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ORIGINAL PAPER

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Physical mapping and putative candidate gene identification of a quantitative trait locus *Ctb1* for cold tolerance at the booting stage of rice

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Abstract *Norin-PL8* is a cold-tolerant variety of rice (*Oryza sativa* L.) that was developed by introgressing chromosomal segments from a cold-tolerant tropical *japonica* variety, *Silewah*, into a template *japonica* variety, *Hokkai241*. We previously identified two closely linked quantitative trait loci, *Ctb1* and *Ctb2*, for cold tolerance at the booting stage of *Norin-PL8* in the long arm of chromosome 4. We report here the physical mapping of *Ctb1* and the identification of the candidate genes. A total of 2,008 segregating individuals were screened for recombination in the *Ctb1* region by a PCR-based screening, and a series of near-isogenic lines (NILs) were developed from progenies of recombinants. A comparison of the degrees of cold tolerance of the NILs indicated that *Ctb1* is located in the 56-kb region covered by a bacterial artificial chromosome clone, OSJNBa0058 K23, that had been sequenced by the International Rice Genome Sequence Project. We found seven open reading frames (ORFs) in the 56-kb region. Two ORFs encoded receptor-like protein kinases that are possibly involved in signal transduction pathways. Proteins that may be associated with a ubiquitin-proteasome pathway were encoded by three ORFs, two of which encoded F-box proteins and one of which encoded a protein with a BAG domain. The other two ORFs encoded a protein with an OTU domain and an unknown protein. We were also able to show that *Ctb1* is likely to be associated with anther length, which is one of major factors in cold tolerance at the booting stage.

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Introduction

Male reproductive development is a dynamic process that includes mitosis/meiosis, the rapid and specific alteration of cell walls, and cellular interactions between developing microspores and tapetal cells (Bedinger 1992), all of which are highly sensitive to environmental stresses such as cold and drought (Satake and Hayase 1970; Sheoran and Saini 1996). As low temperature during the reproductive stage reduces the grain yield of rice plants, a cold-sensitive cereal, by causing sterility, a sterile-based cold injury is a very serious problem for rice producers. The stage most sensitive to low temperature is the booting stage, especially the young microspore stage spanning from tetrad stage to the first contraction phase (Satake and Hayase 1970). In anthers injured by low temperature, microspores are degenerated and tapetal cells are hypertrophied (Nishiyama 1976). Ito (1978) reported that an imbalance in carbohydrate metabolism is associated with cold injury . According to Kawaguchi et al. (1996), stage-specific accumulation of the tetrasaccharide, which has a structure similar to the terminal sequence of an arabinogalactan protein, is reduced by low temperature.

Little is known about the mechanism of cold tolerance. Suzuki (1981) and Tanno et al. (1999) reported that anthers of cold-tolerant varieties are longer than those of cold-sensitive varieties, while Suzuki (1982) found a correlation between anther length and cold tolerance in segregating generations. Saito et al. (2001) suggested that there is an association between the quantitative trait loci (QTLs) for cold tolerance on chromosome 4 and anther length. It has been estimated that 640 pollen grains/anther are necessary for successful pollination (Nishiyama 1983), and it has been established that the amount of pollen is highly correlated with anther length (Oka and Morishima 1967; Suzuki 1981). Therefore, it is likely that anther length is one of the major factors involved in cold tolerance.

Rice varieties vary widely in their degree of cold tolerance. *Japonica* varieties are generally more cold-tolerant than *indica* varieties, however there is a great deal

of variation in cold tolerance even among *japonica* varieties. QTLs for cold tolerance of temperate *japonica* varieties have been mapped on chromosomes 1, 2, 3, 5, 6, 7, 9, and 12 in *japonica* × *indica* crosses (Li et al. 1997; Andaya and Mackill 2003). Saito et al. (1995) showed that segments on chromosomes 3 and 4 introgressed from a tropical *japonica* variety, *Silewah*, into a breeding line, *Norin-PL8*, are associated with cold tolerance. Takeuchi et al. (2001) mapped QTLs for cold tolerance in a cross of a tolerant temperate *japonica* variety, *Koshihikari*, with a sensitive temperate *japonica* variety, *Akihikari*, on chromosomes 1, 7 and 11.

We previously reported the identification of two closely linked QTLs, *Ctb1* and *Ctb2*, for cold tolerance on chromosome 4 (Saito et al. 2001). Map-based cloning of the *Ctb1* and *Ctb2* genes will elucidate the mechanism of cold tolerance introduced from *Silewah* to *Norin-PL8*. Abe et al. (2002) found a single nucleotide polymorphism in an alternative oxidase (AOX) gene linked to *Ctb2* and suggested that the AOX is related to cold tolerance. However, no candidate gene of *Ctb1* has been identified. The objective of the investigation reported here was the physical mapping of *Ctb1* for candidate gene identification. We fine-mapped *Ctb1*, narrowed down the area of interest to 56 kb, and identified seven putative candidate genes.

Materials and methods

Plant materials

Norin-PL8 is a cold-tolerant variety that was developed by backcross breeding in which a cold-tolerant tropical *japonica* variety, *Silewah*, and a temperate *japonica* breeding line, *Hokkai241*, were used as a donor and recurrent parent, respectively (Satake and Toriyama 1979). *Kirara397* is a cold-sensitive temperate *japonica* variety grown in northern Japan. A line designated Syo6 was selected from BC_1F_5 lines of the cross *Kirara397/Norin-PL8//Kirara397* that contained introgressed segments on chromosomes 3 and 4 (Saito et al. 1995). In order to eliminate the effects of genetic factors other than those on chromosome 4, we backcrossed an F_1 plant between Syo6 and *Kirara397* with *Kirara397*. After two backcrosses, we selected a single plant heterozygous for the introgression on chromosome 4 but not containing the introgression on chromosome 3. By self-pollination of the plant, we developed a segregating population, designated BT4 (Saito et al. 2001). The heterozygotes for the introgression on chromosome 4 were selected from BT4, and their progenies were used for the marker-assisted selection of recombinants and development of near-isogenic lines (NILs).

Evaluation of cold tolerance and anther length

Cold tolerance was evaluated by the cool water irrigation method (Futsuhara and Toriyama 1964), which is a commonly used method in cold-tolerance breeding. Although under natural conditions cold injury is caused by a low atmospheric temperature, in the cool water irrigation method cool water is used to lower the temperature of young panicles and cause artificial cold injury. Plants were grown in a greenhouse maintained at a day temperature of 25°C and night temperature of 19°C. Cold treatment was applied at the stages from the beginning of the differentiation of young panicles to the completion of heading by transferring the pots into tanks filled with cool water maintained at 19°C . The depth of the water was about 24 cm—measured from the surface of the soil in the pot. When seeds ripened, cold tolerance was evaluated on the basis of mean spikelet fertility. Twelve and twenty plants from each NIL were used in the fine mapping and physical mapping, respectively. Anthers from spikelets in the middle of panicles were harvested before flowering, and their lengths were measured under a microscope. Twenty fresh anthers from ten panicles and ninety anthers fixed by 50% ethanol from five panicles were measured for each NIL. The computer program STATVIEW ver. 5.0 (SAS Institute, Raleigh, N.C.) was used for the analysis of variance.

Molecular analysis

Total DNA was isolated from leaves by the modified CTAB method (Murray and Thompson 1980). Restriction fragment length polymorphism (RFLP) markers with the prefix 'XNpb' were developed by Saito et al. (1991). RFLP markers with the prefix 'R' or 'C' were obtained from the Rice Genome Research Program, Japan (RGP) (Kurata et al. 1994; Harushima et al. 1998). Southern hybridization was conducted using the ECL system (Amersham Biosciences, Piscataway, N.J.) according to the manufacturer's instruction.

Two sequence-characterized amplified region (SCAR) markers, SCAB11 and SCAM20 (Saito et al. 2001), and two microsatellite markers, OSR15 (Akagi et al. 1996) and RM317 (Temnykh et al. 2000), were used. PCR analyses were carried out in 10-μl volumes of reaction mixture containing 0.2 μ*M* of each primer, 100.0 μ*M* deoxyribonucleotides, 50 m*M* KCl, 10 m*M* Tris-Cl, pH 8.3, 1.5 m*M* $MgCl_2$, 0.001% gelatin, 1 ng/μl template DNA, and 0.02 U/μl AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, Calif.). The thermal cycling protocol used consisted of one cycle of 4 min at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, and a final cycle of 7 min at 72°C. For large-scale screening of the recombinants, we used DNA templates prepared from leaves of young seedlings by the method of Klimyuk et al. (1993). Pieces of the third or fourth leaves (each approx. 5 mm long) were collected in a microtube containing 40 μl 0.25 *N* NaOH, incubated in a boiling water bath for 30 s, neutralized by the addition of 40 μl 0.25 *N* HCl and 20 μl 0.5 *M* Tris-Cl, pH 8.0, 0.25% (v/v) Igepal CA-630 (Sigma, St. Louis, Mo.), and finally incubated in boiling water for 2 min. Although the treated leaf tissue was used as a template in the original method, we used 1 μl of neutralized solution as a template. The amplification products were resolved by electrophoresis in a 2% (w/v) agarose gel or in a 4% (w/v) NuSieve3:1 agarose gel.

Gene annotation

Open reading frames (ORFs) were predicted by a rice genome automated annotation system, RICEGAAS (http://RiceGAAS.dna.affrc.go.jp/), in which coding region prediction programs (GENSCAN, RICEHMM, FGENESH, MZEF), a splice prediction program (SPLICEPREDICTOR), homology search analysis programs (BLAST, HMMER, PROFILE SCAN, MOTIF), a tRNA gene prediction program (TRNASCAN-SE), repetitive DNA analysis programs (REPEATMASKER, PRINTREPEATS), a signal scan search program (SIGNAL SCAN), a protein localization site prediction program (PSORT), and a program for classification and secondary structure prediction of membrane proteins (SOSUI) are integrated.

Results

Fine mapping

In a previous study, we identified two closely linked QTLs, *Ctb1* and *Ctb2*, on the long arm of chromosome 4 (Saito et al. 2001) (Fig. 1a). For the fine mapping of *Ctb1*, 1,255 progenies of BT4 were screened for recombination between the *Ctb1* flanking markers OSR15 and SCAM20. The number of recombinants selected was 117, and our results indicated that the genetic distance between SCAM20 and OSR15 was 4.8 cM. The recombinants were divided into six groups (R1–R6) on the basis of their OSR15 and SCAM20 genotypes (Fig. 1b). It is likely that double crossovers occurred in the *Ctb1* region of R5 and R6. In order to develop NILs without the *Ctb2* gene, we self-pollinated the recombinants belonging to R1 and R2, and selected those individuals that had the *Kirara397* allele for SCAM20 and the *Norin-PL8* allele for OSR15 from the progenies of R1 and R2, respectively. We developed 49 NILs from 54 recombinants. Progenies of five recombinants were discarded due to bad growth under the weak light conditions of the winter.

We divided the NILs into five groups (NS, NA, NB, NC and NT) based on the genotypes of the markers R740, XNpb267, XNpb264, and SCAB11 (Fig. 1c). The two NS lines and 22 NT lines had the *Kirara397* allele and *Norin-PL8* allele for the *Ctb1* region, respectively. The NA lines had the *Norin-PL8* allele for R740 and the *Kirara397* allele for XNpb267, indicating that recombination had occurred between R740 and XNpb267. Similarly, recombination of the lines in NB and NC occurred between XNpb267 and XNpb264 and between XNpb264 and SCAB11, respectively. The number of lines in each group coincided with the genetic distance between markers.

We used 25 lines of NA, NB, and NC for the fine mapping of *Ctb1*. Two NS lines and two NT lines were used as cold-sensitive and cold-tolerant controls, respectively. All of the lines were tested for the absence of the *Ctb2* gene using the microsatellite marker RM317 in the neighborhood of R738. Two NC lines were found to be heterozygous or had the *Norin-PL8* allele for RM317, suggesting the possibility that they harbor the *Ctb2* gene. Therefore, these two lines were not used in further experiments. The other 27 lines, including four control lines, possessed the *Kirara397* allele for RM317.

We evaluated the cold tolerance of the lines and parental varieties (Table 1). The spikelet fertilities of *Norin-PL8* and *Kirara397* were 88.9% and 41.5%, respectively. The spikelet fertility of NS was 40.7%, which was equivalent to that of *Kirara397*. The spikelet fertility of NC was higher than those of NS, NA, and NB and similar to that of NT. These results suggested that *Ctb1* is located around XNpb264. Since in our previous study we showed a possible association between *Ctb1* and anther length (Saito et al. 2001), we compared the anther lengths of the NILs.

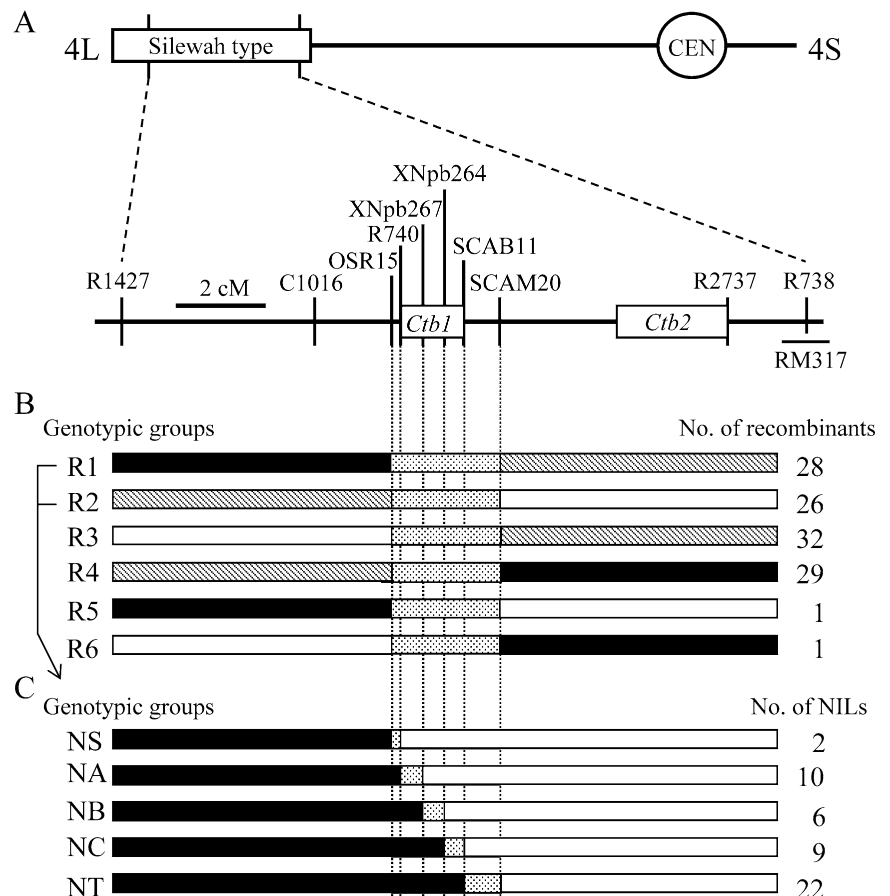

Fig. 1 **a** Chromosomal locations of the introgression from *Silewah* to *Norin-PL8* (*Silewah*-type), the centromere (*CEN*), the QTLs for cold tolerance (*Ctb1*, *Ctb2*), RFLP markers (*C*, *R*, *XNpb*), SCAR markers (SCAB11, SCAM20), and a microsatellite marker (RM317). **b** Genotypes of recombinants (*R1–R6*) selected from advanced backcross progenies between *Norin-PL8* and *Kirara397*. **c** Genotypes of the NILs (*NS*, *NA*, *NB*, *NC*, *NT*) developed from the recombinants with genotypes R1 and R2. **b**, **c** *Solid bars*, *open bars* Intervals homozygous for a *Norin-PL8* allele and *Kirara397* allele, respectively; *hatched bars* heterozygous intervals; *dotted bars* the interval in which a recombination has occurred

Table 1 Mean spikelet fertility and anther length of the genotypic groups of the NILs for the *Ctb1* region (*SD* standard deviation)

Genotypic group		Spikelet fertility (%) ± SD	Anther length (mm) ± SD
Genotype[a]	Number of lines		
NS	2	40.7±4.5	1.92±0.01
NA	10	49.4±8.8	1.95±0.09
NB	6	46.4±9.1	1.96±0.06
NC	7	58.7±6.4	2.02±0.05
NT	2	62.4±1.0	2.01±0.02

[a]The NILs of NS and NT had the *Kirara397* alleles and *Norin-PL8* alleles for the *Ctb1* region, respectively. The NILs of NA, NB and NC are recombinants in the *Ctb1* regions that had the *Norin-PL8* alleles for R740, XNpb267, and XNpb264, respectively

The anthers of NC were longer than those of NS, NA, and NB, thereby supporting our hypothesis that *Ctb1* is associated with anther length.

Physical mapping

A physical map of chromosome 4 was constructed by the International Rice Genome Sequence Project (IRGSP) (http://rgp.dna.affrc.go.jp/IRGSP/). We found that the region between XNpb264 and SCAB11 is covered by two bacterial artificial chromosome (BAC) clones—OSJNBa0093O08 (accession: AL606648) and OSJNBa0058 K23 (accession: AL662970)—from a homology database search using the DNA sequences of XNpb264 and SCAB11 (Fig. 2a). The physical distance between XNpb264 and SCAB11 was 196 kb. Based on the DNA sequences of the BAC clones, we developed six SSLP (simple sequence length polymorphism) markers: BAC1, BAC22, BAC9, PNK2, BAC29, and BAC28.

We analyzed the genotypes of the NILs using the SSLP markers and found that one of the NC NILs, 12-110-7, was heterozygous for the markers between BAC22 and BAC28 (Fig. 2b). In order to test whether *Ctb1* is located between BAC1 and SCAB11, we developed two NILs, 12-110-7 K and 12-110-7P, from the progenies of 12-110-7 and compared their degrees of cold tolerance. The cold tolerance of 12-110-7 K was equivalent to that of the control line without *Ctb1*, Control-S. The cold tolerance of 12-110-7P was significantly higher than that of 12-110-7 K (P=0.0031) and equivalent to that of the control line with *Ctb1*, Control-T. These results indicated that *Ctb1* is located between BAC1 and SCAB11.

To narrow down the *Ctb1* region, we increased the number of NILs in which recombination occurred between BAC1 and SCAB11 by screening 753 progenies of BT4. In this way we developed four more NILs. We genotyped the ten NILs using the SSLP markers and divided them into three groups (NC1–NC3) on the basis of their genotypes (Fig. 2c). The NILs of NC1 (18-170-2, 29-

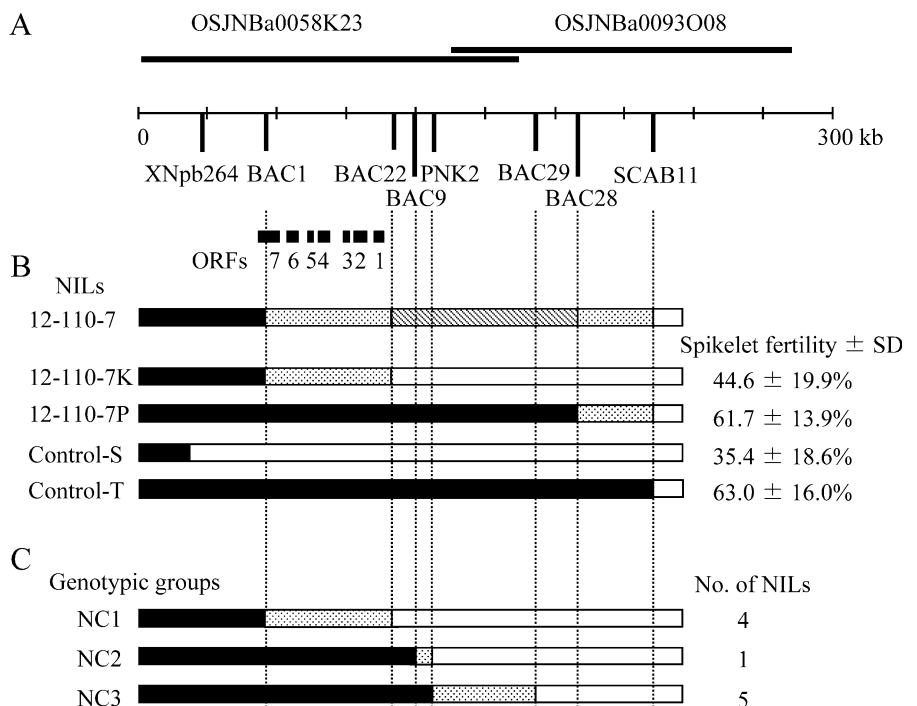

Fig. 2 **a** BAC clones covering the *Ctb1* region (OSJNBa0058 K23 and OSJNBa0093O08), ORFs annotated, and locations of RFLP (XNpb264), SCAR (SCAB11) and SSLP (*BAC* and *PNK*) markers. **b** Cold tolerance and genotypes of NILs 12-110-7 K, 12-110-7P, the control line without *Ctb1* (*Control-S*) and the control line with *Ctb1* (*Control-T*). **c** Genotypes of the NILs (NC1–NC3) for the physical mapping of *Ctb1*. NILs 12-110-7 K and 12-110-7P were developed from 12-110-7 that was heterozygous for the markers between BAC22 and BAC28. **b**, **c** *Solid bars*, *open bars* Intervals homozygous for a *Norin-PL8* allele and a *Kirara397* allele, respectively; *hatched bars* heterozygous intervals; *dotted bars* interval in which a recombination has occurred

Table 2 Spikelet fertility of the NILs whose recombination occurred between BAC1 and SCAB11 and differences in spikelet fertility between each NIL and the control line without *Ctb1* (Control-S) (*SD* standard deviation)

NIL	Genotype[a]	Spikelet fertility (%) ± SD	Difference from Control-S[b]	
			t	*P*
18-170-2	NC1	35.7±22.4	1.479	0.1475
29-279-6	NC1	33.0±17.7	1.211	0.2334
12-195-1	NC1	39.3±18.7	2.227	0.0320
27-355-3	NC1	47.4±15.0	3.919	0.0004
27-486-2	NC2	54.9±22.7	4.342	0.0001
8-104-2	NC3	45.5±20.4	3.114	0.0035
8-123-6	NC3	53.5±18.9	4.554	<0.0001
22-250-5	NC3	48.5±22.1	3.441	0.0014
23-580-9	NC3	44.4±13.6	2.686	0.0120
26-349-8	NC3	50.1±16.4	4.263	0.0001
Control-S[c]		25.9±19.4		
Control-T[c]		53.4±20.8		

[a]The NILs of NC1, NC2 and NC3 had the *Norin-PL8* alleles for BAC1, BAC9 and PNK2, respectively
[b]Significance of the difference was evaluated by the *t*-test
[c]Control-S and Control-T had the *Kirara397* allele and the *Norin-PL8* allele for the *Ctb1* region, respectively

279-6, 12-195-1 and 27-355-3) had *Norin-PL8* alleles for BAC1 and *Kirara397* alleles for BAC22. The NIL of NC2 (27-486-2) had *Norin-PL8* alleles for BAC9 and *Kirara397* alleles for PNK2. The NILs of NC3 (8-104-2, 8-123-6, 22-250-5, 23-580-9 and 26-349-8) had *Norin-PL8* alleles for PNK2 and *Kirara397* alleles for BAC29. Table 2 shows the range of cold tolerance of the NILs. The cold tolerance of 27-486-2 was significantly higher than that of Control-S, indicating that *Ctb1* is located between BAC1 and PNK2. The cold tolerances of 18-170-2 and of 29-279-6 were not significantly higher than that of Control-S, while the cold tolerance of 27-355-3 was significantly higher than that of Control-S. The results indicated that *Ctb1* is located between BAC1 and BAC22. The physical distance between BAC1 and BAC22 was 56 kb.

Table 3 Candidate genes in the *Ctb1* region

ORF	Protein	Rice ESTs[a]	EST source	
			Cultivars	Tissue
1	Hypothetical protein	AK063487[b]	Nipponbare	Callus treated by cold
2	F-box protein with Kelch repeats	AK065137[b]	Nipponbare	Shoot
		AK061943[b]	Nipponbare	Green shoot
		CB673687	Nipponbare	Leaf infected by *M. grisea*
		CB673686	Nipponbare	Leaf infected by *M. grisea*
3	Ser/thr protein kinase	AK099667[b]	Nipponbare	Shoot
		AK066337[b]	Nipponbare	Shoot
		CB648387	Nipponbare	Leaf infected by *M. grisea*
4	Hypothetical protein with OTU domain	AK066247[b]	Nipponbare	Shoot
		CB657467	Nipponbare	Leaf infected by *M. grisea*
		CB657468	Nipponbare	Leaf infected by *M. grisea*
		AU030332	Nipponbare	Immature leaf
		AU030331	Nipponbare	Immature leaf
5	Receptor-like protein kinase	AK065956[b]	Nipponbare	Shoot
		AK069252[b]	Nipponbare	Flower
		CB650844	Nipponbare	Leaf infected by *M. grisea*
6	F-box protein with WD40	CB622818	IR36	Leaf
		CB669292	Nipponbare	Leaf infected by *M. grisea*
		BI305332	Nagina 22	Root treated by drought
		CB622819	IR36	Leaf
		CB669293	Nipponbare	Leaf infected by *M. grisea*
7	BAG domain containing protein	AU183232	Nipponbare	Immature leaf
		AU183229	Nipponbare	Immature leaf
		AU101420	Nipponbare	Panicle shorter than 3 cm

[a]Rice ESTs found in GenBank that are at least 99% identical with predicted ORFs are shown
[b]Full-length cDNAs developed by the rice full-length cDNA consortium (http://cdna01.dna.affrc.go.jp/cDNA/)

Candidate gene analysis

There were nine ORFs between BAC1 and BAC22, two of which were relatively short (261 bp and 624 bp, respectively) and showed no hits to expressed sequence tags (ESTs). The seven remaining ORFs did show hits to ESTs (Fig. 2a, Table 3), of which ORF5 and ORF7 showed hits to ESTs expressed in the reproductive tissue. The amino acid sequence predicted from ORF1 did not show hits to any protein by a BLASTP search. The ORF2 coded for an F-box protein with Kelch repeats, the latter reported to be associated with protein-protein interaction (Kuroda et al. 2002). The amino acid sequence of ORF3 was similar to that of putative ser/thr receptor-like protein kinases, which bear a weak similarity to self-incompatibility (S)-locus receptor kinases of *Brassica* (expected value of 2e-69). Although the amino acid sequence of ORF4 did not show any hits to the protein database, it contained an ovarian tumor (OTU)-like cysteine protease domain. ORF5 coded for a receptor-like protein kinase that is similar to a *Pto* kinase for disease resistance (expected value of 1e-97). The protein of ORF6 had an F-box domain and WD40 repeats, the latter reported to be associated with protein-protein interaction (Kuroda et al. 2002). ORF7 coded for a protein containing a Bcl2-associated athanogene (BAG) domain, which is included in molecular chaperone regulators (Bariknarová et al. 2002).

Discussion

In this study, we fine-mapped the QTL *Ctb1* for cold tolerance at the booting stage to a 56-kb region. The region contained nine ORFs, seven of which showed hits to ESTs from rice. The seven ORFs contained two receptor-like ser/thr protein kinases, two proteins associated with protein-protein interaction, a protein in a molecular chaperone modulator, and two hypothetical proteins of unknown function.

Protein phosphorylation plays an important role in plant signal transduction related to biotic stresses (i.e., pathogen infection and insect herbivority) and abiotic stresses (i.e., high or low temperature, drought and salinity). Interestingly, we found two receptor-like protein kinases in the *Ctb1* region. The protein kinase encoded by ORF3 showed a slight similarity to S-locus receptor protein kinases of *Brassica*. Receptor-like protein kinase genes that share sequence homology with S-locus protein kinases appear to be ubiquitous in plants; S-related protein kinase genes have been identified in maize (Walker and Zhang 1990) and in self-fertilizing *Arabidopsis* (Walker 1993; Dwyer et al. 1994). Some of the genes have been reported to be predominantly expressed in vegetative tissues, suggesting their variable functions in plant development. ORF3 showed hits to the ESTs from vegetative tissues and although the possibility that it is also expressed in reproductive tissues can not be excluded, this protein kinase is less likely to be involved in cold tolerance at the reproductive stage. The receptor-like protein kinase encoded by ORF5 showed a similarity to the disease resistance gene *Pto*, suggesting a possible association between ORF5 and disease resistance. However, since Li and Gray (1997) identified a pollination-related receptor-like protein kinase with a similarity to *Pto* in tobacco, it is possible that the product of ORF5 plays another role in plant functions other than disease resistance.

The products of ORF2 and ORF6 had F-box motifs. The F-box is involved in the ubiquitin-proteasome pathway, which plays a key role in many cellular processes, including cell-cycle control, circadian rhythm, flower development, and hormonal signal transduction (Kuroda et al. 2002). Kuroda et al. (2002) discovered 568 F-box proteins in the *Arabidopsis* genome. Distinct F-box proteins provide specific recognition of the target to be polyubiquitinated (Patton et al. 1998). Therefore, F-box proteins function in many processes, including flower formation (Samach et al. 1999), auxin response (Ruegger et al. 1998), jasmonic acid signal transduction (Xie et al. 1998), circadian rhythm (Kiyosue and Wada 2000; Nelson et al. 2000; Somers et al. 2000), and phytochrome A-specific light signal transduction (Dieterle et al. 2001). The F-box motif is generally followed by protein-protein interaction domains, such as leucine-rich repeats (LRRs), WD40 repeats, and Kelch repeats—the F-box domains of ORF2 and ORF6 are followed by Kelch repeats and WD40 repeats, respectively. Both WD40 and Kelch repeats predict a similar tertiary structure, a β-propeller, suggesting their functional similarity, although they have no similarity at the sequence level (Andrade et al. 2001). The C-terminal domains of ORF2 and ORF6 probably define the function of the proteins.

The ORF7 protein contained a BAG domain that binds to the ATPase domain of the molecular chaperones Hsp70 and Hsc70 (the constitutively expressed cytosolic isoform of Hsp70) (Sondermann et al. 2001). Interestingly, it contained a ubiquitin domain at its amino terminus. Lüders et al. (2000) reported that BAG-1, which is a BAG protein possessing a ubiquitin domain, plays a role in the physical linkage between the Hsc/Hsp chaperone system and the proteasome. According to Townsend et al. (2003), BAG-1 is a multifunctional protein that interacts with a wide range of cellular targets to regulate growth control pathways in mammals. However, to the best of our knowledge, a BAG protein with a ubiquitin domain has not yet been reported in plants. On the other hand, Sung and Guy (2003) reported that altered expression of Hsp70/Hsc70 genes has pleiotropic effects on growth, development, thermotolerance, and regulation of the heat shock response. The function of the ORF7 protein may be associated with the pleiotropic functions of the Hsp70/Hsc70 family.

ORF4 showed hits to ESTs from rice, wheat, barley and maize, suggesting that it is specific to monocots. It contained an OTU domain whose function has not been investigated in detail. Recently, Evans et al. (2003) demonstrated that Cezanne, which belongs to the OTU superfamily, is associated with deubiquitination. The product of ORF5 may be involved in the ubiquitin-

proteasome pathway. ORF1 did not show any hits to the EST database except for AK063487. Since AK063487 is the EST from callus, it may not be expressed in differentiated tissues.

In a previous study we showed that *Ctb1* is possibly associated with anther length (Saito et al. 2001). This association was confirmed in the present study (Table 1). The amount of pollen produced in rice varieties varies under normal conditions, ranging from 1,000 pollen grains to 2,500 pollen grains per anther (Suzuki 1981), with 640 pollen grains per anther reported to be necessary for successful pollination (Nishiyama 1983). Therefore, the amount of pollen is an important factor in tolerance to sterile type or cold injury. Since the amount of pollen is highly correlated with anther length (Oka and Morishima 1967; Suzuki 1981), anther length is likely to be associated with cold tolerance. Of the seven ORFs identified in the *Ctb1* region, five ORFs (ORF2, ORF3, ORF5, ORF6 and ORF7) appeared to be associated with a signal transduction pathway or a ubiquitin-proteasome pathway. Since both pathways function in various aspects of growth and development, it is difficult to speculate about the ORF responsible for cold tolerance or anther length. Expression analyses of the ORFs using the NILs for *Ctb1* and transformation experiments of the candidate genes are necessary for the identification of the *Ctb1* gene.

Until recently, little had been known about the loci for cold tolerance at the booting stage of rice because it is a quantitatively inherited trait. However, the development of molecular maps have enabled QTL analysis, and several QTLs for cold tolerance have been identified (Saito et al. 2001; Takeuchi et al. 2001; Andaya and Mackill 2003). Physical mapping of the QTLs for cold tolerance and identification of the candidate genes will be helpful in understanding the genetic mechanism of cold tolerance and ultimately in developing more tolerant rice cultivars.

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