

Trapping and characterization of cold-responsive genes from T-DNA tagging lines in rice

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Abstract

To identify low-temperature-responsive genes in rice, we screened *gus*-trapped T-DNA tagging lines that had been subjected to cold stress at 5 °C. Out of 15,586 lines, 81 (0.52%) showed cold-responsive GUS activity. Among the 62 lines we selected for further study, 53 showed increased GUS activity while 9 showed less. In addition, 16 of those 62 lines were influenced by abscisic acid (ABA), suggesting an ABA-dependent cold response. We used inverse PCR or thermal asymmetric interlaced (TAIL) PCR to identify 37 tagged genes from those lines, and further characterized two of them at the molecular level. The *OsRLK1* gene, which encodes a putative leucine rich repeat (LRR)-type receptor-like protein kinase, was inducible by cold and salt stresses. The *OsDMKT1* gene, encoding a putative demethylmenaquinone methyltransferase (DMKT), was also inducible by low temperatures, but not by high salt or drought. The T-DNA-tagged *OsDMKT1* gene also co-segregated with the cold-inducible *gus* gene. Our results indicate that the T-DNA tagging lines are useful in obtaining the stress-responsive genes in rice.

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1. Introduction

Temperature is a major factor that limits the geographical locations suitable for growing particular plant species. For example, plants raised in temperate or subtropical climates may often be exposed to cold stress. Rice, for example, is adversely affected by chilling (i.e. 10–20 °C) compared to the response of cold-tolerant crops, such as wheat and barley [1]. All modern cultivars of rice are believed to have been derived from a tropical ancestor and, therefore, are adapted to development at warmer temperatures. A number of injuries can result from exposure to low temperatures, depending on the growth phase in which it occurs. For this crop species, the most cold sensitive periods are the seedling stage and the early microspore stage of pollen development [2,3]. During the seedling phase, cold-stress symptoms may include delayed germination, poor growth, necrosis, slow chlorophyll synthesis in the plumule, and chlorosis, result-

ing in a failure to ripen during grain-filling [4,5]. In the reproductive stage, low temperatures can dramatically reduce grain yield by inducing spikelet sterility. This phenomenon is either due to the lack of microspore development [6] or a reduction in the number of pollen grains per anther [7]. In fact, exposure to 15 °C/10 °C (day/night) can decrease both spikelet number and fertility by up to 90% for cold-sensitive genotypes [8]. This can, consequently, affect overall grain and eating quality [9].

Cold-regulated gene expression is important in plants to provide chilling tolerance [10,11] and cold acclimation [12–14]. These cold-responsive genes encode diverse proteins, such as enzymes related to the metabolism of carbohydrates, lipids, antioxidants, compatible solutes; chaperones, antifreezing proteins, and others related to water stress [13,15,16]. Various approaches for identifying these genes have been taken. For example, in *Arabidopsis*, large-scale gene identification has been pursued via SAGE, microarray, and DNA chip analyses [17–19]. In rice, several cold-responsive genes have been isolated by differential screening, coupled with expressed sequence tag (EST) analysis [20]. Low-temperature-induced genes, *lip5*, *lip9*, and

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lip19, were first identified from rice by screening more than 1500 subtracted cDNAs [21]. The *lip19* gene encodes a bZIP-type protein [22] whose expression is induced specifically at 4 °C, but not at 12 °C [23]. A LEA-like protein gene *wsi18* as well as genes for alternative oxidase (AOX1a and AOX1b) and glutathione reductase (RGRC2) are also induced by cold stress [24–26]. Moreover, expression of the omega-3 fatty acid desaturase gene, *OsFAD3*, is highly induced by low temperatures in the roots but not in the leaves [27]. The *OsP5CS* gene involved in the biosynthesis of proline is induced by cold as well as other abiotic stresses, such as high levels of salt [28].

The cellular mechanisms that perceive changes in temperature and the signal transduction processes are poorly understood. Expression of stress-responsive genes is believed to be regulated by complex signaling through either an ABA-dependent or -independent pathway [29,30]. Those pathways responsible for the activation of such genes are becoming more clear through the use of genetic and biochemical approaches. A group of transcription factors belonging to the AP2/EREBP family has been extensively studied [31–33]. For example, when *CBF1/DREB1B* is overexpressed, the *COR* gene can be induced, resulting in greater tolerance to freezing, salt, and drought stresses [34,35]. The *hos1* mutant also shows enhanced expression of cold-responsive genes (including *CBF/DREB1*) under normal growing conditions, which indicates that *HOS1* is a negative regulator of cold-stress signaling [36]. Moreover, Chinnusamy et al. [37] have shown that expression of *CBF* and the *CBF*-regulated genes is decreased in the *ice1* mutant, where *ICE1* encodes a MYC-like bHLH transcriptional activator. In rice, several signaling-related protein genes have also been investigated. A calcium-dependent protein kinase gene, *OsCDPK7*, is activated by cold stress; its overexpression enhances cold tolerance in transgenic plants [38]. Moreover, overexpression of the rice calreticulin1 gene (*CRO1*), which encodes a CDPK-interacting protein, improves activity of the cold-responsive 47 kDa CDPK [39]. Mitogen-activated protein kinase genes, *OsMEK1* and *OsMAP1*, have been isolated by subtractive screening of 12 °C-treated rice anthers, and also show cold-specific induction [23]. Likewise, other MAPKs genes (*OsMAPK4*, *OsWJUMK1*, and *OsMAPK5*) are influenced by treatment with low temperatures [40–42]. For example, the cold-responsive AP2/EREBP-type transcription factor genes, *OsDREB1A* and *OsDREB1B*, have been isolated; transgenic expression of the former in *Arabidopsis* has resulted in higher tolerance to cold stress. Nonetheless, some, but not all, of the target genes of *Arabidopsis DREB1A* are also induced in the transgenic plants, suggesting that the cold-responsive DREB pathway is at least partially conserved in these model plants [43]. The nearly completed rice genome sequence is currently available, as well as comprehensive full-length cDNA and EST databases [44–47]. T-DNA and transposon-tagging populations have been generated for large-scale functional analysis of those

genes [48,49]. In some research, T-DNA insertional mutagenesis employs the *gus* reporter gene under alternative donor/acceptor sites for splicing [49–52]. When T-DNA is inserted in a suitable orientation, transcripts of *gus* and the endogenous plant gene can be fused, generating a fusion transcript. The advantage of the reporter gene trap is that expression of the reporter gene may reflect the time and location of endogenous gene expression and the gene trap itself may cause a mutant phenotype that reflects the function of the corresponding gene. T-DNA-tagged genes can be identified via PCR amplification of genomic DNA at the insertion site. In *Arabidopsis*, the *Ac/Ds* transposon-tagging system has been successfully used to find the genes involved in plant development (reviewed in [53,54]) as well as those regulated by abiotic stress [55]. T-DNA tagging rice lines are also used to study genes that encode Mg-chelatase [56], poly(A) binding proteins [57], and other proteins [50]. In this study, we identified cold-inducible genes by screening the T-DNA tagging rice population for cold-responsive GUS expression. To our knowledge, this is the first report on the identification of stress-responsive genes using such an approach.

2. Materials and methods

2.1. Plant materials, growth conditions, and stress treatments

Rice seeds (*Oryza sativa* ssp. *japonica* cv. Dongjin and Hwayoung) were surface sterilized, rinsed with tap water, and germinated in Petri dishes at 30 °C in the dark. After 2 days, the seedlings were transplanted to soil and raised in either a greenhouse or a growth chamber (typically 30 °C, 60% RH, 16 h daylength, 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$). To produce our mutants, sterilized, dehusked seeds were germinated in an MS [58] agar medium containing 0.2% phytigel (Sigma, USA) at 30 °C under continuous light. After 1 week, the resultant seedlings were transplanted to soil in the greenhouse. For our wounding- and cold-stress treatments, the shoot or root were damaged by cutting them into pieces and floating them on MS media for various intervals at either 26 or 5 °C. The treatments for salt or ABA stress involved floating the leaf fragments on MS media containing 300 mM NaCl or 100 μM ABA for various lengths of time. For drought stress, the leaf fragments were dried in the air until 30% loss of fresh weight. After the treatments, the tissues were immediately frozen in liquid N₂ and stored at –80 °C.

2.2. Screening of T-DNA tagging rice for abiotic-stress response

We screened T-DNA-tagged lines that had been transformed by *Agrobacterium* harboring either the pGA2144 or the pGA2707 vector [49]. Shoots from the youngest tiller on T0 plants were cut into 2–3 cm-long stem segments, and floated overnight in an MS liquid medium at 5 °C. The

cold-treated segments were then incubated in an X-gluc solution according to the method of Jeon et al. [49], and the chlorophyll was removed with 70% ethanol. Unstressed control samples were immersed into the X-gluc solution directly, and the shoot-wounding experiment was performed at 26 °C. GUS staining was examined under a dissecting microscope. The GUS-positive lines were selected and further screened with wounding (26 °C) for 12 h, cold (5 °C) for 12 h, salt (300 mM NaCl) for 6 h, or ABA (100 μM) for 12 h.

2.3. Identification of T-DNA-tagged genes

Genomic DNA was extracted from the mature leaves of selected lines according to the method of Chen and Roland [59] after the samples were ground with an MM300 Mixer Mill (Retsch, Hann, Germany). To isolate the T-DNA flanking sequence, inverse PCR (IPCR) was performed as described previously [50,56,57]. For *EcoRI* cutting in our pGA2144-tagged lines, the primers for the first PCR included GUS2 and IP-GUS. Primers for the second PCR were GUS1 and IP-Tnos. For *HindIII* cutting, the first-PCR primers were GUS2 and H3IPCR1; the second-PCR primers were GUS1 and H3IPCR2. For *PstI* cutting in the pGA2707-tagged lines, the first-PCR primers were Hph-3F and TubR3; second-PCR primers were 2707LP2 and TubR4. Samples were amplified for 35 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 5 min. The PCR product was directly sequenced with the proper primers. For TAIL-PCR, cDNA templates were synthesized using total RNA and reverse transcriptase with the RT-gus-650-re primer. Primary, secondary, and tertiary PCR were conducted as described by Liu et al. [60], with minor modifications. The primary PCR was performed in 50 μl containing a 2 μl aliquot of cDNA, 0.2 μM GUSH9 primer, three arbitrary degenerate primers (0.6 μM for AD1, 0.6 μM for AD2, and 0.75 μM for AD3), 0.2 mM dNTPs, and 1.5 units of Ex-Taq polymerase (Takara, Japan). Aliquots (2 μl) from 40-fold dilutions of the primary PCR products were used for the secondary PCR reaction (50 μl), containing 0.2 μM GUSH7-specific primer, the same AD primers used in the primary reaction, 0.2 mM dNTPs, and 1.5 units Ex-Taq polymerase. Tertiary PCR was performed in a 50 μl solution containing a 2 μl aliquot from 40-fold dilutions of the secondary PCR products, 0.2 μM of GUSH5-specific primer, the same AD primers in the primary reaction, 0.2 mM dNTPs, and 1.5 units Ex-Taq polymerase. The PCR products were directly sequenced using the GUSH5 primer. Primers for IPCR and TAIL-PCR are as follows: (GUS1) 5'-GGATACAAGTCTGTACCTTG-3'; (GUS2) 5'-CTGCA-TATAACCTGCACATTAGC-3'; (IP-GUS) 5'-CAGCAGG-GAGGCAAACAAT-3'; (IP-Tnos) 5'-GCGCGGTGTCAT-CTATGTTACT-3'; (H3IPCR1) 5'-CGAGACAACGCAGAGAAAG-3'; (H3IPCR2) 5'-TTCGTACTCGCCTCTCTCC-3'; (Hph-3F) 5'-GATCGTTATGTTTATCGGCACTT-3'; (TubR3 primer) 5'-GGTGAATGGCATCGTTTGAA-3'; (2707LP2) 5'-AGTGCTTGACATTGGGGAATTCAG-3';

(TubR4 primer) 5'-ACAAGCCGTAAGTGCAAGTG-3'; (RT-gus-650-re) 5'-TAGTCTGCCAGTTCAG-3'; (GUSH5) 5'-ATCCAGACTGAATGCCACAGG-3'; (GUSH7) 5'-TCTGCATCGGCGAACTGATCG-3'; (GUSH9) 5'-CATC-ACTTCTGATTATTGACC-3'; (AD1) 5'-GTNCGA(G/C)(A/T)CAN(A/T)GTT-3'; (AD2) 5'-NGTCGA(G/C)(A/T)GANA(A/T)GAA-3'; (AD3) 5'-(A/T)GTGNAG(A/T)ANC-ANAGA-3'.

Genomic sequences containing the tagged sequence were retrieved from the RiceGD (<http://www.btn.genomics.org.cn/rice/>; [46]) and the Monsanto rice blast database, and were annotated using the Rice Genome Automated Annotation System (RiceGAAS; <http://www.ricegaas.dna.affrc.go.jp>), the Softberry program (<http://www.softberry.com/berry.phtml>), and the BLASTP program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

2.4. Quantitative RT-PCR analysis

The gene-specific primers of the *OsRLK1* gene in Line 0-162-67 and the *OsDMKT1* gene in Line 1C-051-22 were designed and used for RT-PCR analysis. Specific primers for the coding region of each gene included the following: (*OsRLK1*-forward) 5'-TCTGTACCACGCGAGAAACC-3'; (*OsRLK1*-reverse) 5'-CCAGTGCCATGGATGAGG-3'; (*OsDMKT1*-forward) 5'-GCGACGCAAACCTCAAATCC-3'; (*OsDMKT1*-reverse) 5'-TCACACGGTCAATTCAGTCC-3'.

As a control, we used the primers specific to rice actin gene *Rac1* [61], 5'-CATGCTATCCCTCGTCTCGACCT-3' (forward) and 5'-CGCACTTCATGATGGAGTTGTAT-3' (reverse). For the control of cold- and salt-response expressions, the following specific primers were used: to *GF14-c* (U65957), 5'-AGTAATCCCTTAATTGGTC-3' (forward) and 5'-TTAAATATTGCTCAATAAAAC-3' (reverse); and to *Salt* (S45168; [62]), 5'-TAAGCGACCACGAAGAGTATGA-3' (forward) and 5'-AGTGATACCAATATGAGAAACACATAA-3' (reverse). Ten microgram of total RNA was used for the RT-PCR analysis, according to the method of Takakura et al. [63]. The PCR cycles numbered 30 for *OsRLK1*, 26 (leaves) or 22 (stems) for *OsDMKT1*, 22 for *Rac1*, 28 for *GF14-c*, and 25 for *Salt*.

2.5. Northern blot analysis

Total RNA was isolated using Tri reagent (Molecular Research Center, USA). Thirty micrograms of total RNA was resolved on a 1.3% formaldehyde agarose gel, and blotted onto a Hybond-N membrane. To prepare the probe, *OsDMKT1* was labeled with [α -³²P] dCTP (3000 Ci mmol⁻¹), using the random priming method of Feinberg and Vogelstein [64]. After hybridization [65], the membrane was washed with 2× SSC, 0.1% SDS at RT for 15 min; 1× SSC, 0.1% SDS at RT for 15 min; and 0.1× SSC, 0.1% SDS at RT for 15 min. Hybridization signals were detected with a BAS-1500 image analyzer (Fuji, Japan) and exposed on HyperfilmTM MP (Amersham, UK).

2.6. Genotyping for *OsDMKT1*

To distinguish the genotypes of the progeny from our T-DNA-tagged lines, we used genomic DNAs of the T1 progeny from 1C-051-22 for PCR-based genotyping. This PCR reaction was carried out with 250 ng of genomic DNA as template and 0.5 unit of Ex-Taq polymerase, and involved 35 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1.5 min. The forward (P1) and reverse primers (P2) were 5'-ACACGTCCGGTAGAATACCC-3' (1.6 kb downstream of the ATG start codon) and 5'-AAACCGTGTCAAAAGGAACC-3' (0.1 kb downstream of the TGA stop codon); the forward primer (P3) of T-DNA was 5'-GGTGAATGGCATCGTTTGAA-3' on the left-border.

3. Results

3.1. Screening cold-responsive T-DNA tagging lines

To identify T-DNA-tagging lines that were cold-responsive, we assayed young tillers and leaf sheaths for GUS expression after stress treatment at 5 °C. Among the 15,586

lines studied, 81 (0.52%) displayed differential GUS activity. Due to growth defects or sterility, 19 lines were lost at the next generation; the remaining 62 generated seeds and were used for further analysis. Of these, 53 lines showed increased GUS activity after cold stress, whereas 9 showed decreased activity. Among the cold-responsive lines, treatment with ABA caused GUS activity to rise in 12 lines but lessen in 4 others. We categorized the tagging lines into three classes, according to their inducibility by low-temperature or ABA treatment (Fig. 1): Class I, containing 41 lines with cold-induced GUS activity; Class II, with 12 lines, showed GUS activity induced by both cold stress and ABA treatment; while the remaining 9 lines which comprised had suppressed GUS activity brought on by chilling.

3.2. Isolation of flanking sequence and identification of tagged genes

To identify the trapped genes, we extracted genomic DNAs from the cold-responsive lines and isolated the T-DNA flanking regions by either IPCR or TAIL-PCR amplification. Sequencing of the amplified DNA plus the BLAST search of the rice genomic sequences resulted in

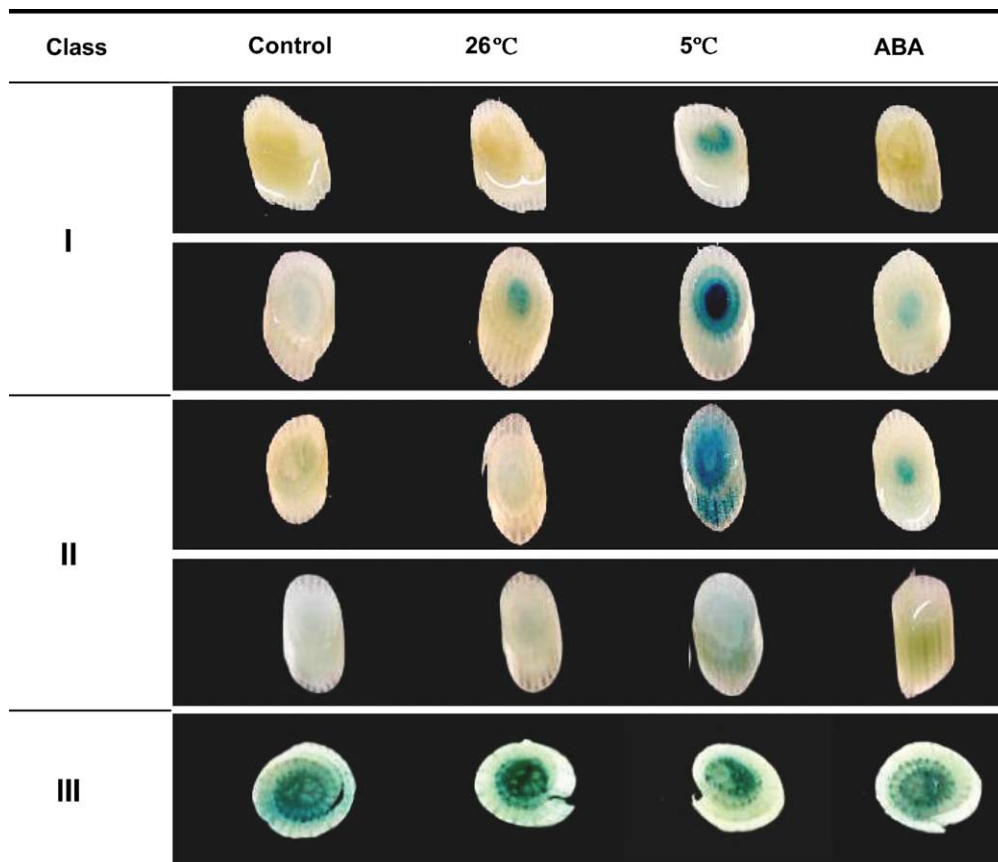


Fig. 1. GUS activity in shoots of gene-trap rice plants. T-DNA tagging lines were examined for inducibility by cold and ABA. After stress treatments, shoot segments were stained in X-gluc solution and chlorophyll was removed. Class I shows lines where GUS activity was induced only by 5 °C; Class II indicates GUS induction by both 5 °C and ABA; and Class III shows constitutive GUS expression, which is down-regulated by 5 °C.

Table 1
Characteristics of the gene-trap lines with the GUS expression pattern in response to low temperature

Line	GUS staining ^a					Chromosome (accession no)	Putative tagged gene	T-DNA location(s) ^b
	First 5 °C (24 h)	Second screening (12 h)						
		C	W	5 °C	ABA			
0-138-33	++	–	–	+	–	1 (AP003215)	Hypothetical protein	ATG –0.4 kb
0-143-03	+	+	+	–	+	4 (AL606682)	Hypothetical protein	Stop +1.1 kb
0-144-49	+	–	–	+	–	1 (AP003240)	Hypothetical protein	Stop +0.3 kb
0-149-58	+++	–	–	++	–	8 (AP004015) 1 (AP003350)	Ubiquitin-specific proteinase homologue Putative carboxymethylenebutenolidase	Stop +0.3 kb 6th intron
0-151-21	+	–	–	++	–	7 (AP005195)	Hypothetical protein	3rd intron
0-152-28	++	–	–	+	–	10 (AE017082)	PS II chlorophyll a-binding	1st exon
0-158-25	+	–	–	++	–	7 (AP004349) 1 (AP003735)	Putative chitinase Putative glutathione S-transferase	ATG –0.6 kb ATG –0.3 kb
0-162-67	+	–	–	+	–	3 (AC099732)	Receptor-like protein kinase	ATG –0.2 kb
0-165-52	+	–	–	+	–	5939 ^c	Hypothetical protein	ATG –83 bp
0-168-38	+	–	–	+	–	8 (AP004459)	Phosphate/phosphoenolpyruvate translocator	ATG –0.9 kb
0-175-70	+++	–	+	++	++	3 (AC084282)	Gibberellin-stimulated transcript	ATG –50 bp
0-180-71	+	–	–	++	–	8431 ^c	Chloroplastic triosephosphate isomerase	Stop +0.7 kb
0-192-05	+	+	–	++	–	3 (AC134236) 1 (AP004767)	Hypothetical protein Unknown protein	ATG –1.1 kb 2nd intron
0-205-39	+	–	+	++	++	1 (AP004331)	Hypothetical protein	Stop +0.5 kb
0-205-60	+	+	–	++	–	13328 ^c	Protein kinase	ATG –2 bp
0-227-35	+	–	–	++	++	8 (AP004761)	Unknown protein	1st intron
0-230-29	+	–	–	++	++	18744 ^c	Hypothetical protein	ATG –1.1 kb
0-230-51	+	–	–	++	++	13328 ^c	Protein kinase	ATG –2 bp
0-230-55	+	–	–	++	++	13358 ^c	Ser/thr protein kinase	ATG –0.3 kb
1C-051-22	+	++	+	++	+	10 (AE017096) 12547 ^c	Tubulin beta-1 S-adenosylmethionine:2-demethyl menaquinonemethyltransferase	2nd exon 1st intron
1C-051-44	++	+	+	++	++	513 ^c	Hypothetical protein	11th intron
1C-052-38	+	++	++	+	–	1 (AP003794)	Unknown protein	1st exon
1C-053-24	+	++	+	+++	+	4 (AL662985)	Hypothetical protein	ATG –0.3 kb
1C-054-05	+	+	+	++	+	8 (AP003877)	Unknown protein	4th intron
1C-061-57	+	++	++	+	+	4 (AL606632) 3 (AC137930) 1 (AP001383)	Hypothetical protein Putative peptide transport protein Hypothetical protein	ATG –1.0 kb 3rd exon ATG –0.4 kb
1C-070-04	+	++	++	–	++	10 AC087547)	Putative diphenol oxidase	ATG –0.4 kb
1C-076-54	++	+	+	++	++	10 AE017066)	Hypothetical protein	1st exon
1C-108-23	+	+	+	++	+	7 (AP005719)	Hypothetical protein	1st intron
1C-118-30	+	++	+	++	++	4 (AL606632) 3 (AC137930)	Hypothetical protein Putative peptide transport protein	ATG –1.0 kb 3rd exon
1C-146-13	+	+	+	++	+	8 (AP004137)	Hypothetical protein	3rd exon

^a +++: strong; ++: moderate; +: weak; –: no staining.

^b ATG: start codon; stop: stop codon; +: down stream; –: upstream.

^c China contig number.

the identification of 37 tagged genes. These genes, their putative functions, and the location of the T-DNA insertion in each gene are presented in Table 1. Genes that encoded for putative signaling-related proteins were the most abundant, and metabolism-related genes were next in number. However, more than half of the tagged genes could not be classified.

3.3. Characterization of the cold-responsive genes trapped by the gus reporter gene

Line 0-162-67 showed GUS activity that was induced by cold stress but not by wound or ABA. This line possesses a T-DNA insertion 222 bp upstream from the start codon of *OsRLK1*, where the *gus* gene is inserted in the same

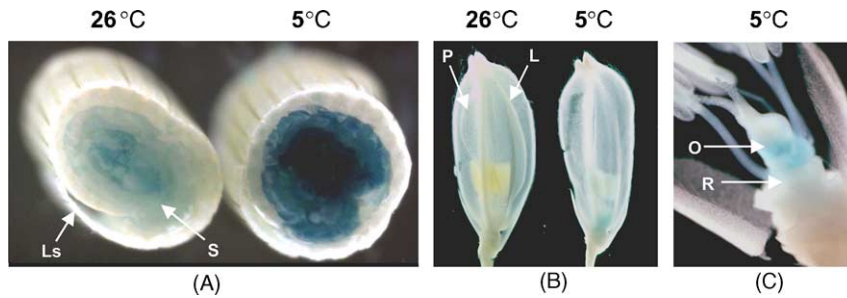


Fig. 4. GUS histochemical assay of 1C-051-22 under wounding (26°C) or cold stress (5°C). (A) Shoot segment of the youngest tiller from mature T1 heterozygote plant. (B) Young flower before heading stage. (C) Flower in (B) with palea and lemma removed to show internal floral organs. Ls, leaf sheath; S, stem; P, palea; L, lemma; O, ovary; R, rachilla.

orientation as the *OsRLK1* transcript (Fig. 2A). We predicted that *OsRLK1* encodes a leucine-rich repeat (LRR)-type receptor-like protein kinase. This gene is 3006 bp long, and consists of two exons. A corresponding EST clone (C98920) with exon 2, including 3'-UTR, is present in DDBJ. Our BLASTN search indicated that the gene is located on chromosome 3 (AC099732). The *OsRLK1* consists of 1001 amino acids that comprise 3 transmembrane regions, 20 extracellular LRRs, and a cytoplasmic kinase domain. This protein shares 63, 32, and 31% homology with the rice LRR-type protein kinases, OsLRK1 (AF193835), OsBRI1 (P048C01), and Xa21 (A57676), respectively.

Line 1C-051-22 showed GUS induction by wounding and further induction by cold stress. This line possesses a T-DNA insertion into the 1st intron of *OsDMKT1*, which is predicted to encode a SAM-dependent demethylmenaquinone methyltransferase (DMKT) (Fig. 2B). The *OsDMKT1* protein shares 77 and 72% similarity with a rice DMKT-like protein (BAB84438.1) and an *Arabidopsis* DMKT, AtDMKT2 (Q9FFE0), respectively. Two motifs common to a variety of SAM-dependent methyltransferases [66] are conserved in *OsDMKT1* (Fig. 3). An EST clone (BM419407) matching the 3'-UTR of *OsDMKT1* was found in DDBJ, confirming that *OsDMKT1* is an expressed gene. However, the homology of *OsDMKT1*, as shared with DMKTs that are characterized in other organisms, e.g. *Escherichia coli* (UbiE; [67]), *Bacillus subtilis* (MenG; [68]), *Lactococcus lactis* (MenH; [69]), and yeast (COQ5; [70]), is only ca. 10%. Therefore, we must still determine whether *OsDMKT1* indeed has DMKT activity. We examined the GUS expression pattern of the tagged line after cold stress, and found that it was barely detectable in untreated leaves, and was also unchanged by cold treatment in that tissue type. In the inner layer of the stems, however, GUS activity was increased by cold treatment (Fig. 4). Young spikelets exhibited cold-inducible GUS activity in the ovaries.

3.4. Induced expression of tagged genes by cold stress

We used semi-quantitative RT-PCR (QPCR) and northern blot analyses to examine whether the cold-responsive GUS expression patterns of the tagged lines were corre-

lated with the tagged gene in wild-type plants. Identity of the PCR bands was confirmed by DNA sequence analysis. Expression of the rice actin gene, *RAC1*, served as an internal control for PCR. Here, expression of *OsRLK1* was induced by cold after 3 h, and continuously increased until 24 h (Fig. 5). Salt stress also induced its expression. In the stems, the transcript level of *OsDMKT1* increased after 3 h of low-temperature exposure, reaching a maximum at 12 h. However, its responsive expression was not detected in the leaves (Fig. 6A) or in the roots. Cold inducibility was confirmed by northern blot analysis (Fig 6B).

3.5. Co-segregation analysis of GUS expression with the *OsDMKT1* gene

More than one copy of T-DNA is often integrated into rice chromosomes (data not shown). Therefore, we examined whether cold-inducible GUS expression was due to T-DNA tagging in *OsDMKT1*. Fifteen plants of Line 1C-051-22 were grown, and the genotypes of their progeny were determined using *OsDMKT1*-specific primers and the T-DNA primer gene (Fig. 7). We found that plant numbers 6, 14, and 15 were T-DNA homozygotes, while 5, 7, 8, 10, 11, and 12 were heterozygotes. All of these were GUS positive

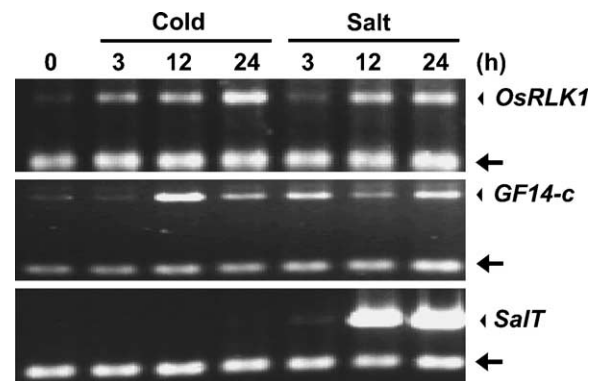


Fig. 5. Expression pattern of *OsRLK1* induced by cold and salt stress. Total RNAs from stress-treated samples served as templates for quantitative RT-PCR with gene-specific primers. *GF14-c* and *Salt* were used for the cold- and salt-response controls, respectively. Arrow indicates rice actin gene *RAC1* for PCR control.

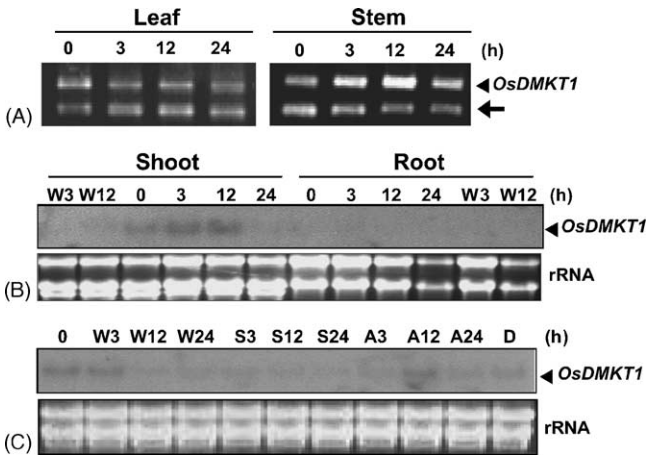


Fig. 6. Expression pattern of *OsDMKT1* induced by abiotic stresses. (A) Quantitative RT-PCR analysis of *OsDMKT1*. RNAs were isolated at specified intervals from cold-treated leaves and stems. Transcript of the rice actin gene, *Rac1* (indicated by arrow), shows an internal control for PCR analyses. (B) Northern blot analysis of *OsDMKT1* for cold stress. Each lane was loaded with 30 µg of total RNA isolated from shoots and roots that had been wounded (W) and treated with cold stress for the indicated time. (C) Northern blot analysis of *OsDMKT1* for other stresses. Each lane was loaded with 30 µg of total RNA isolated from shoots that had been periodically treated with salt (S), drought (D), or ABA (A). Membranes were hybridized with [³²P]-labeled *OsDMKT1* as probes. Ethidium bromide-stained gels shown at the bottom demonstrate equal RNA loading.

and inducible by cold. In contrast, the wild-type segregants, numbers 1, 2, 3, 4, 9, and 13 were GUS-negative. These results indicate that GUS expression was due to that T-DNA insertion.

4. Discussion

In this study, we employed T-DNA-tagged lines to identify cold-inducible genes. Here, the promoterless GUS reporter gene was inserted into rice chromosomes so that, when the T-DNA was placed in the proper orientation, the *gus* gene and the endogenous plant gene produced a fusion transcript [49,50]. Of the 15,586 lines that were screened, we observed 81 (0.52%) of them to show alteration (both positively or negatively) in GUS activity by cold stress. Seki et al. [71] have revealed, through microarray analysis of 7000 independent cDNA clones, that 0.3% of the *Arabidopsis* genes are inducible by low temperature, high-salt, and drought stresses. In a similar analysis, Fowler and Thomashow [19] have shown that 306 genes (3.8%) out of a total of 8000 are cold-responsive. Because 48.6% of the rice T-DNA is integrated into the genic region [72], 0.14–1.8% of the T-DNA tagging population should be cold-responsive, if the response frequency is similar between rice and *Arabidopsis*. Our screening of rice T-DNA tagging lines has led to the identification of cold-responsive genes at a frequency within the estimated range of values.

We have identified 37 genes trapped by the *gus* reporter gene, and have confirmed that some of these are truly cold-responsive. The *OsRLK1* gene tagged in Line 0-162-67 has high homology with other LRR-type receptor-like protein kinases, such as OsLRK1 and OsBRI1, both of which are involved in the diverse processes of meristem development, brassinosteroid signaling, and disease resistance [73]. At present, we do not know whether OsRLK1 protein is indeed involved in stress signaling, although *OsRLK1*

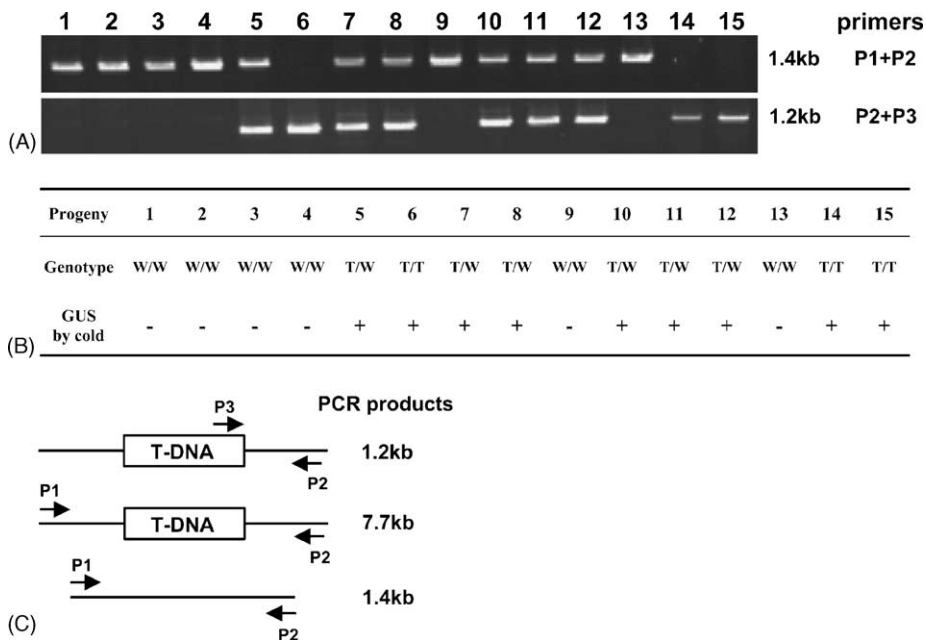


Fig. 7. Comparison of *OsDMKT1* genotype and GUS expression. (A) Fifteen plants at T2 generation were genotyped with primers P1 and P2 (upper panel) or primers P2 and P3 (lower panel). Lanes 1, 2, 3, 4, 9, and 13 are wild-type (W/W); lanes 5, 7, 8, 10, 11, and 12 are heterozygous (T/W); lanes 6, 14, and 15 are homozygous (T/T). (B) Comparison of genotypes with GUS activity. (+), GUS-positive; (-), GUS-negative. (C) Schematic diagrams of genotyping; P1, forward primer in *OsDMKT1*; P2, reverse primer in *OsDMKT1*; P3, forward primer in T-DNA.

expression is induced by cold as well as salt stress. In *Arabidopsis*, Hong et al. [74] have reported that this type of kinase gene is responsive to several environmental stresses, including low temperatures. We could not find any morphological change in the knockout progeny of the *OsRLK1*-tagged line, perhaps because of the large number of genes present in the genome. To illustrate, *Arabidopsis* possesses 417 genes of this type [75] while rice has more than 500. Because Line 0-162-67 carries two copies of T-DNA (data not shown), we could not exclude the possibility that the other tagged gene is responsible for cold-induced GUS expression. Moreover, it is possible that the coincidence of induced GUS activity and *OsRLK1* expression by both cold and salt stress indicates that GUS activity in that line is due to the *OsRLK1*-tagged GUS expression.

From the cold-responsive Line 1C-051-22, two tagged genes were identified. One is a putative tubulin β -1 gene where T-DNA was inserted in the 2nd intron with the same orientation. We could not detect any responsiveness of the gene to cold stress, whereas the other tagged gene, *OsDMKT1*, evidenced cold-responsive expression. In addition, genotyping of the T1 progeny coupled with an examination of GUS activity confirmed that *OsDMKT1* is the gene responsible for cold induction. Thus, we concluded that GUS expression, induced by low temperatures, was a reflection of the T-DNA insertion in the *OsDMKT1* gene. Cold-responsive GUS expression was confined to the stem region, a finding that matches the expression pattern for *OsDMKT1*. DMKT catalyzes the terminal step of MK biosynthesis, transferring methyl groups to DMK [76]. Although MK has an essential role as an electron carrier in prokaryotes and eukaryotes [77], its corresponding gene has not been reported in plants. It would be interesting to investigate whether *OsDMKT1* is indeed responsible for MK biosynthesis. *OsDMKT1* has the conserved motifs of SAM-dependent methyltransferase, which contribute to the binding of SAM and/or *S*-adenosyl homocysteine [66]. The glycine residue that is important for methyl transferase function [67] is present in *OsDMKT1*. In this study, we were able to retrieve seven *OsDMKT*s from the NCBI rice database using the amino acid sequence of *OsDMKT1*. Among them, the least homologous member showed 27% similarity with *OsDMKT1*. Therefore, the functional role of *OsDMKT1* in the cold-stress response, as well as in the expected metabolism, should be investigated in the future.

In conclusion, our approaches for isolating cold-responsive genes demonstrate the effectiveness of gene-trap mutagenesis as a means for discovering novel genes that are regulated in response to low temperatures in rice. The *gus* trapping method provides an advantage in analyzing the expression pattern of trapped genes. By analyzing the GUS staining pattern in our tagged lines, we could locate the places where the gene is expressed and examine their responsiveness to various stimuli. These knockout plants will be useful in studying the functional roles of the tagged gene during cold stress. As an example, their tolerance might be

directly assayed by monitoring chlorophyll fluorescence or ion leakage after exposure. These in-planta assays would give pivotal information on the cold-stress response in this model species that is so prone to chilling damage. Furthermore, characterization of the tagged genes would provide insights into the general cold-response mechanism in plants.

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