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# Assessment of cold tolerance at early developmental stages and allelic variation at candidate genes in South American rice germplasm

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**Abstract** Rice is susceptible to cold during early developmental stages. Most tolerant cultivars have been developed for other conditions than those occurring in South America's rice growing areas, or their grain type is not suitable for the local markets. If locally adapted varieties were available, growers could anticipate sowing date, making flowering time coincide with the moment of maximum solar radiation and increasing yields. In this work, 116 rice inbred lines and varieties of diverse origin within the South American gene pool were tested for seedling survival and germination percentage under low temperature in controlled conditions. As a result, lines used as controls responded as expected, whereas lines with similar behavior to controls, intermediate between susceptible and tolerant controls and more extreme than controls were detected at both seedling and germination stages. Allelic variation at candidate genes *OsGSTZ1*, *OsGSTZ2* and *OsCDPK13* was analyzed in a subset of ten contrasting lines. Ten out of thirty-four polymorphisms detected in all three genes were associated with cold tolerance in these lines. A functional marker, designed from an amino-acid-changing SNP found in gene *OsGSTZ2*, was tested in the 116 lines. As a result, a tight association was observed between cold tolerance and marker score. In conclusion, wide variability for cold tolerance at early developmental stages has been detected in rice advanced breeding materials that are adapted to local growing conditions. Also, a functional marker tightly associated with the trait is available for performing marker assisted selection.

**Keywords** Rice cold tolerance · Seedling · Germination · Candidate genes · Functional marker

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## Introduction

Rice (*Oryza sativa* L.) is one of the most important food crops, and a staple food for more than half the world's population (FAO 2004). It is grown on $\sim$154 million ha year$^{-1}$ or $\sim$11 % of the world's cultivated land (Khush 2005; FAO 2010; FONTAGRO 2010), in every continent except Antarctica. As it is the case in other tropical and subtropical crops, one of the main factors limiting rice production is the occurrence of low temperatures during the growing season. This is especially critical in areas where the most widely grown cultivars are of the *indica* ssp., which generally exhibit greater sensitivity to low temperatures than those of the *japonica* ssp. (Glaszmann et al. 1990; Mackill and Lei 1997; Baruah et al. 2009; Kim and Tai 2011).

Low temperatures may affect rice crop growth and development from germination to grain filling, and greatly reduce yield (Yoshida et al. 1996; Mackill and Lei 1997; Nakagahra et al. 1997). Germination, seedling establishment, booting and grain filling are the crop stages with highest risk of chilling injury (Ye et al. 2009). Some chilling-sensitive plants species can undergo acclimation, i.e. they increase cold tolerance after being gradually exposed to low temperatures (Moynihan et al. 1995). Rice, however, lacks this acclimation capacity (Sato et al. 2001). Cold injury symptoms in rice include poor germination, seedling mortality, leaf chlorosis and necrosis, reduced tillering, delayed heading, spikelet sterility and grain filling inhibition (Kaneda and Beachell 1974; Vergara 1976; Mackill and Lei 1997; Jiang et al. 2008; Kim and Tai 2011).

Developing rice cultivars with greater tolerance to low temperatures at seedling and reproductive stages would have a major impact on the crop (Andaya and Tai 2006). Whereas such cultivars are available in some rice growing areas, they do not necessarily show wide adaptation, or their grain type is not suitable for the local market. This is the case in Argentina and other South American countries, where most of the rice under cultivation is of the long-grain *indica* type, and no cold tolerant cultivars are available so far. As a result, sowing date is often delayed until temperatures are mild enough to escape chilling injury risks. If, on the other hand, locally adapted, cold tolerant varieties were available, growers could anticipate sowing date, making flowering time coincide with the moment of maximum solar radiation and increasing yields (Akita 1989; Okawa et al. 2003).

Breeding for rice cold tolerance in the field can be very challenging, because environmental conditions are unpredictable, and differences in phenology among genetic materials might lead to scoring errors due to escape. Assessment of cold tolerance in growth chambers under controlled conditions, on the other hand, allows simultaneous screening of large numbers of individuals at the same phenological stage in a reduced space, but it is laborious and time-consuming, and results obtained under such conditions might not be correlated with performance in the field. These obstacles could be circumvented if molecular markers for marker-assisted selection were available.

Genetic analysis of cold tolerance at early developmental stages in rice has resulted in the identification of a large number of quantitative trait loci (QTL) associated with the trait (Qian et al. 2000; Misawa et al. 2000; Miura et al. 2001; Andaya and Mackill 2003; Fujino et al. 2004, 2008; Zhang et al. 2005; Lou et al. 2007; Jiang et al. 2008; Wang et al. 2009; Ji et al. 2010, among others). Most of these studies have been carried out using mapping populations derived from *indica* x *japonica* crosses.

Andaya and Tai (2006) saturated the genomic region in which Andaya and Mackill (2003) had detected the QTL designated as *qCTS12*, and proposed two glutathione-S-transferase-coding genes present in this region as candidate genes for the trait (*OsGSTZ1* and *OsGSTZ2*). Furthermore, Kim et al. (2011) showed that naturally occurring *OsGSTZ2* isoforms differed in their enzymatic properties, which may contribute to the differential response to chilling stress generally exhibited by the two major rice subspecies, *indica* and *japonica*. Takesawa et al. (2002) observed an increase in tolerance to low temperature stress during germination and seedling growth of rice when they overexpressed *OsGSTZ1*. Similar results were found by Abbasi et al. (2004) when overexpressing a kinase-encoding gene, *OsCDPK13*.

Little is known about the degree of genetic diversity that South American rice breeding germplasm may harbor for cold tolerance at early developmental stages. Under the increasing occurrence of extreme

meteorological events due to climate change and the need for producing higher yields in the same land area, breeding for rice cold tolerant varieties should aid in raising crop yield and stability in the region. In the present paper we report (1) the response to low temperature at early developmental stages of a collection of advanced lines and cultivars of diverse origin within the South American gene pool, obtained from the rice breeding program of the Estación Experimental Agropecuaria (EEA) Corrientes, Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina; (2) the assessment of allelic variation at candidate genes *OsGSTZ1*, *OsGSTZ2* and *OsCDPK13* in a subset of these lines; (3) the association between plant response to low temperature at early developmental stages and allelic variation, and (4) the development of a functional marker for cold tolerance at the seedling stage.

## Materials and methods

### Plant material

One hundred and sixteen South American accessions including advanced breeding lines and commercial cultivars (Supplementary Table 1) were used in phenotypic evaluations. Accessions were chosen on the basis of pedigree information, to ensure that ample variability was represented in the collection. This collection included six cultivars with known response to low temperatures at the seedling stage: four tolerant (Quilla 158502, Quilla 166002, Quilla 132703, and CT6748-8-CA-17, respectively termed "104", "105", "107" and "115") and two susceptible ones (Oryzica 1 and FL05383-1P-11-1P-2P-M, respectively termed "119" and "120").

Allelic variation at candidate genes was investigated in the ten lines with the most contrasting response to low temperature at the seedling stage (i.e. five tolerant and five susceptible lines). Based on pedigree information, a few lines from within the most tolerant and the most susceptible groups were previously discarded from this analysis because they shared an ancestor with another line within the same group. This was done to avoid the occurrence of spurious associations (false positives) when establishing the association between allelic variation and phenotype.

### Phenotypic evaluation

#### *Seedling stage*

Seedling cold tolerance phenotyping was performed as described previously by Andaya and Tai (2006) using a growth chamber (STH–020, Sanshu Sangyo Co. Ltd) at 9 °C constant temperature, 12 h photoperiod, and a photosynthetic photon flux density of 300 μmol $m^{-2}$ $s^{-1}$.

A randomized complete block design with three replications was used. At least 25 seedlings per accession were grown in outdoor bins. Of these, five seedlings per accession with three completely expanded leaves, similar size and healthy aspect were picked and used as the experimental unit. The plants were contiguously placed in speedling trays and the lines (treatments) were completely randomized into the growth chamber. Symptom evaluations were carried out at 8, 10, 12 and 14 days after transferring plants to the growth chamber, using a 1–9 cold injury score (International Rice Research Institute (IRRI) 1996; Supplementary Table 2) where 1 = no injury and 9 = seedling death. This was repeated three times, and each batch constituted a block.

#### *Seed germination stage*

Assessment of cold tolerance at the seed germination stage was performed following the protocol proposed by the Fondo Latinoamericano para Arroz de Riego (FLAR; Corredor et al. 2007; Supplementary Text). A randomized complete block design with three replications was used. Seeds were placed in sterilized paper soaked in a fungicide solution (2 ppm Benomyl) and rolled into a 2 cm-diameter cylinder. Cylinders with seeds of 116 lines were put in a plastic tray. Trays were simultaneously placed in a growth chamber at constant 12 °C in the dark. Percent germination was determined when coleoptile length in one of the tolerant control lines (Quila 132703) reached 4 mm. This was repeated three times, and each batch constituted a block. Along with this experiment, we carried out a germination test with the same technique but at 30 °C, to assess each line's germination potential. With this value we calculated an index (I) according to the following equation:

**Table 1** Characteristics of primers designed

| Gene | Fragment number | Designation | Primer denomination | Sequence (5′–3′) | Tm (°C) | %GC |
|---|---|---|---|---|---|---|
| *OsGSTZ1* | 1 | P1 | Os200(3)R1F | ATGAGCTCGTGCTCCTACAGGG | 60.7 | 59 |
| | | | Os200(2)R1R | TCCCCATCTACTAATGCTGGCACA | 59.9 | 50 |
| | 2 | P2 | Os200(2R2F | TGCCAGCATTAGTAGATGGGGA | 57.9 | 50 |
| | | | Os200(2)R2R | CTGCGACATCCTTCCAGAAGTT | 57.2 | 50 |
| | 3 | P3 | Os200(2)R3F | AACTTCTGGAAGGATGTCGCAG | 57.2 | 50 |
| | | | Os200(2)R3R | TCCCTAGGTACTGGAGTACTGGAT | 57.9 | 50 |
| *OsGSTZ2* | 1 | P4 | Os300(2)R1F | AAGCCAATCCTGTACGGTGCCT | 61.1 | 54.5 |
| | | | Os300(2)R1R | CACCAATGCAATGGCGAGAGAGT | 59.8 | 52.1 |
| | 2 | P5 | Os300(2)R2F | TCTGACTCTCTCGCCATTGCAT | 58.4 | 50 |
| | | | Os300(2)R2R | TTGAACTTCGTCCCCAGTAGCA | 58.3 | 50 |
| | 3 | P6 | Os300(2)R3F | ATGCTACTGGGGACGAAGTTCA | 58.1 | 50 |
| | | | Os300(2)R3R | GTGACTGAGCACTTGAGTTGAGC | 58 | 52.1 |
| *OsCDPK13* | 1 | P7 | Os700(2)R1F | CGCGAGGTCTCGTAAGGTCCAT | 60.8 | 59 |
| | | | Os700(4)R1R | CACCGCTTGCCCATCCTCATAG | 60.1 | 59 |
| | 2 | P8 | Os700(2)R2F | AAGCGCAAGCTCATCACCAAGG | 60.5 | 54.5 |
| | | | Os700(2)R2R | CTTCAAACGCTCCGAAGGGCAA | 60.3 | 54.5 |
| | 3 | P9 | Os700(2)R3F | GTTTGAAGGCCCATGAAGTGCTAA | 57.9 | 45.8 |
| | | | Os700(2)R3R | AGGTGCTCCTCACGTTCTATCTTG | 58.3 | 50 |
| | 4 | P10 | Os700(4)R4F | GCYGAGCGTCTTTCAGAGGAGG | 60.8 | 61.3 |
| | | | Os700(2)R4R | CTAGGCGGCCTTGCAACTACAT | 59.6 | 54.5 |

Number and designation of gene fragments, primer name, sequence, melting temperature (Tm) and %GC. The letters F and R at the end of the primer name indicate forward or reverse primers

$$I = CGS.100/(GP.n/100)$$

where CGS = number of cold-germinated seeds, GP = germination potential, and n = number of seeds tested. This index was used to adjust percent germination at low temperature as relative to each line's germination potential.

### *Statistical analysis*

Analysis of variance (ANOVA) of phenotypic data from the seedling stage was performed using the MIXED procedure of the SAS Software Package (SAS 1999), whereas ANOVA of data from the germination stage was carried out with the GLM procedure of the same package. Subsequently, a multivariate analysis was done for both stages using the program InfoStat (Di Rienzo et al. 2010). Also, the Pearson correlation coefficient between the score at the seedling stage and germination percentage was calculated using the CORR procedure of SAS.

## Allelic variation at candidate genes

### *Primer design*

Specific primers were designed to PCR-amplify the coding region of genes *OsGSTZ1*, *OsGSTZ2* (Andaya and Tai 2006) and *OsCDPK13* (Abbasi et al. 2004). cDNA sequences were downloaded from Genbank and aligned with the corresponding *japonica* and *indica* genomic DNA sequences, in order to design consensus primers. Global alignments were performed with MEGA (Molecular Evolutionary Genetics Analysis) software, version 4.0.2. (http://www.megasoftware.net, Tamura et al. 2007), and manually edited when necessary.

To facilitate further sequencing, primers were designed to amplify genes into ~1,000 bp overlapping fragments. Primer-BLAST tool of NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) was used for primer design and in

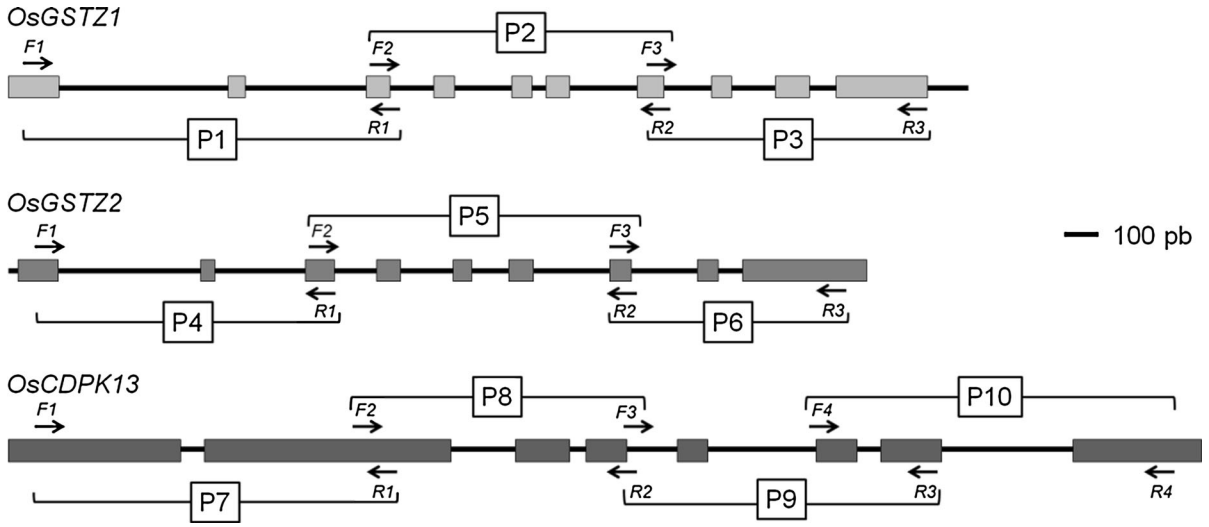


**Fig. 1** Diagram of the genes analyzed. The primers are indicated by *arrows*, and designated with a number (*1–4*) that indicates the fragment within each gene amplified by the primer pair, and a letter: *F* (forward) or *R* (reverse). *P1* to *P10* designate gene fragments. *Full boxes* represent exons and *lines* between *boxes* represent introns or untranslated regions

silico PCR against the full genome sequence of *Oryza sativa* (*indica* and *japonica* ssp.), to ensure that a single PCR fragment of the expected size would be amplified. Primer sequences and characteristics are shown in Table 1, and their location in the genes is depicted in Fig. 1.

### *DNA extraction, PCR amplification and fragment purification*

Genomic DNA was isolated according to Haymes (1996), from ca. 100 mg leaf tissue of 1 week-old seedlings. Extracted DNA was resuspended in TE buffer to a final concentration of 100 ng $\mu l^{-1}$. PCR amplifications were performed in a reaction mix composed of 11.05 µl ultrapure water, 6.25 µl of 5× GoTaq™ reaction buffer (Promega), 1 µl of dNTPs (25 mM), 0.25 µl of each forward and reverse primers (prepared at 100 pmol $\mu l^{-1}$), 1 U GoTaq™ DNA Polymerase (Promega) and 1 µl of DNA sample, to obtain a final volume of 20 µl per reaction. Amplifications were carried out in two thermocyclers, Eppendorf Mastercycler™ and MJResearch PTC-100™, with the following program: initial denaturation at 95 °C (3 min), 35 cycles of 94 °C (30 s), 60 °C (30 s) and 72 °C (1 min 30 s), and final extension at 72 °C (5 min). Amplicons were separated in 1.5 % agarose, 1× TBE buffer gels, run at constant 65 V for ∼90 min in a horizontal electrophoresis cell. SybrSafe™ (Molecular BioProbes) was used for DNA staining. Gels were visualized in a Safe Imager™ Blue-Light Transilluminator (Invitrogen). Bands of expected size were cut from the gel and purified with the Silica Bead Gel DNA Extraction Kit # K0513 (Fermentas), following the manufacturer's instructions.

### *Sequencing of PCR fragments*

Sequencing was carried out at the Laboratorio de Agrobiotecnología, EEA INTA Balcarce (Argentina), in a MegaBACE automatic sequencer (Amersham Biosciences) and at the Unidad de Genómica, Instituto de Biotecnología, CNIA-INTA Castelar (Argentina), in an ABI3130XL automatic sequencer (Applied Biosystems). Sequencing reactions were prepared as follows: 4 µl of "premix" sequencing reagents (BigDye™, Molecular BioProbes), 0.5 µl of each primer (3 µM), 100 ng DNA, and water to a final volume of 10 µl. Reactions were run in an MJResearch PTC-100™ thermocycler, with the following program: 35 cycles of 95° C (20 s), 50° C (15 s) and 60° C (1 min), and a final step at 16° C (5 min). In order to check for sequencing quality, the same control sample was included in every sequencing run. Also, all fragments were sequenced in duplicate from independent PCR reactions.

**Table 2** Characteristics of primers used in marker development

| Primer name | Sequence | Tm (°C) | Expected amplicon size (bp) |
|---|---|---|---|
| LS_F | 5′ CGCCATTGCATTGGTGAGTA 3′ | 63 | 1,160[a] |
| LS_R | 5′ GGCAAGCCAAGATGGAACTG 3′ | 63 | |
| NLS_F | 5′ ACTTTCAGTGTTTTGTTTGC 3′ | 49 | 163[b] |
| NLS_R | 5′ TGGATGCTTGAACAAAGTAT 3′ | 49 | |

Primer name, sequence, melting temperature (Tm) and expected amplicon size. The letters F and R at the end of the primer name indicate forward or reverse primers

[a] Amplicon not detected in electrophoresis, under the PCR conditions used in the protocol

[b] Amplicon only present in individuals with the A allele

*DNA polymorphism detection*

Sequence analysis was performed with the BioEdit software (version 5.0.6). Poor quality regions were trimmed out manually, and only the resulting high quality sequences were used for further analyses. In a few cases, high-quality sequences coming from two independent reactions of the same line were not 100 % identical and were hence discarded. Sequences of a given gene fragment were aligned with their respective reference sequences (cDNA and genomic *indica* and *japonica* sequences), using MEGA. Alignments were manually inspected, and the location and type of polymorphisms found was recorded.

Marker development

An aminoacid-changing SNP found in an exon of gene *OsGSTZ2*, polymorphic between tolerant and susceptible lines (A/G transition; see "Results"), was chosen as target for marker development. A functional marker for cold tolerance at the seedling stage was thus developed using the Temperature Switch PCR (TSP) technique proposed by Tabone et al. (2009). Primers were designed with Primer-BLAST using the full gene sequence as target. Two pairs of primers, termed locus-specific (LS) and nested locus specific (NLS), were designed (Table 2).

PCR amplifications were performed in a 20 µl mix consisting of 10.15 µl ultrapure water, 6.25 µl 5× GoTaq® buffer (Promega), 1 µl dNTP mix (25 mM), 0.25 µl of each LS_F and LS_R primers (100 pmol $\mu l^{-1}$), 0.75 µl of each NLS_F and NLS_R primers (100 pmol $\mu l^{-1}$), 1 U GoTaq® DNA Polymerase (Promega) and 0.5 µl DNA (100 ng $\mu l^{-1}$). Reactions were run in an Eppendorf Mastercycler™ thermocycler with the following program: (1) initial denaturation at 95 °C (3 min), (2) 15 cycles of 95 °C (30 s), 53 °C (30 s) and 72 °C (60 s), (3) 5 cycles of 95 °C (10 s) and 45 °C (30 s), (4) 15 cycles of 95 °C (10 s), 53 °C (30 s) and 72 °C (5 s), and (5) a final extension step at 72 °C (5 min). Amplicons were separated in 1 % agarose—1× TBE gels, as described previously.

Marker analysis was carried out in the 116 lines evaluated in the cold tolerance assays. A 1/0 score system for band presence/absence was used for genotyping. Then, a goodness-of-fit, Chi square test was performed to determine the association between genotype and cold tolerance at the seedling stage. Furthermore, a Student's t test was performed to compare the mean of cold injury score of individuals showing the 163 bp band versus that of individuals for which no band was detected.

The factibility of designing a CAPS marker from this SNP was established by in silico analysis of the full gene sequence with the Restriction Enzyme Map Analysis Tool of GenScript (http://www.genscript.com).

## Results

Phenotypic evaluation

*Seedling stage*

ANOVA of the cold injury score revealed statistically significant effects ($p < 0.0001$) of blocks (batches) and treatments (lines). The frequency distribution of cold injury score is presented in Fig. 2a. Tolerant and susceptible control lines showed an overall average

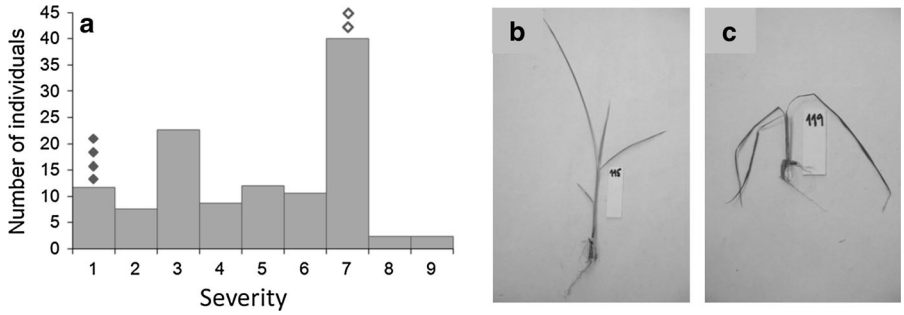


**Fig. 2** **a** Frequency distribution of the cold injury score (0 = no injury; 9 = seedling death; IRRI 1996) of 116 lines evaluated at the seedling stage in controlled conditions at 9 °C. *Full diamonds* indicate the average score of tolerant controls, and *empty diamonds* indicate the average score of susceptible controls. **b** Tolerant control (cultivar CT6748-8-CA-17) 14 days after cold treatment initiation. **c** Susceptible control (cultivar Oryzica 1) 14 days after cold treatment initiation

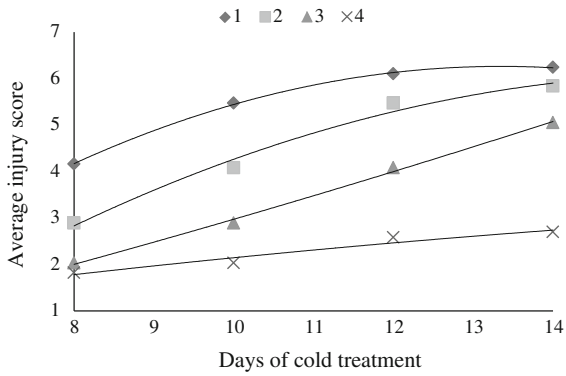


**Fig. 3** Average seedling injury score (1 = no injury; 9 = seedling death) of *lines* grouped by multivariate analysis into four susceptibility groups (*1*: susceptible, N = 18; *2*: moderately susceptible, N = 43; *3*: moderately tolerant, N = 39; *4*: tolerant; N = 16) over time under cold treatment

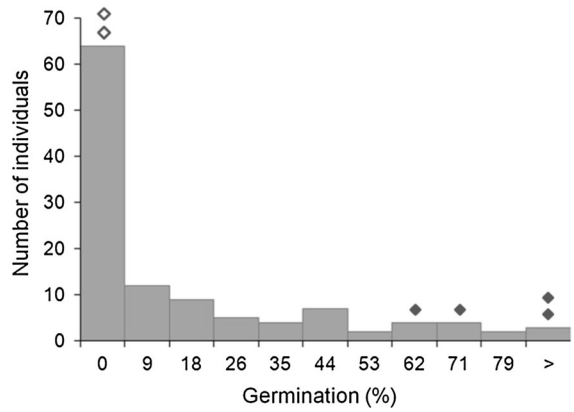


**Fig. 4** Frequency distribution of seed germination percentage at 12 °C. *Full* and *empty diamonds* indicate the germination percentage of those *lines* respectively used as tolerant and susceptible controls in the seedling cold tolerance assay

cold injury score of 1.7 ± 0.5 and 7.7 ± 0.05, respectively (Fig. 2a–c). Among the remaining lines used in the study, there were some with intermediate, similar and more extreme response than that of the controls (Fig. 2a). Some lines exhibited symptoms of cold damage already in the first observation date. Early symptoms included slight curling and discoloration of leaves, and wilting and necrosis were the predominant visual symptoms in later observation dates.

Multivariate analysis yielded a classification of lines into four groups: group 1, defined as susceptible (18 lines), group 2 as moderately susceptible (43 lines), group 3 as moderately tolerant (39 lines), and group 4 as tolerant (16 lines; data not shown). All groups showed increased severity symptoms over time, although in different magnitude (Fig. 3).

### *Seed germination stage*

ANOVA of seed germination percentage at 12 °C revealed significant differences between treatments (i.e. lines; $p < 0.0001$). Over 50 % of the lines did not germinate at the temperature tested (Fig. 4). Even though the control lines employed had not been specifically selected for this stage, but for the seedling stage, those used as susceptible controls did not germinate, whereas tolerant controls had variable response, but always showed over 60 % seed germination.

**Table 3** Lines selected for investigating allelic variation at candidate genes *OsGSTZ1*, *OsGSTZ2* and *OsCDPK13*

| Line denomination | Cold tolerance[b] | Ssp.[c] | Average cold injury score | | | | Group average cold injury score |
|---|---|---|---|---|---|---|---|
| | | | Date 1 | Date 2 | Date 3 | Date 4 | |
| 15 | S | *J*I* | 2.9 | 4.1 | 6.7 | 7.7 | 6 ± 1.4 |
| 86 | S | *I* | 3.9 | 5.3 | 6.1 | 7 | |
| 95 | S | *J*I* | 5.1 | 5.8 | 6.1 | 6.5 | |
| 119[a] | S | *I* | 6.2 | 6.7 | 7 | 7.7 | |
| 120[a] | S | *I* | 4.1 | 6.6 | 7.4 | 7.8 | |
| 103 | T | *Jt* | 1.3 | 1.3 | 1.9 | 2.1 | 2.2 ± 0.8 |
| 104[a] | T | *J* | 1.7 | 2.3 | 2.3 | 2.3 | |
| 112 | T | *J* | 1.9 | 2.4 | 2.6 | 2.6 | |
| 114 | T | *J* | 2.9 | 3.2 | 3.6 | 3.6 | |
| 115[a] | T | *J*I* | 1.1 | 1.2 | 1.3 | 1.5 | |

Seedling cold tolerance group into which each line was assigned, subspecies of origin, cold injury score at the seedling stage (vector) per line, and susceptible and tolerant group average score and standard deviation

[a] Lines used as controls in the seedling assay

[b] S = susceptible; T = tolerant

[c] *I* = *indica*; *J* = *japonica*; *J*I* = *japonica* × *indica cross*; *Jt* = *tropical japonica*

### *Correlation analysis between seed germination and seedling stage*

There was no correlation ($r = -0.11$; $p = 0.25$) between data obtained from the seedling stage assay and those resulting from the germination stage assay. Moreover, grouping of lines by their response to cold had no concordance when compared between stages (data not shown).

## Allelic variation at candidate genes

Information on origin and phenotypic behavior of the ten selected lines used to investigate allelic variation at genes *OsGSTZ1*, *OsGSTZ2* and *OsCDPK13* is shown in Table 3. Successful PCR amplification was attained in most cases, yielding conspicuous bands of the expected size. However, sequences from several fragments did not meet the high quality standard we had established and were hence discarded from further analyses (Supplementary Table 3).

We found a total of 34 polymorphic regions in all three genes −9 in *OsGSTZ1*, 9 in *OsGSTZ2* and 16 in *OsCDPK13*. SNPs and indels respectively accounted for 82.5 % and 17.5 % of the polymorphisms (Table 4). Also, nine SNPs and one indel differed between susceptible and tolerant lines: two of these SNPs were located in exons, one in the 3′ UTR, and the remaining SNPs and the indel, in introns. Of the two SNPs found in exons, only one resulted in an aminoacid change (isoleucine/valine) at position 99 of the predicted protein (GST enzyme) in gene *OsGSTZ2*. The same SNP had been reported by Kim et al. (2011) as being associated with rice seedling cold tolerance (G allele: susceptible, A allele: tolerant), through a significant reduction in the enzyme's catalytic activity.

## Marker development

Successful PCR amplification with primers designed to target the aminoacid-changing SNP was attained in all cases, and the same band pattern was observed for each line in electrophoresis runs of two independent PCR reactions. As an example, a partial gel is shown in Fig. 5. A very good association between allele constitution and seedling cold tolerance was detected ($\chi^2 = 0.504$; $p = 0.52$; Supplementary Table 4), i.e. lines that amplified the 163 bp band had been classified as cold tolerant or moderately tolerant (groups 3 and 4) in the seedling stage assay, whereas the remaining lines, for which no such band was detected showed, on average, a higher susceptibility to cold than the first group ($p < 0.0001$; Supplementary

**Table 4** Polymorphisms detected in genes *OsGSTZ1*, *OsGSTZ2* and *OsCDPK13*

| Gene | Amplified fragment | | | Length of sequenced fragment (bp) | | | Number of individuals analyzed | No. SNPs | | No. indels | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Number | Designation | Length (bp) | Total | Introns | Exons | | Introns | Exons | Introns | Exons |
| *OsGSTZ1* | 1 | P1 | 1,314 | 1,210 | 1,167 | 43 | 7 | 2 (1/22) | 0 | 0 | 0 |
| | 2 | P2 | 936 | 481 | 351 | 130 | 2 | 1 | 0 | 2 (1/176) | 0 |
| | 3 | P3 | 1,081 | 971 | 541 | 430 | 7 | 3 (1/180) | 1 | 0 | 0 |
| *OsGSTZ2* | 1 | P4 | 1,126 | 1,026 | 922 | 104 | 9 | 1 | 1 | 0 | 0 |
| | 2 | P5 | 1,085 | 895 | 693 | 202 | 3 | 3 (1/231) | 1 | 0 | 0 |
| | 3 | P6 | 857 | 849 | 431 | 418 | 8 | 2 (1/213) | 1 | 0 | 0 |
| *OsCDPK13* | 1 | P7 | 1,263 | 1,034 | 108 | 926 | 3 | 0 | 2 (1/463) | 0 | 1 |
| | 2 | P8 | 1,012 | 965 | 364 | 601 | 9 | 1 (1/364) | 0 | 1 | 0 |
| | 3 | P9 | 1,039 | 841 | 518 | 323 | 5 | 5 (1/104) | 2 (1/162) | 2 (1/259) | 0 |
| | 4 | P10 | 1,308 | 1,160 | 581 | 579 | 3 | 1 | 1 | 0 | 0 |

SNP and indel density (1/number of base pairs) in each region sequenced are indicated in parenthesis

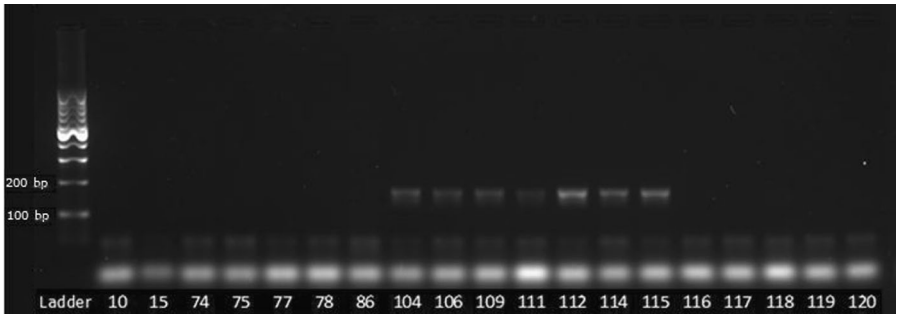


**Fig. 5** Agarose gel electrophoresis of a 163-bp SNP marker. *Line identification numbers* are shown at the *bottom*. *Lines* which show the 163 bp band (*104*, *106*, *109*, *111*, *112*, *114* and *115*) had been classified as cold tolerant (group 4) in the seedling stage assay, whereas the remaining *lines* had been classified as susceptible, moderately susceptible or moderately tolerant (groups 1, 2 and 3)

Table 5). Only three exceptions to this association were detected, as two lines that had been previously classified as moderately susceptible and one classified as susceptible showed band amplification in two independent PCR reactions.

*In silico* restriction enzyme map analysis indicated that the PCR-based marker developed here could be converted into a CAPS marker, by using LS primers for PCR amplification (Table 2) and further digestion of the 998 bp amplicon with one of the following restriction enzymes: *Mae*II, *Tai*I, *Tsc*I or *Tsp*49I, which recognize sequence ACGT. For example, digestion with *Tai*I would yield two fragments in the case of presence of the A allele (of 927 and 71 bp), or three fragments in the case of presence of the G allele (of 470, 457 and 71 bp).

## Discussion

Despite the vast number of references available in the literature on the assessment of cold tolerance at early

developmental stages in rice, results are often difficult to compare due to differences in the evaluation methods and the genetic materials used, and the complexity of symptoms caused by cold stress. In this paper, we used a collection of advanced lines and cultivars from a breeding program, selected for agronomic type, yield and grain quality, but with unknown response to cold temperature at early developmental stages (except for the cultivars used as controls). We obtained a similar range of seedling injury score values than the one reported by Andaya and Tai (2006), who followed the same protocol for phenotypic evaluation in the fine mapping of QTL and the subsequent identification of two of the candidate genes studied in this work. These results are also in line with those reported by Lou et al. (2007) and Andaya and Mackill (2003). In addition, Kim and Tai (2011) compared this visual symptoms scale with the determination of a number of physiological variables associated with response to cold and found a strong positive correlation between the two, which further supports the use of the former methodology.

Injury score values in the control lines used here were very similar to the ones found by Andaya and Mackill (2003) and Andaya and Tai (2006, 2007) in M202 (tolerant) and IR50 (susceptible), the parental lines used in the mapping of cold tolerance QTLs, indicating that our choice of control lines was adequate.

The fact that the lines used in this study displayed a wide range of cold tolerance/susceptibility, even more extreme than the controls, revealed the existence of wide variability for seedling cold tolerance in local germplasm, at least under the experimental conditions used. We also detected variability in the response of these lines at the seed germination stage, although its magnitude was lower than that observed at the seedling stage. The absence of a correlation between germination and seedling stages is consistent with what was found by Zhang et al. (2005) and Jiang et al. (2008). Like other stress-related traits, cold tolerance appears to depend on the plant developmental stage, so its genetic control may differ, at least partly, between stages (Foolad and Lin 2001; Andaya and Mackill 2003; Baruah et al. 2009).

Analysis of allelic variation at candidate genes revealed slight differences in SNP density, as 2.6, 3.2 and 3 SNPs per kb were found in *OsGSTZ1*, *OsGSTZ2* and *OsCDPK13*, respectively. Each sequence was analyzed meticulously and many apparent polymorphisms were discarded due to the stringent quality threshold used; hence, the number of polymorphisms could have been underestimated.

Our findings are consistent with previous reports on the effect of *OsGSTZ2* on cold tolerance at the seedling stage in rice (Takesawa et al. 2002; Andaya and Tai 2006). Indeed, Kim et al. (2011) reported that the distribution of the A/G SNP from which we developed a functional marker, was significantly associated with sensitivity to low temperatures at the seedling stage in 76 rice genotypes.

In our study, the association between the response to cold at the seedling stage and variation at this SNP was investigated and detected in a very small set of lines, as it was described earlier. In order to ascertain whether this association was consistent in the remaining lines, a PCR-based functional marker was designed and analyzed in the panel of 116 lines that had been used in the phenotypic assays. *In silico* analysis showed that this marker would be amenable to conversion into a CAPS marker as well.

The highly significant association found between the presence of the A allele and cold tolerance at the seedling stage is in agreement with the one reported by Kim et al. (2011). It is noteworthy that we independently found the same SNP, and we did so in a completely different and larger germplasm collection, which further supports the role of *OsGSTZ2* in cold tolerance at the seedling stage.

Analysis of the PCR-based functional marker we have developed showed a low percentage (2.6 %) of false positives (i.e. lines that showed band amplification but were susceptible). Aside from the possibility of occurrence of cross-contamination or PCR artifacts, this may be due to the action of additional cold tolerance/susceptibility genes. Nevertheless, these lines can be later detected and discarded in the field, whereas several susceptible lines will have been previously discarded by marker analysis and hence not taken to the field.

Preliminary data from 2 years of field evaluation of the lines used here (Pachecoy and Marin, unpublished results) show a strong correlation with the data obtained under controlled conditions in the present study. This provides evidence for the use of the functional marker we have developed, in marker-assisted selection for cold tolerance at the seedling stage in breeding programs.

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