosphorus and potassium active ingredient in the form of fertilizer; this was done by adjusting the amount of phosphorus potassium fertilizer (P, 20%; K, 20%: Coop Chemical Co.).

Twenty seedlings were planted in a paddy field in 2001, in Kashimadai, Miyagi, Japan. These were given 0 g, 0.6 g, 1.2 g, or 2.4 g active ingredient of nitrogen fertilizer per square meter in the form of fertilizer (N, 16%; P, 16%; K, 16%: Coop Chemical Co.), designated 0N, 1/4N, 1/2N and 1N, respectively. Phosphorus and potassium fertilizer were supplied at 2.4 g active ingredient per square meter by adjusting the level of the phosphorus potassium fertilizer (P, 20%; K, 20%: Coop Chemical Co.).

For the selection of substituted lines (SLs), the seeds were germinated and sown on a synthetic culture soil in a small container as described above. These seeds were the next generation of 7 of 42 inbred progeny.

For delimitation of the target QTL, Koshihikari, the selected NIL, and SLs were germinated and sown on synthetic culture soil in a cell (2.5×2.5×4.5 cm) tray (60×30 cm). These plants were grown in chambers (continuous 25°C) under natural irradiation with supplementary lighting. Ten seedlings of cv. Koshihikari or of each corresponding line were planted in a paddy field at Kashimadai, Miyagi, Japan in 2003 and supplied with 1.2 g of nitrogen, 2.4 g each of phosphorus and potassium as an active ingredient per square meter (N, 10%; P, 16%; K, 16%: Coop Chemical Co. Ltd, Tokyo, Japan)

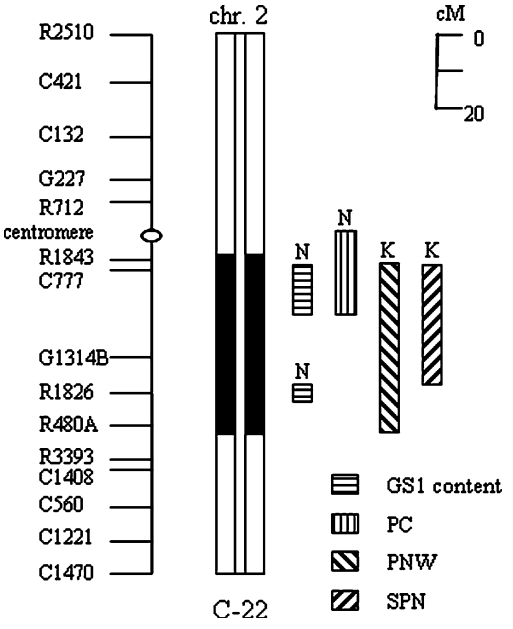

Fig. 1 Graphical presentation of the genotype of NIL C-22 with respect to chromosome 2. *Open and filled regions* represent segments derived from Koshihikari and Kasalath, respectively. The QTLs for GS1 and soluble protein content in senescing leaf blades and for agronomic traits are indicated: *GS1 content* GS1 protein content in senescing leaf blades, *PC* soluble protein content in senescing leaf blades, *PNW* panicle weight on the main stem, *SPN* spikelet number on the main stem. *N* or *K above* the individual column indicates the positive allele from Nipponbare and Kasalath, respectively. Genetic distance, in centiMorgans, is based on linkage analysis of 98 BILs (Lin et al. 19981998). A probability of less than 0.05 was used to define the borders of confidence intervals for QTLs (modified from Obara et al. 2001)

Genotype mapping and NIL selection

DNA was extracted as follows. The region 1 cm from the tip of the leaf blade was placed into a microtube and homogenized with a pestle in an extraction buffer [200 m *M* Tris-HCl (pH 8.0), 250 m *M* NaCl, 25 m *M* EDTA, 0.5% (w/v) sodium dodecyl sulphate (SDS)]. After incubation for 60 min at 60°C, DNA was precipitated with isopropanol and then resupended in 50 µl of TE buffer [10 m *M* Tris-HCl (pH 8.0), 1 m *M* EDTA].

Three sequence tagged sites (STS) and two cleaved amplified polymorphic sequence (CAPS) markers for genotype mapping of each line were obtained from a database established by the Rice Genome Research Program (http://rgp.dna.affrc.go.jp/publicdata/caps/index.html). Three STS and nine CAPS markers were designed (Table 1) based on each cDNA sequence or genome sequence database of Nipponbare established by the Rice Genome Research Program (http://rgp.dna.affrc.go.jp/publicdata/geneticmap2000/index.html or http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/status.pl). Fragments amplified by PCR were digested with the

Table 1 STS and CAPS markers for genotype mapping

Marker name	Type	Enzyme	Forward primer sequence	Reverse primer sequence
2-S116	STS		5′-gggaagtgcgtacgtttgtt-3′	5′-ttttcgtaagttgggggatg-3′
2-S125	STS		5′-ggatgcacatttggattgtc-3′	5′-ttaggtcggtccacacacaa-3′
2-S150	STS		5′-taagggatgggcttgaattg-3′	5′-ggtttcgtcgtccttgatgt-3′
2-S101	CAPS	*Hae*III	5′-ccatagctgatctgcttcg-3′	5′-gccccacacctagcttcata-3′
2-S136	CAPS	*Hin*fI	5′-aagtttaaacgcgctgttgc-3′	5′-cgccatgtcgacaacagtta-3′
2-S152	CAPS	*Hin*fI	5′-tgggtaaatctgccctcttg-3′	5′-ttctgcacttgccattcttg-3′
2-S159	CAPS	*Xho*I	5′-ctgagtccatccttgccaat-3′	5′-ccacaagtcctccacacctt-3′
2-S167	CAPS	*Hinc*II	5′-cccctattgaatgggttgtcca-3′	5′-gatgtgggcatcaaaagaaggg-3′
2-S173	CAPS	*Eco*RI	5′-tgcatccaggtaggcaactcaa-3′	5′-aagcagtgtccgatatgcacca-3′
2-S303	CAPS	*Dra*I	5′-cgaactaaacagggcccata-3′	5′-tcggccaaatgtccttaaac-3′
2-S307	CAPS	*Hpa*I	5′-cccagagatcccttaccaca-3′	5′-cgaaagagagcggcattaac-3′
R480A	CAPS	*Hae*III	5′- attctggtgttgggaaatcg-3′	5′-atttgcagctgcctaaccat-3′

appropriate restriction enzymes, and then separated by electrophoresis on a 0.8% agarose gel (2-S116, 2-S125, 2-S150, 2-S136, 2-S303, 2-S307 and R480A) or a 2% agarose gel (2-S101, 2-S152, 2-S159, 2-S167 and 2-S173) (Goto et al. 1998). A total of 17 STS and CAPS markers were used to estimate the substitution status of the chromosome segments in all selected plants. These markers were mapped using MAPL98 (Ukai et al. 1995) in 200 plants with a segregated genotype. In 99KF1-14-1, the region (approximately 50 cM) was heterozygous and other regions were homozygous for the Koshihikari allele on chromosome 2 (Fig. 1); almost all of other chromosome regions were homozygous for the Koshihikari allele. These 42 inbred progeny were used for NIL selection. Five STS (C777 and C10005) and CAPS (R1843, S10844 and R480A) markers covering the 50-cM segment from the Kasalath chromosome of C-22 were used to estimate the substitution status of chromosome segments in the first selection. The genotypes of 103 of 703 plants were confirmed to have the Koshihikari or Kasalath chromosome by all marker loci tested. In addition, 17 STS and CAPS markers were used to estimate the substitution status of chromosome segments in second selection. The genotypes of the next generation derived from the first selected plants were confirmed as carrying the Koshihikari or Kasalath chromosome, respectively, for all marker loci tested. Finally, 92 plants in which a 50-cM segment from the Kasalath chromosome of C-22 was substituted were selected, and these plants were classified into nine SLs based on MAS. These plants were planted in a paddy field in 2002, at Kashimadai, Miyagi, Japan. Linkage analysis was performed using the QGENE program (Nelson 1997).

Quantification of GS1 protein by immunoblotting

When the 12th leaf blade from the base of the main stem of each plant had just emerged from the 11th leaf sheath, the 8th to11th leaves were harvested. Fresh leaves were weighed, frozen in liquid nitrogen, and stored at −80°C until used. Three independent samples at all leaf positions were provided for the quantification of GS1 protein. The frozen leaf samples were homogenized with a mortar and pestle in the presence of washed sand in an extraction buffer as described previously (Yamaya et al. 1995). The crude protein fraction was prepared from the homogenate, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and immunoblotted with affinity-purified GS1 IgG as described previously (Yamaya et al. 1992). Following the immunoreaction, GS1 polypeptides were visualized and quantified densitometrically (Hayakawa et al. 1993). Soluble protein content was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Evaluation of physiological traits

The number of developing tillers and leaf length were measured at 14, 21, 35, 42 and 49 days after planting. Leaf age was estimated on the basis of leaf length. For the total nitrogen measurements, whole shoots at planting were harvested, dried in an oven at 70°C for several days, weighed, and powdered. Total nitrogen was determined following Kjeldahl digestion as described previously (Makino et al. 1984). At 40 days after heading, the panicle was harvested and dried for 30 days in a well-ventilated room with an electric fan. Panicle weight, shoot weight, and panicle number were then measured. The ratio of developing tiller formation in the second axil (RDT) was measured using SLs at 24 days after sowing.

The data were analyzed using the Excel statistical analysis 97 package (SSRI Co., Tokyo, Japan), and the differences in mean values between Koshihikari and C-22 were determined by paired Student's *t* test.

Results

Development of the NIL C-22

The segregating population developed for NIL selection was an inbred progeny derived from 99KF1-14-1, in which small segments of a chromosome from Kasalath, an *indica* cultivar, were substituted in a Koshihikari, a *japonica* cultivar, genetic background (T. Ebitani et al., unpublished data). The line 99KF1-14-1 is heterozygous on parts of chromosomes 2 and 11. QTLs for GS1 protein content in senescing leaves as well as agronomic traits, such as panicle weight and spikelet number, had been previously found in the heterozygous region of chromosome 2 (Obara et al. 2001). MAS resulted in a plant in which, with respect to chromosome 2, the region around these QTLs was homozygous for Kasalath while almost all of the other regions were homozygous for Koshihikari (Fig. 1). Other chromosomes were almost entirely homozygous for Koshihikari (data not shown). This line was designated C-22 and used for subsequent analyses.

Comparison of GS1 and soluble protein at various leaf positions

The QTL from Kasalath contributes to a decrease in both GS1 and soluble protein content (Obara et al. 2001). This contribution of the Kasalath allele on chromosome 2 was confirmed in the current study using lines from Koshihikari crossed with Kasalath. Under 1/2N and 1N conditions GS1 protein levels in C-22 were 12–37% lower than those in Koshihikari at all leaf positions (Fig. 2a, b). Soluble protein content in C-22 was also 6–46% lower than that in Koshihikari. How-

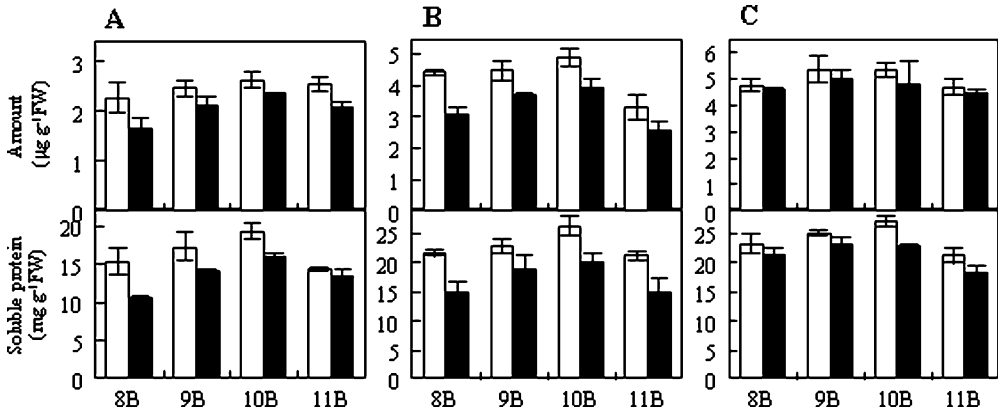

Fig. 2 Changes in GS1 protein content (*upper panels*) and soluble protein content (*lower panels*) in leaf blades of various leaf positions from Koshihikari (*open bars*) and C-22 (*filled bars*). Plants were grown under conditions of 1/2N (**a**), 1N (**b**), and 2N (**c**), respectively, in the greenhouse. The contents are expressed as micrograms of GS1 protein on a fresh weight basis, using purified GS1 as the standard. Independent triplicate analyses with different samples were performed and the mean values plotted. *Vertical bars* represent the standard deviation ($n=3$), *numbers* indicate leaf positions

ever, under the 2N condition, the contents of GS1 and soluble protein in C-22 were identical or slightly lower than those in Koshihikari (Fig. 2c). Because the level of leaf soluble protein varies with leaf age (Kamachi et al. 1991), GS1 content estimated on a basis of leaf fresh weight was plotted against the level of leaf soluble protein to roughly eliminate any variation resulting from leaf age. In all of the leaves of Koshihikari and C-22 grown with different levels of nitrogen, soluble protein showed a good correlation ($r^2=0.84$) with GS1 protein content (data not shown). Thus, the changes in GS1 protein content brought about by the presence of the Kasalath allele were mainly caused by changes in soluble protein content.

Effect of the Kasalath allele on yield and its components under various levels of nitrogen

The total panicle number of C-22 grown in the greenhouse in 2001 and supplied with 1/2N, 1N, and 2N was 36.8%, 29.0%, and 18.7% higher than that of Koshihikari, respectively (Table 2). This was reproduced in the greenhouse in 2002. The total panicle weight of C-22 grown in the greenhouse in 2001 and supplied with 1/2N and 1N was 50.3% and 20.3% higher than that of Koshihikari, respectively (Table 2). Under the 2N condition, there was no significant difference in total panicle weight between C-22 and Koshihikari. Total panicle weight of C-22 supplied

Table 2 Differences in total panicle number and panicle weight between Koshihikari and C-22 grown in the greenhouse and in the field under various levels of nitrogen

Growth condition	Mean								
	Panicle number (plant^{-1})			Total panicle weight (g)			Panicle weight on the main stem (g)		
	Koshihikari	C-22	P^d	Koshihikari	C-22	P^d	Koshihikari	C-22	P^d
2001[a]									
1/2N	5.4	7.4	0.005	7.5	11.3	<0.0001	1.9	2.0	0.10
1N	6.9	8.9	0.02	13.3	16.1	0.0005	2.7	2.3	0.06
2N	10.1	12.0	0.02	20.3	22.4	0.15	2.8	2.6	0.17
2002[b]									
1/2N	5.4	8.1	<0.0001	11.0	13.0	<0.0001	2.2	1.9	0.003
1N	8.2	9.4	0.02	18.3	18.6	0.21	2.7	2.3	0.009
2N	13.3	16.1	0.001	27.7	27.9	0.28	2.5	2.1	0.009
2001[c]									
0N	17.4	21.8	<0.0001	39.1	44.1	0.003	3.0	2.7	0.001
1/4N	21.3	24.0	0.003	45.2	47.4	0.24	3.0	2.7	0.0005
1/2N	22.4	26.6	0.0002	49.8	51.1	0.44	3.1	2.7	0.0001
1N	25.8	28.9	0.01	55.1	58.0	0.24	3.0	2.7	0.0001

[a] Plants grown in the greenhouse in 2001 were supplied with 1/2N, 1N, or 2N nitrogen. Data are means from nine plants
[b] Plants grown in the greenhouse in 2002 were supplied with 1/2N, 1N, or 2N nitrogen. Data are means from 12 plants
[c] Plants grown in the field in 2001 were supplied 0N, 1/4N, 1/2N, or 1N nitrogen. Data are means from 20 plants
[d] *P* values from paired Student's *t* tests for the difference of means between Koshihikari and C-22 at each growth condition

with 1/2N also increased in the greenhouse in 2002. Thus, the QTLs for total panicle number and total panicle weight were confirmed to be present in the substituted region of Kasalath chromosome, particularly when plants were grown at low and normal levels of nitrogen. The gene activity for this phenotype in the substituted region was designated as *Pnn1* (*panicle number 1*).

Under the various level of nitrogen, panicle weight on the main stem of Koshihikari plants was significantly higher than on that of C-22 in the greenhouse in 2002, but it was not significant in 2001. The Kasalath allele had no effect on single-spikelet weight at any the nitrogen concentrations tested (data not shown).

The contribution of the Kasalath allele to an increase in total panicle number and total panicle weight obtained in the greenhouse experiment was confirmed in the paddy field. The total panicle number of C-22 grown with 0N, 1/4N, 1/2N and 1N was 25.3%, 13.7% 18.9%, and 12.0% higher than that of Koshihikari, respectively (Table 2). Without any additional nitrogen supply, C-22 had a 13.1% higher total panicle weight than Koshihikari, which is a significant difference at the 1% level. When nitrogen was applied, there was no significant difference in total panicle weight between Koshihikari and C-22. Panicle weight on the main stem in Koshihikari was significantly higher than on that of C-22, although providing nitrogen at different amounts had no influence on panicle weight on the main stem of these plants.

Total panicle weight of plants grown under various nitrogen levels in the greenhouse in 2001 was plotted against shoot dry weight (Fig. 3). All plants of Koshihikari and C-22 grown in the greenhouse and in the field showed a positive correlation ($r^2 = 0.97$ in 2001 greenhouse, $r^2 = 0.94$ in 2002 greenhouse, $r^2 = 0.98$ 2001 field)

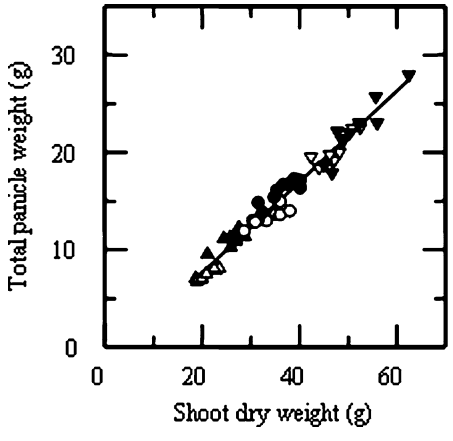

Fig. 3 Relationship between total shoot dry weight and total panicle weight of Koshihikari (*open symbols*) and C-22 (*filled symbols*) grown in the greenhouse and supplied with various levels of nitrogen: 1/2N (*triangle*), 1N (*circle*), 2N (*inverse triangle*)

between total panicle weight and total shoot dry weight, indicating little difference in yield per unit of shoot dry weight.

Effect of the Kasalath allele on plant growth under various levels of nitrogen

The life span of a rice plant consists of basically two distinct sequential growth stages—vegetative and reproductive. The vegetative stage refers to the period from germination to the initiation of panicle primordia, while the reproductive stage is subdivided into the preheading—i.e., from the initiation of panicle primordia to heading— and postheading (ripening)—i.e., from heading to maturity—stages (Yoshida 1981). At 44 days after planting in the greenhouse, internode elongation was observed in Koshihikari and C-22. All plants of Koshihikari and C-22 reached heading between 75 days and 77 days after planting. These phenotypic profiles were identical even when the plants were supplied with different concentrations of nitrogen. The two lines could be distinguished by their growth from the vegetative stage (sowing to 44 days after planting) to the reproductive stage, which varied from 44 days to 76 days. Thus, the Kasalath allele did not contribute to the growth phase under the growth and nutrient conditions of this experiment.

Typical phenotypes of Koshihikari and C-22 plants in the greenhouse at 25 days after planting and at the mature stage are shown in Fig.4a–d when grown under the 1/2N (minimum) and 2N (maximum) conditions. At the early vegetative stage, C-22 showed more vigorous growth than Koshihikari under all nitrogen conditions (Fig. 4a, b). At 21 days after planting, tiller number per plant in C-22 grown under the 1/2N and 2N conditions was 37.2% and 40.3% higher than that in Koshihikari, respectively (Fig. 4e, f). In the greenhouse, leaf expansion of C-22 was faster than that of Koshihikari. When these lines were grown in the paddy field, C-22 also had more tillers at the vegetative stage than Koshihikari. At the vegetative stage (33 days after planting), the tiller number of C-22 grown under the 0N, 1/4N, 1/2N and 1N conditions was 82%, 74%, 69%, and 83% higher, respectively, than that of Koshihikari grown under the same conditions, and the differences were significant ($P < 0.0001$, $n = 20$).

At transplanting (39 days after sowing), total nitrogen content of the shoot was 0.268 mmol and 0.321 mmol in Koshihikari and C-22, respectively. Dry weight of shoot was 0.151 g and 0.198 g, respectively, in Koshihikari and C-22. Significant differences ($P < 0.01$, $n = 3$) were observed in both nitrogen content and dry weight of the shoot. These data suggested that the Kasalath allele contributed to the increase in the rate of absorption for nitrogen at the early stage of vegetative growth.

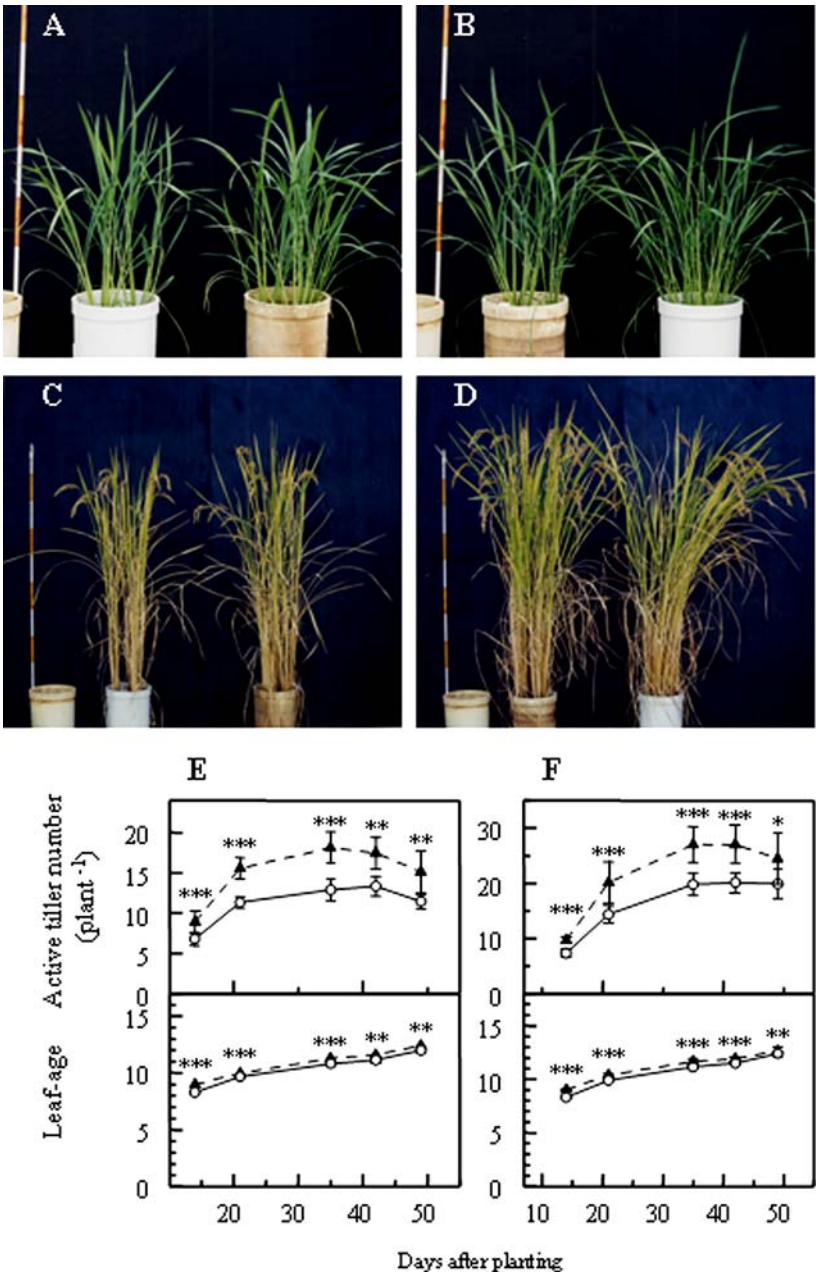

Fig. 4 Typical phenotype of Koshihikari and C-22. Morphological characters of Koshihikari (*left*) and C-22 (*right*) at 25 days after planting (**a**,**b**) and at the mature stage (**c**,**d**). Changes in active tiller number (*upper panels*) and leaf age (*lower panels*) of Koshihikari (*open circle*) and C-22 (*filled triangle*). Plants were grown under conditions of 1/2N (**a, c, e**) and 2N (**b, d, f**) in the greenhouse. *Vertical bars* indicate the standard deviation($n = 9$). *, **, and *** represent significance differences between Koshihikari and C-22 at the 5%, 1%, and 0.1% level, respectively (paired Student's *t* test)

Delimitation of *Pnn1* using substituted lines at the Kasalath chromosome in C-22

Nine SLs, in which the 50-cM segment from Kasalath chromosome in C-22 was substituted (Fig. 5), were used for the delimitation of *Pnn1*. The ratio of developing tiller formation in the second axil (RDT) of those SLs was determined because panicles were normally produced from tillers developed at the early vegetative stage (Yoshida 1981). The RDT of Koshihikari and C-22 were, respectively, 46% and 79% at 24 days after sowing. Those nine SLs were segregated to either the Koshihikari type or the C-22 type (Fig. 5). The Koshihikari type was seen in C30-3, C6-6, C4-14, and C6-5, for which the RDT was lower than that in Koshihikari. The C-22 type was confirmed in C42-3, C42-9, C33-2, C34-3 and C2-7, for which the RDT was higher than that in C-22. Tight linkage was observed between tiller number at an early stage and panicle number at the mature stage. There were no significant differences in total panicle weight among the nine SLs under the growth conditions applied in 2003. Linkage analysis showed that *Pnn1* was tightly linked to markers 2-S152 and 2-S173. These data indicated that *Pnn1* could be localized between markers 2-S152 and 2-S173. The genetic distance of this region was 6.7-cM. A positive correlation ($r^2 = 0.90$) was observed between RDT and leaf age at 4.0–4.6 in all lines.

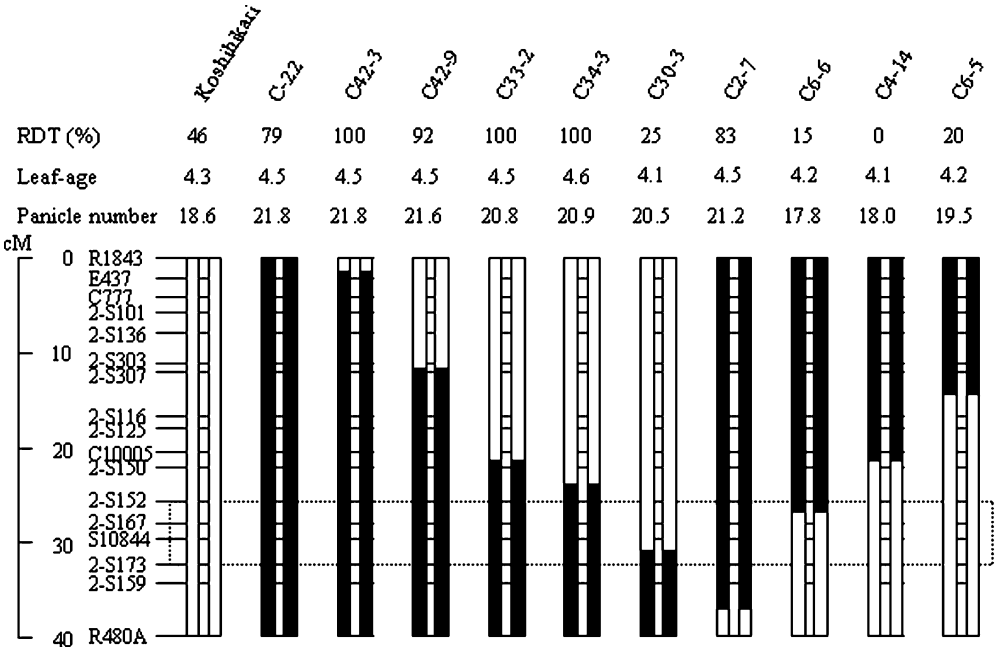

Fig. 5 Delimitation for *Pnn1* as determined by linkage analysis. The 50-cM segment from the Kasalath chromosome in C-22 was substituted. Koshihikari, C-22 and nine SLs were examined for ratio of developing tiller formation in the second axil (RDT), leaf age at 24 days after sowing ($n = 11$–15), and panicle number at the mature stage ($n = 10$). *Open and closed regions* represent segments derived from Koshihikari and Kasalath, respectively. The genetic map was reconstructed with 17 DNA markers, and *vertical bars* indicate the DNA marker loci examined. All DNA markers are shown on the *left*. The *dotted line* indicates the region in which at *Pnn1* was located

Discussion

GS1 content and total panicle weight

The results of the present investigation clearly show that the 50-cM fragment of Kasalath chromosome in chromosome 2 contains regulatory gene(s) for controlling GS1 protein content, panicle number, and panicle weight in rice. These results are in good agreement with those of our previous work in which a QTL for GS1 protein content was mapped in the same chromosome area with QTLs for those agronomic traits (Obara et al. 2001). Because the structural gene for GS1 could not be located in any QTL region for GS1 protein content on the rice chromosomes (Obara et al. 2001), the *GS1* gene itself probably does not strongly affect the regulation of GS1 protein content in senescing leaves. Since biochemical and agronomic traits are determined by the integration of both positively and negatively acting multigenes, total gene activities in the putative seven QTLs for GS1 content (Obara et al. 2001) could be important in the regulation of GS1 protein content. The structural gene for GS1 was mapped in the QTL region for single-spikelet weight, suggesting that *GS1* function in senescing leaves is tightly related to grain filling, probably via its capacity for nitrogen export, as has been suggested previously (Sakurai et al. 1996; Tobin and Yamaya 2001).

Physiological studies combined with genetic approaches on the regulation of nitrogen metabolism and nitrogen use efficiency have been reported in *Arabidopsis thaliana* (Rauh et al. 2002; Loudet et al. 2003), barley (Mickelson et al. 2003), and maize (Hirel et al. 2001; Limani et al. 2002). Hirel et al. (2001) showed that two out of five GS1 structural genes as well as a QTL for GS1 activity are located in the vicinity of QTL regions for yield, thousand kernel weight, and kernel number. Co-localization studies of QTLs for GS1 and various agronomic traits in rice and maize strongly suggest that the GS1 protein itself is important in improving nitrogen use efficiency and increasing the productivity of cereals. However, it was difficult to examine whether the change in GS1 content affects the panicle weight as well as the panicle number because the Kasalath allele in the substituted region on chromosome 2 of C-22 decreased the levels of both GS1 protein and soluble proteins. Another four putative QTLs for GS1 content on chromosomes 8 and 11 also overlapped with some of the QTL regions for rate of leaf discoloration, panicle weight, and spikelet number (Obara et al. 2001). Therefore, studies using approaches similar to those used here are necessary for examining the effects of a specific change in leaf GS1 content on these candidate traits.

Genetic manipulation of *GS1* has been used to either over-express or inhibit its expression in various plants, including tobacco (Brugière et al. 1999; Fuentes et al. 2001; Oliveira et al. 2002), alfalfa (Temple et al. 1998; Ortega et al. 2001), and *Lotus corniculatus* (Vincent et al. 1997). However, the results obtained in those experiments were not always consistent with respect to the function of GS1. Unlike the introduction of new properties such as herbicide tolerance, transgenic studies for the analysis of the function of gene products in plant metabolism have several disadvantages. These problems are largely caused by a lack of reproducibility, unsolved co-suppression or gene silencing, and somatic variation during the callus

induction and regeneration processes (Bourque 1995). The limited reproducibility is partly caused by the lack of methodology at present to control the copy number and position of the transgene in the chromosomes (Bourque 1995; Stam et al. 2000).

Whole plant growth and panicle weight

At all the levels of applied nitrogen, a QTL for total panicle number wasconfirmed at the substituted region for Kasalath on chromosome 2, based on the observation that the panicle number of C-22 was significantly higher than that of Koshihikari. Under low nitrogen levels (1/2N in the greenhouse and 0N in the paddy field), the increase in total panicle weight was caused primarily by the increase in total panicle number, without a change in individual panicle weight (Table 2). Panicles are normally produced from tillers developed at the early vegetative growth stage (Yoshida 1981). There are two stages in the formation of tillers, namely (1) the differentiation and (2) the subsequent development of tiller buds. The tiller bud is anatomically a type of axillary bud. The *Pnn1* allele from Kasalath increased tiller number development at the early vegetative growth stage, leading to an increase in panicle number at maturity.

Under high nitrogen levels (2N in the greenhouse), increases in the tiller and panicle numbers due to the presence of the *Pnn1* allele from Kasalath were also observed—as in the case of low nitrogen supply—but to a lesser extent. However, the increase in total panicle number did not contribute to the increase in total panicle weight, particularly in the field conditions. These results indicate that the function of *Pnn1* is apparently masked when plants grow vigorously in the presence of ample nitrogen. *Pnn1* derived from the Kasalath allele would pleiotropically contribute to the development of tillers at the vegetative stage and that of each panicle at the ripening stage. Zhuang et al. (1997) showed that the location and direction of the genetic effect of QTLs on panicle number per area coincided with that on spikelet number per panicle, which is related to panicle weight. Our results suggest that *Pnn1* has a more important role in the growth of tillers than in the development of panicles, because the stage of tiller development precedes that of panicle development in the life span of rice (Yoshida 1981). Tiller development is known to be affected by spacing, light, nutrient supply, and other environmental conditions (Yoshida 1981). Changes in tiller number, in response to spacing and nitrogen supply, prevented the development of each panicle at the ripening stage. Takeda et al. (2003) showed that *OsTB1*, a transcriptional factor, is a negative regulator for the development of lateral tillers in rice, as is the *TB1* gene in maize (Doebley et al. 1997). The tiller number in the transformed plants, in which *OsTB1* was overexpressed, was apparently lower than that of the transformed control plants. Loss-of-function of *OsTB1* with a fine culm in a *fine culm 1* mutant produced many tillers. Li et al. (2003) identified *MONOCULM 1* (*MOC1*) in rice, a putative GRAS family nuclear protein, as a master regulator in the control of tiller development. Komatsu et al. (2003) recently identified *LAX*, a transcriptional factor, and *SPA* as major regulators of shoot branching in rice using various mutants. *OsTB1* is located on the long arm of chromosome 3, *MOC1* is located on the long arm of chromosome 6, and *LAX* is located on the long arm of chromosome 1 in rice; hence, these genes are different from *Pnn1*, which is located on the long arm of chromosome 2. Thus, tiller development in rice is controlled by several regulatory genes. We have shown that *Pnn1* increased yield, nitrogen use efficiency, and biomass production under a low input of nitrogen fertilizer. The function of *Pnn*1 could be useful in other cereals because of the synteny in cereal genomes (Ahn and Tanksley 1993).

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