

EXPRESSION ANALYSIS OF SOME COLD RESPONSIVE ESTS IN SEEDLING-STAGE COLD-TOLERANT QTL IN RICE (*ORYZA SATIVA* L)

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Accepted: 04th March, 2013

ABSTRACT

Recombinant inbred lines (RILs) derived from a *Japonica* parent Hygokithanishiki (HGKN) and *Indica* parent Hokuriku (HOK) were used to identify quantitative trait loci (QTLs) controlling cold tolerance at the seedling stage in rice (*Oryza sativa* L). Genetic map with 95 SSR markers were used for QTL analysis and 163 RILs were subjected to two consecutive cold stresses at 4 °C for cold tolerance evaluation. Five major QTLs were identified in two different cold stresses and among these QTLs, QTL on chromosome 11 scored 9.39 and 5.43 LOD values at the first and the second cold stresses respectively. In rice expression sequence tags (EST) data base, cold responsive ESTs were found in the QTL region of chromosome 11 and they were analyzed by reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR was done under cold stress and control conditions in parental rice cultivars and in selected cold tolerant RILs with relevant chromosome fragment from HGKN parent and in selected cold susceptible RILs with relevant chromosome fragment from HOK parent. Results showed that ESTs numbered CI047516.1, CI049555 and CI049229 expressed highly in cold tolerant parent and in selected cold tolerant RILs at cold stress giving evidence on possible candidate ESTs in cold tolerant QTL.

Key words: Rice, Cold tolerance, QTL, EST expression

INTRODUCTION

Cold tolerance is a complex trait involving a number of biochemical and physiological events, which are controlled by major and minor quantitative trait loci (QTLs). Cold tolerant QTLs at seed germination, vegetative and booting stages have also been identified in different populations (Fujino *et al.* 2004; Saito *et al.* 2001; Andaya and MacKill 2003a; Andaya and MacKill 2003b; Andaya and Tai 2006; Andaya and Tai 2007; Zhang *et al.* 2005). Cold tolerance is developmentally regulated and growth stage-specific (Foolad, 2001). Two cold tolerant QTLs, *Cts1(t)* and *Cts2(t)*, were first identified for cold tolerance at the seedling stage in rice (Kwak *et al.* 1984; Nagamine *et al.* 1991) and later, Misawa *et al.* (2000) Fujino *et al.* (2004) Saito *et al.* (2001) Andaya and MacKill (2003a, 2003b) Zhang *et al.* (2005) Andaya and Tai (2006) Andaya and Tai (2007) identified more major cold tolerant QTLs in rice at different growth stages.

Genomic approaches for identification of expressed genes, namely expressed sequence tag (EST; Adams *et al.* 1995), serial analysis of gene expression (SAGE; Velculescu *et al.* 1995), and massively parallel signature sequencing (MPSS; Brenner *et al.* 2000), have been widely used in gene expression studies in various organisms. EST sequencing was the first method used for the rapid identification of expressed genes in organisms (Adams *et al.* 1995). cDNA sequences, known as ESTs, can be used to analyze gene structure, expression, and function. Further, ESTs can be used for genome study to understand chromosomal composition, organization, and structure. With a genome size of ~430 Mb, rice is estimated to have 30,000 to 50,000 genes (Yamamoto and Sasaki, 1997). ESTs have been utilized to identify the genes that are expressed in various tissues, cell types, or developmental stages (Michalek *et al.*, 2002; Ogihara *et al.* 2003; Ronning *et al.* 2003). ESTs provide a direct approach for discovering genes associated with a stress response. Many ESTs have been generated for rice, and these have been valuable in

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confirming and cataloguing genes (Sasaki *et al.* 1994; Uchimiya *et al.* 1992; Umeda *et al.* 1994; Yamamoto and Sasaki, 1997; Reddy *et al.* 2002a; Zhang *et al.* 2005).

In the present study cold responsive ESTs identified in the candidate region of the major seedling stage cold tolerant QTL were further studied to understand their contribution towards cold tolerance.

MATERIALS AND METHODS

Plant Material: A mapping population consisting of 163 RILs at F₆ generation was derived from a cross between a cold tolerant *Japonica* rice cultivar, “Hyogo-Kitanishiki” (abbreviated as HGKN), and a cold susceptible *Indica* rice cultivar, “Hokuriku-142” (abbreviated as HOK). HOK was bred from a cross between a Korean cultivar “Milyang 21” and an IRRI line “IR-2061-214-31” in Hokuriku Agricultural Experimental Station, Japan. These RILs were used in the QTL analysis for cold tolerance at the seedling stage with the parental cultivars as controls (Unpublished data).

Bio-Assay Conditions to Evaluate Cold Tolerance in RILs: Seeds were surface-sterilized by dipping in 70 % ethanol for 1 min and in NaOCl solution for 1 h, followed by washing in sterilized distilled water. Breakage of dormancy and acceleration of uniform germination were performed by keeping surface-sterilized seeds at 35 °C for 6 days in distilled water. Germinating seeds were planted in trays (24 cm x 24 cm) filled with soil. For each RIL, 20 germinating seeds were planted according to the randomized complete block design with 4 replicates each with 5 seeds, and they were grown for 7 days under normal growth conditions at 25 °C under 16 h light and 8 h dark. Seedlings were watered daily with 0.001 % Hyponex (N : P : K = 5 : 10 : 5 by volume, Hyponex, Japan) solution. Cold stress was given at 4 °C for 3 days under the same photoperiod. After this first period of cold stress, seedlings

were returned to the normal growth conditions and kept for 5 days for recovery. The level of cold tolerance was recorded according to an arbitrary five-point scale (first rating); where 1- a whole seedling became completely withered and dead, 2- 1st and 2nd leaves became withered but a stem remained green, 3 - only a stem and 3rd leaf remained green, 4 - a stem and two leaves remained green, and 5 - normal growth with all the leaves remained green. After the first rating, the same set of seedlings were kept under the normal growth conditions for another 3 days and then subjected to the second 3-day period of cold stress at 4 °C. The second rating was done at the 5th day of recovery. An arbitrary rating scale was; 1- a whole seedling became withered, 2 - only bottom part of a stem remained green, 3 - only a whole stem remained green, 4 - a stem and one leaf remained green, and 5 - a stem and more than one leaf remained green. The whole experiments were repeated three times.

Map Construction and QTL Mapping: Linkage analysis was performed with MAPMAKER/EXP 3.0 (Lincoln *et al.* 1992). Kosambi map function was set to the map construction. The linkage map was constructed using MAPMAKER 3.0 (Lander *et al.* 1987), a version of the linkage program that runs on Macintosh computers. The frequencies of observed recombinations between two markers were converted to genetic distance, using the map function of Kosambi. The bioassay ratings of cold tolerance averaged over three replicates were used for QTL mapping. Both single marker analysis and interval analysis were carried out to locate QTLs using the computer program QTL cartographer 2.5(Wang *et al.*, 2007). A LOD score of 3.0 was taken as the threshold to declare the presence of QTLs. Genetic parameters associated with significant QTLs were collected both at the single marker analysis and interval analysis. Data were further analyzed by WinQTL CARTOGRAPHER for determination of threshold LOD value by 1000 permutation test at composite interval mapping.

Selection of Cold Tolerant and Cold Susceptible RILs: Constructed genetic map of the RILs was closely observed to select the cold tolerant RILs with the chromosome fragment of the QTL region from cold tolerant *Japonica* parent and cold susceptible RILs with the chromosome fragment of the QTL region from the cold susceptible *Indica* parent.

Preparation of Seedlings for Cold Stress:

De-hulled seeds of a *Japonica* rice cultivar Hyogokithanishiki (HGKN) and *Indica* rice cultivar Hokuriku (HOK) were subjected to overnight imbibition to stimulate synchronous germination. Imbibed seeds were surface-sterilized with 1 % (w/v) solution of sodium hypochlorite (NaClO) for 10 min and rinsed in distilled water. Sterilized seeds were allowed to germinate on wet blotting papers in glass petri dishes (70-mm-diameter) in a dark incubator adjusted at 35 °C for 3 days. Germinated seeds were planted in soil-filled trays and kept at 28 °C with 16h/8 h light/dark for one week.

Cold Stress for Expression Analysis: For low temperature stress, one-week-old seedlings were subjected to cold treatment at 4 °C in the dark. The same procedure was applied to the cold tolerant inbred lines with

the chromosome fragment from HGKN parent and cold susceptible inbred lines with chromosome fragment from cold susceptible inbred parent HOK.

RT-PCR Analysis: Primers for ESTs were designed by DNASIS. Seedling leaves were collected, frozen with liquid nitrogen and then were subjected to RNA extraction using guanidine thiocyanate. The amount of transcripts was determined by RT-PCR analysis using a first strand cDNA synthesis kit (TOYOBO, Osaka, Japan). The total template RNA samples for the cDNA synthesis were treated with DNaseI to remove contaminated DNA. RT-PCR was performed with specific primers, which were designed and synthesized by Invitro Lifetech Oriental (Nacalai). Primer information is shown in Table 1.

For each sample 4 µl c-DNA template was added to 16 µl reaction mixture containing 1 µl each of forward and reverse primers, 2 µl 10 x buffer, 0.8 µl MgCl₂, 1 µl dNTPs, 10 µl Q water and 0.2 µl rTaq Polymerase to make a 20 µl PCR mixture. RT-PCR was carried out by amplification with 22 cycles under conditions described in Table 1 using a thermal cycler, Gene Amp PCR System 9700 (Applied Biosystem). For each sample, 4 µl c-DNA template was added to 16 µl reaction

Table 1: Primers designed for cold induced ESTs

EST Number	Forward primer	Backward primer
CI304905.1	5'AGT CCC TCC GAT TCC GAT GG3'	5'GGA GGA GGA CGA CGA CGA GG3'
CI305110.1	5'CTC CTC CCC AAA CCC TAG CC3'	5'GTA AAG CCG ACG TAG GAG GG3'
CI304308.1	5'TCC GTT CCA CAG CCA AGA TG3'	5'GAA CCT ATC CAC CAT GCC CC3'
CI304488.1	5'GAA GGA ATC CAA GTT GAA GGC3'	5'AAC CTA TCC AGA TCT CGT CCA3'
CI052424.1	5'GAG ATT GAG AAG ATG GTC CAG3'	5'TCT TGT CAT CGA ACT CAT CAG3'
CI 050328	5'GGA CTC AAT TCA GAT CAA AC3'	5'ACT GAA TTG ATG GGT AGA AC3'
CI051012.1	5'ACG AGA TGA AGG AGC TGG AG3'	5'TTC AAT TCA AAA GGA CTG CC3'
CI051955.1	5'ACA AGA AGA AGG TGG AGT CC3'	5'GCA TGT TGT AGG CGT AGT TC3'
CI050653.1	5'GAG ATG TAC AAG CAG TGG CAG3'	5'TTT TTT TTT GGT CAG TGG TCA3'
CI048067.1	5'CAG AGG ATG AGG AGC ACA AG3'	5'CGC AGT TCT TCA ATT CAA AA3'
CI047516.1	5'GAA CTA CGC CTA CAA CAT GC3'	5'CGC AGT TCT TCA ATT CAA AA3'
CI047016.1	5'CTG GTG GAG GGA AGT GGT GT3'	5'GCC AGG CAG TAT TAG CAA CC3'
CI047164.1	5'TCG ATG ACA AGA TGA AGG AG3'	5'GCA AGC ACC ACA ATA ATT AA3'
CI049555	5'CGG ACA AGA AGA AGA TCG AG3'	5'TTC AAT TCA AAA GGA CTG CC3'
CI048802.1	5'GGA AGG AGA ACT AAG AGC TG3'	5'GGG TAG GTA GCT TTA TGT GG3'
CI049229	5'ATA TCA GAA AAA GGA CCT CG3'	5'CAG GAT AAA AAT GTC AAG CA3'
CI049063.1	5'GCA CCA GCA TGA CTC AAG TT3'	5'TTA TCA GTT GTA TCC CGG CA3'
CI047503.1	5'CAT C AT TC G GGC C TT AA G AGA3'	5'CAT C AC GC A TTC AGC ATC TTG3'

mixture. Rice actin gene was used as a control. RT-PCR products were resolved on 1.2 % or 2.0 % agarose gel, stained with ethidium bromide, and pictures of images were taken under UV light.

ESTs, having differential expression patterns in cold stressed parental cultivars, were used for further expression analysis in selected cold tolerant RILs and cold susceptible RILs at control conditions and at cold stress, by RT-PCR. Primers designed for cold induced ESTs are given in the [Table 1](#).

RESULTS AND DISCUSSION

Two QTLs found at the first and the second cold treatments are given in Table 2 (unpublished data). The EST data base was mined in this particular chromosomal fragment to find cold responsive ESTs.

Eighteen cold responsive ESTs were found in the QTL region of chromosome 11 in rice EST data base. Primers for the cold responsive ESTs were designed using software DNASIS. The primer sequences of forward and backward primers are given in the table 1.

Primer No.	HOK Con	HGKN Col	HOK Con	HGKN Col	PCR cycle No	Primer No.	HOK Con	HGKN Col	HOK Con	HGKN Col	PCR cycle No
Actin						Actin					26cycles
26cycles CI304905.1						CI052424.1					25cycles
25cycles											22cycles
25cycles					CI305110.1	CI051012.1					25cycles
CI304308.1					25cycles						22cycles
CI304488.1					25cycles	CI051955.1					25cycles
CI052424.1					25cycles						22cycles
CI 050328					25cycles	CI050653.1					25cycles
CI051012.1											22cycles
25cycles CI051955.1						CI048067.1					25cycles
25cycles CI050653.1											22cycles
25cycles CI048067.1						CI047164.1					25cycles
25cycles CI047516.1											22cycles
25cycles CI047016.1						CI047503.1					25cycles
25cycles					CI047164.1						22cycles
25cycles											
CI049555					25cycles						
CI048802.1					25cycles						
CI049229					25cycles						
CI049063.1					25cycles						
CI047503.1					25cycles						

Figure 1: Expression levels of cold induced ESTs in parental rice cultivars under control condition and under cold stress at 4°C for three days HGKN: Hyogokithanishiki, cold tolerance Japonica rice cultivar; HOK: Hokuriku-142, cold susceptible Indica rice cultivar Con: under control condition Col: Under cold stress

ESTs those expressed in higher levels under cold stress in cold tolerant rice cultivar Hyogokithanishiki compared to that of cold susceptible rice cultivar Hokuriku were further studied in selected cold tolerant and cold susceptible inbred lines under both control conditions and cold stress conditions. ESTs with data base numbers CI047516.1, CI049555, and CI049229 were expressed significantly higher levels in cold tolerant RILs under cold stress compared to that of cold susceptible RILs (Fig. 1).

According to the results of EST expression analysis, ESTs numbered CI047516.1, CI049555, and CI049229 showed significantly higher expressions in cold tolerant parent Hyogokithanishiki (Fig. 1) and in cold tolerant inbred lines under cold stress (Fig. 2). These ESTs expressed both in cold tolerant parent and cold susceptible parent under cold stress. They were not R specific but at least they are responsible for cold tolerance in the QTL on chromosome 11 among other un-

known cold responsible genes. This is a potential QTL region to find the candidate genes for cold tolerance. As an example in another study two candidate genes both encoding ζ glutathione S-transferase have been predicted at a major QTL, *qCTS12*, on rice chromosome 12 for cold tolerance at the seedling stage (Andaya and Tai 2006). This was supported by the report that over expression of the ζ glutathione S-transferase gene enhanced cold tolerance at both germination and seedling stages (Takesawa *et al.* 2002). Likewise, this QTL region must be studied in systematic way to find R specific cold responsive ESTs in future studies.

ACKNOWLEDGEMENTS

The first author wishes to acknowledge Monbukagakusho (Ministry of Education, Culture, Sports, Science and Technology, Government of Japan) for the financial support for the postgraduate studies in Japan

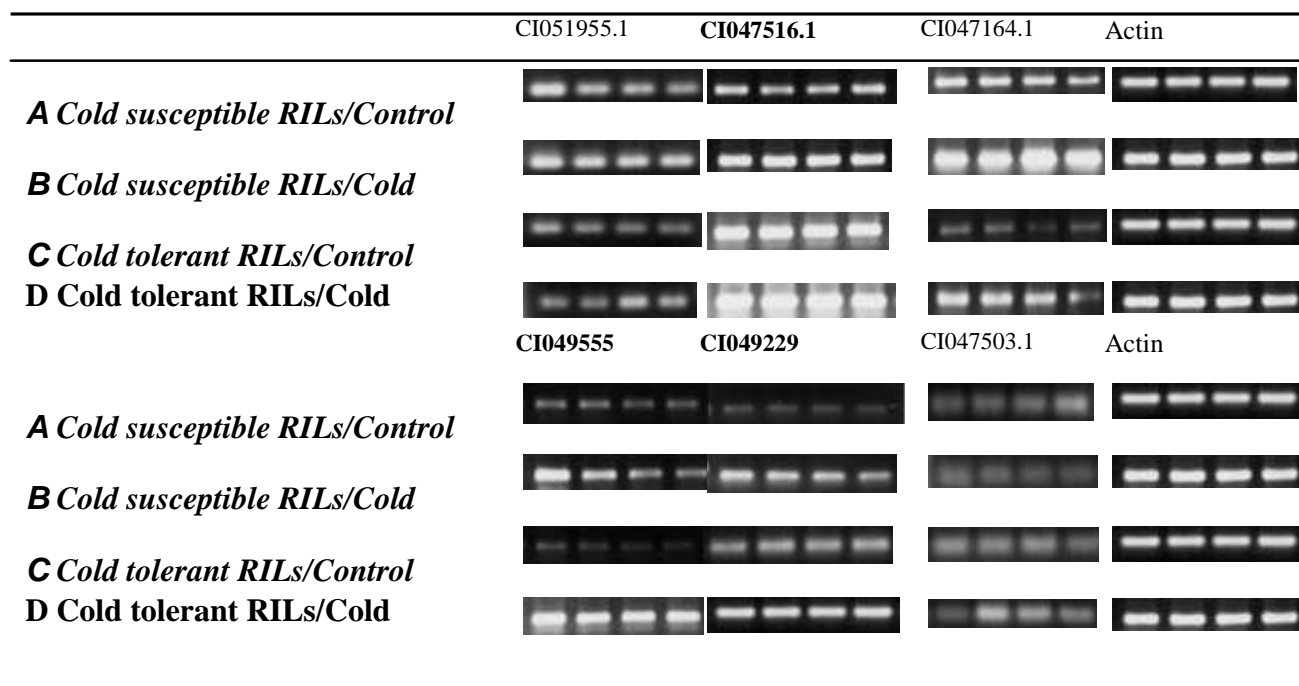


Figure 2: Expression level of cold induced ESTs in selected cold tolerant and cold susceptible RILs under control condition and under cold stress at 4°C for three days **A:** four cold susceptible RILs under control conditions, **B:** four cold susceptible RILs under cold stress; **C:** cold tolerant RILs under control conditions, **D:** cold tolerant RILs under cold stress.

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