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Map-based cloning of the rice cold tolerance gene *Ctb1*

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ABSTRACT

Low temperatures during the booting stage reduce rice yields by causing cold-induced male sterility. We previously mapped a quantitative trait locus for cold tolerance, *Ctb1*, to a 56-kb region containing 7 putative genes. In this study we further mapped *Ctb1* to a 17-kb region containing two genes that encode an F-box protein and a ser/thr protein kinase. The F-box protein gene was preferentially expressed in young panicles, while the ser/thr protein kinase gene was expressed in leaves and young panicles. Both genes were cloned from a cold-tolerant variety, Norin-PL8, and introduced into a cold-sensitive variety, Hokkai241, and a cold-sensitive line, BT4-74-8. The cold tolerance of T_2 and T_3 progenies was assessed by measuring the degree of spikelet fertility in plants treated with cool water irrigation (19 °C, 25 cm) or cool air (12 °C, 4 days). The results indicated that the F-box protein gene confers cold tolerance. Cold tolerance is associated with greater anther length, and the transgenic plants had longer anthers than non-transformed controls. The F-box protein interacts with a subunit of the E3 ubiquitin ligase, Skp1, suggesting that an ubiquitin–proteasome pathway is involved in cold tolerance at the booting stage.

1. Introduction

Rice is a staple crop cultivated worldwide. However, since it is a cold-sensitive plant that evolved in tropical and subtropical areas, rice yields are greatly reduced under low temperature conditions. One of the factors reducing rice yields is cold-induced male sterility (CIMS). In CIMS, the growth stage that is sensitive to cold is the booting stage, especially the young microspore stage just after meiosis [1]. Low temperatures at the young microspore stage cause the degeneration of microspores and hypertrophy of tapetal cells [2].

Despite extensive studies, the physiological mechanisms underlying CIMS are still unclear. It is reported to be associated with an imbalance in carbohydrate metabolism [3]. Low temperatures result in an accumulation of sucrose in the anthers, and this is accompanied by a decrease in the activity of cell wall-bound invertase [4]. This reduced activity indicates a reduction in the supply of sucrose from the anther wall apoplast to the tapetum and young microspore. The phytohormone ABA affects cell wall invertase expression, suggesting that ABA may be a potential signal for CIMS [5]. A tetrasaccharide with a structure similar to that of a glycan chain on an arabinogalactan protein accumulates in anthers during microsporogenesis, and this accumulation is reduced by low temperatures [6].

Gene expression in anthers is responsive to low temperatures. The components of a signal transduction pathway, *OsMEK1* and *OsMAP1*, are induced by cold treatment in anthers [7], suggesting the existence of a molecular mechanism for cold response in anthers. Furthermore, a gene expression profile analysis with a cDNA microarray containing 8987 expressed sequence tags (ESTs) from rice indicated that 38 ESTs are up-regulated, and 122 ESTs are down-regulated, by cold treatment [8]. Although the global expression pattern of anther proteins is largely unaltered after cold treatment, the abundances of 37 out of more than 4000 anther proteins were changed by more than 2-fold after cold treatment [9]. However, since these cold-responsive genes and proteins have a broad spectrum of potential functions, it is difficult to speculate about the molecular mechanism underlying CIMS based on these results.

Cold tolerance at the booting stage is commonly evaluated on the basis of mean spikelet fertility under cold stress [10]. Rice varieties vary widely in their levels of cold tolerance at the booting stage, and many genes are associated with this variation. *Japonica* subspecies are generally more cold-tolerant than *indica* subspecies. Quantitative trait loci (QTLs) for cold tolerance were mapped on chromosomes 1, 2, 3, 5, 6, 7, 9, and 12 in a population from a cross between a *japonica* variety, M202, and an *indica* variety, IR50 [11]. There is a great deal of variation in cold tolerance even among the *japonica* varieties. Takeuchi et al. [12] identified QTLs on chromosomes 1, 7, and 11 based on a doubled-haploid population from a cross between a cold-tolerant *japonica*, Koshihikari, and a cold-sensitive *japonica*, Akihikari. QTLs for cold tolerance were identified on chromosomes 1, 4, 5, 10, and 11 using near-isogenic

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Table 1
SSLP markers in the *Ctb1* region.

Marker	Forward primer (5′–3′)	Reverse primer (5′–3′)	Type/SSR motif
BAC1	CTGCCTGAATTAAGCTTGCC	ACAAACGATCGCAACGTGCC	(ct)4
PNK5	TGATAAGTAGAGGCCAAATTGCTG	CCTTACCTTCCTTCACAGATCGAT	INDEL
PNK7	GACGGTACTACTCACGGATCAAAT	GCGTTACGTCACGTACTCAAATGG	INDEL
PNK10	CGTGCATCCCTCTTGTAGCATATG	GTGTCTGAACATCTCATATGACACC	INDEL
BAC22	TTCGTGCTGCTCGGAGCTAATCAA	CATCGTCGACTACCTCTACCACAA	(ccg)7

lines between a cold-tolerant *japonica*, Kunmingxiaobaigu, and a cold-sensitive *japonica*, Towada [10,13]. QTLs of a cold-tolerant *japonica*, Norin-PL8, were identified on chromosomes 3 and 4 [14], while a QTL of a cold-tolerant *japonica*, Hokkai-PL9, was identified on chromosome 8 [15].

We previously reported on the identification of two closely linked QTLs, *Ctb1* and *Ctb2*, on chromosome 4 of Norin-PL8 [16]. *Ctb1* was mapped to a 56-kb region containing seven putative candidate genes [17]. The objective of the current study was to identify which of these genes is *Ctb1*. Here we describe the high resolution mapping of *Ctb1* using recombinants in the 56-kb region, and our analysis of candidate gene expression. We also created transgenic plants that exhibit cold tolerance at the booting stage, and thus identified the *Ctb1* gene. The possible function of this cold tolerance gene is discussed.

2. Materials and methods

2.1. Plant materials

Norin-PL8 is a cold-tolerant variety that was developed by backcross breeding in which a cold-tolerant variety, Silewah, and a cold-sensitive breeding line, Hokkai241, were used as the donor and recurrent parent, respectively [18,19]. Kirara397 is a cold-sensitive commercial variety. The near-isogenic lines (NILs) for fine mapping of the cold-tolerant QTL, *Ctb1*, were developed by backcrossing Kirara397 to Norin-PL8 [14,16,17].

2.2. Marker analysis

Simple sequence length polymorphism (SSLP) markers (Table 1) were used for genotyping the NILs. Polymerase chain reaction (PCR) was carried out in 10-μl reaction mixture volumes containing 5 μl of GoTaq Master Mix (Promega, Madison, WI), 0.2 μM of each primer and 1 μl of DNA template solution. DNA templates were prepared as described by Klimyuk et al. [20] with some modification. A small leaf tip (approximately 5 mm long) was ground in a 2-ml microtube containing zirconia beads (Assist, Tokyo, Japan) by vigorous shaking for 5 s using the CapMix (ESPE, Seefeld, Germany). After adding 40 μl 0.25 N NaOH, the sample was incubated at 100 °C for 30 s, then neutralized by the addition of 40 μl 0.25 N HCl and 20 μl of a solution containing 0.5 M Tris–HCl, pH 8.0 and 0.25% (v/v) Igepal CA-630 (Sigma, St. Louis, MO), and finally incubated at 100 °C for 2 min. The PCR conditions were as follows: a 4-min hold at 94 °C; 45 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and a final extension step of 72 °C for 7 min. The amplification products were resolved by electrophoresis in a 4% (w/v) MetaPhor agarose gel (Lonza, Rockland, ME).

2.3. RNA analysis

Total RNA was extracted from young panicles at the booting stage and mature leaves using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA). Reverse transcriptase (RT)-PCR was carried out using the mRNA Selective PCR Kit (Takara-Bio, Shiga, Japan) in 25-μl reaction mixture volumes containing 10 ng/μl of RNA and 0.4 μM of each primer. Primers for the F-box protein gene (FB) were forward 5′-GGCTCTCATCTGGTTTCTTCATGG-3′ and reverse 5′-ACGAAAAGGGGGGTGTCATTTTTC-3′. Primers for the ser/thr protein kinase gene (PK) were forward 5′-GGCTGAGATCATCGATGCTGACCT-3′ and reverse 5′-GCAATACGCTGCCTATCTTAAACA-3′. The RT-PCR conditions were as follows: reverse transcription at 42 °C for 30 min; followed by 25 cycles of 85 °C for 1 min, 45 °C for 1 min, 72 °C for 2 min; and a final extension step of 72 °C for 7 min. The amplification products were resolved by electrophoresis in a 1.2% (w/v) agarose gel. Primers for ACTIN gene (forward 5′-GGTAGAAGATGGCTGACG-3′ and reverse 5′-GTCATCTTCTCACGATTGGC-3′) were used a reference.

2.4. Plant transformation

Genomic DNA of Norin-PL8 was isolated from leaves according to the modified CTAB method [21]. DNA fragments containing the *Ctb1* candidate genes were amplified from the DNA using the Expand Long Template PCR System (Roche Diagnostics, Mannheim, Ger.). The primers (forward 5′-GGCTATATATGTGAATTGTGTTGGTTGCTG-3′ and reverse 5′-CAAAAGTAGCTTTTTACCGCCAATTGTACC-3′ for FB and forward 5′-CAGCCGCGTTCGTCAGCAACTTTGTTTATT-3′ and reverse 5′-ACGCCATGGCATACGTACAGCTTCGAGCTA-3′ for PK) were designed using the Nipponbare genomic DNA sequence (AL662970). The PCR conditions were as follows: a 2-min hold at 94 °C; 10 cycles of 94 °C for 30 s, 62 °C for 30 s, 68 °C for 8 min; 20 cycles of 94 °C for 30 s, 62 °C for 30 s, 68 °C for 8 min plus 20 s/cycle; and a final extension step of 68 °C for 7 min. The amplification products were cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and then recombined to the plant transformation vector pCAMBIA1300 (CAMBIA, Canberra, Australia). The constructs were used to transform ElectroMAX *Agrobacterium tumefaciens* LBA4404 cells (Invitrogen). The cold-sensitive rice variety Hokkai241 and the NIL BT4-74-8 were used for *Agrobacterium*-mediated transformation according to the method of Hiei et al. [22]. Inheritance of the transgenes in T_1 and T_2 plants was assessed by PCR using primers specific to the hygromycin-resistance gene (forward 5′-ATCTTAGCCAGACGAGCGGG-3′ and reverse 5′-GATGCCTCCGCTCGAAGTAG-3′).

2.5. Evaluation of cold tolerance and anther length

Cold tolerance in the T_2 lines was evaluated using the cool water irrigation method [23], in which deep cool water is used to cool the young panicles and cause artificial cold injury. T_2 seeds of T_1 plants harboring the transgene were germinated, and progeny that had inherited the transgene were selected. T_2 progeny that did not inherit the transgene were used as controls. For the Hokkai241 transformants, 8 plants/line were used. For the BT4-74-8 transformants, 20 plants/line were used. Plants were grown in a greenhouse with day and night temperatures of 25 °C and 19 °C. Cold treatment was applied at stages from the beginning of panicle differentiation to the completion of heading, by keeping the pots in tanks filled with cool water maintained at 19 °C for about 5 weeks. The depth of the water was about 25 cm above the soil surface. At maturity, pan-