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Transcriptome profiling of rice seedlings under cold stress

Luciano C. da Maia[A,D], *Pablo R. B. Cadore*[A], *Leticia C. Benitez*[B], *Rodrigo Danielowski*[A], *Eugenia J. B. Braga*[B], *Paulo R. R. Fagundes*[C], *Ariano M. Magalhães Jr*[C] *and Antonio Costa de Oliveira*[A]

[A]Universidade Federal de Pelotas, Department of Plant Sciences, Campus Universitário, S/N - CEP 96160-000 - Capão do Leão, RS - Brazil.
[B]Universidade Federal de Pelotas, Department of Botany, Campus Universitário, S/N - CEP 96160-000 - Capão do Leão, RS - Brazil.
[C]EMBRAPA – Brazilian Agricultural Research Corporation, BR-392 Road, Km 78, 9° Distrito, Monte Bonito, Pelotas/RS - Brazil.
[D]Corresponding author. Email: lucianoc.maia@gmail.com

Abstract. Rice (*Oryza sativa* L.) is one of the most important species for food production worldwide, besides being an excellent genetic model among the grasses. Cold is one of the major abiotic factors reducing rice yield, primarily affecting germination and reproduction phases. Currently, the RNAseq technique allows the identification of differential expressed genes in response to a given treatment, such as cold stress. In the present work, a transcriptome (RNAseq) analysis was performed in the V3 phase for contrasting genotypes Oro (tolerant) and Tio Taka (sensitive), in response to cold (13°C). A total of 241 and 244 M readings were obtained, resulting in the alignment of 25.703 and 26.963 genes in genotypes Oro and Tio Taka respectively. The analyses revealed 259 and 5579 differential expressed genes in response to cold in the genotypes Oro and Tio Taka respectively. Ontology classes with larger changes were metabolic process ~27%, cellular process ~21%, binding ~30% and catalytic activity ~22%. In the genotype Oro, 141 unique genes were identified, 118 were common between Oro and Tio Taka and 5461 were unique to Tio Taka. Genes involved in metabolic routes of signal transduction, phytohormones, antioxidant system and biotic stress were identified. These results provide an understanding that breeding for a quantitative trait, such as cold tolerance at germination, several gene loci must be simultaneously selected. In general, few genes were identified, but it was not possible to associate only one gene function as responsible for the cultivar tolerance; since different genes from different metabolic routes were identified. The genes described in the present work will be useful for future investigations and for the detailed validation in marker assisted selection projects for cold tolerance in the germination of rice.

Additional keywords: abiotic stress, chilling, expression, functional genomics, transcripts.

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Introduction

Rice (*Oryza sativa* L.) is one of the most important cultivated species for food production worldwide, being consumed by over half of the world's population (Yang *et al*. 2015). Due to a large increase in the globe's population forecasted for the next decades, an increase in rice yield and production will be needed. Rice, besides its importance in food security and economy, is also a genetic model among the grasses.

Cultivated species are frequently exposed to adverse conditions during their cultivation, also called abiotic stresses. Among the abiotic stresses, cold is one of the suboptimal conditions that are more harmful to rice. Similar to other tropical and subtropical species, rice is sensitive to low temperatures (Andaya and Tai 2006). Genotypes belonging to the japonica group generally present better levels of cold tolerance when compared with those from the indica group, whereas among javanica (tropical japonica), tolerant and sensitive genotypes can be found (Mackill and Lei 1997).

In general, the optimal temperature for rice germination is 25−35°C. Temperatures below 15°C at germination stage result in damages such as reduction in germination and vigour, delay in seedling emergence, delay in initial growth and high seedling mortality (Fujino 2004). In the reproductive phase, during the beginning of pollen production, low temperatures can disturb meiosis and mitosis, affecting the formation of mature microspores, leading to sterility (Shinada *et al*. 2013).

The genetic control of cold tolerance is a quantitative and complex trait, with low heritability, presenting additive, dominance and epistatic gene actions. Genetic mapping efforts revealed several quantitative trait loci (QTLs) involved in cold response: *qCTS4*, *qCTS11*, *qCtss12*, *qLTG3-1*, *qLTG-3-2* and *qLTG-4* associated to the control of tolerance to cold during

germination. However, no marker associated to these QTLs was efficient for use in marker assisted selection (MAS), due to deviations caused by genotype (G) × environment (E) and epistatic effects on the segregation of these loci (Fujino 2004).

From the molecular point of view, many mechanisms and metabolic routes are involved in the induction of plant acclimatisation and tolerance. A large number of genetic, molecular, biochemical, physiological and phenotypical changes do occur during acclimatisation, including upregulation of antioxidant mechanism proteins (superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) among others), synthesis and accumulation of osmoprotectants (polyamines and prolin), sugars (sucrose, maltose, glucose and fructose) and proteins (Lissarre *et al*. 2010).

It is not yet known whether plants have a sensor for perception of the cold; however, it is well known that changes in membrane fluidity and lipid composition are responsible for sensing cold and inducing the first signals in the presence of the stress. Cold induces changes in the plasma membrane and rearrangements in the cytoskeleton that modulate calcium channels, increasing Ca^{2+} content in the cytosol, which is followed by induction of *COR* (*cold-responsive*) genes. Changes in calcium content act as a secondary messenger in the signal transduction system, modulating phospholipase C (PLC) and D (PLD), which regulate IP_3 and phosphatidic acid respectively (Chinnusamy *et al*. 2010).

Calcium influx is sensed by many protein families such as calcium-dependent protein kinases (CDPKs), calmodulins (CaMs) and Salt overly sensitive 3-like (SOS3-like) that act in the transduction signal system. Besides calcium influx, the accumulation of reactive oxygen species (ROS) in the cytosol can also be detected by other protein groups such as Mitogen-activated protein kinases (MAPK, MAPKK and MAPKKK), *C*-repeat BindingFactor/Dehydration Response Element Binding (*CBF*/*DREB*), *ICE1*, *MYB* and *ZAT* among others (Chinnusamy *et al*. 2010).

Recent advances in the large scale sequencing of RNA (RNA-Seq) provide a high efficient and low cost way of generating high throughput transcriptome data such as tissue specific mRNA, new genes, SNPs and alternative splicing (Trapnell *et al*. 2010).

Based on the importance of a better understanding of the genetic control and the small amount of RNAseq information for cold tolerance during rice germination, the goal of this work was to evaluate differential expressed genes in the transcriptome of two contrasting rice cultivars (cold tolerant and sensitive) in response to cold in the germination phase and to identify potential new markers.

Materials and methods

Plant material for RNA

The rice (*Oryza sativa* L.) genotypes were chosen based on their contrasting response to cold at the germination stage under field conditions. For the experiment, the genotypes Tio Taka (subspecies *indica* – cold sensitive at germination stage) and Oro (subespecies *japonica* – cold tolerant at germination stage) were used. Seeds from both genotypes were germinated in plastic boxes (*gerbox*) in growth chamber type biological oxygen demand (BOD), with photoperiod of 16 h light/8 h dark. For the control treatment, both genotypes were kept at 25 ± 2°C and the seedlings were maintained until S3. For the cold treatment, genotypes were kept in BOD at 13 ± 2°C until S3 stage (emergence of prophyll). The experimental design was completely random, with three replications per treatment, where each replication consisted of one *gerbox* (50 seeds), being the experimental unit. When plants reached S3, leaf tissues were collected, frozen in liquid nitrogen and stored in ultrafreezer at −80°C for later RNA extraction and analyses.

Library preparation

Total RNA was extracted from 100 mg leaf tissue (bulk of 50 seedlings), using the Plant RNA Reagent Purelink (Invitrogen). RNA quality and concentration were checked in spectrophotometer NanoDrop ND-1000 (GE Healthcare) and its integrity checked in 1% agarose gel. The libraries were prepared using the kit TruSeq RNA Sample Preparation V 2 (Illumina), according to manufacturer instructions. The quality of libraries was evaluated with the aid of an Agilent 2100 BioAnalyser (Agilent Technologies) using the kit Agilent DNA 1000 (Agilent). Library sequencing was performed as *paired-end* 2 × 100 bp on the platform HiSEqn 2500 (Illumina).

Reads analysis and identification of differential expressed genes

For the analysis and visualisation of read quality, the software FastQC was used. After, low quality bases and library adaptors were removed from each library using Trimmomatic ver. 0.32. Reads were mapped against the reference genome of *Oryza sativa* cv. Nipponbare (IRGSP build 1.0-RAP-DB). In the first phase, TopHat ver. 2.0.11 and Bowtie ver. 0.12.7 software packages were used for the mapping of reads on the pseudomolecules of the rice genome (available at http://rapdb.dna.affrc.go.jp/index.html, accessed 1 February 2016).

Later, the Cufflinks ver. 2.1.1 software was used in the transcript assembly and Cuffdiff ver. 2.1.1 was used to estimate the differential expression of each locus. For both packages, default parameters were used as indicated in other reports (Trapnell *et al*. 2010; do Amaral *et al*. 2016).

Differential expression and gene description

Initially, a multidimensional scaling (MDS) graph was obtained in, R ver. 3.1.0 (R Development Core Team) and the package classic edgeR ver. 3.2.4 according to the parameters indicated by Anders *et al*. (2013), with the goal of verifying sample repeatability and the overall difference between treatments.

Using the software cummRbund (Trapnell *et al*. 2010), only transcripts with FPKM (*Fragments Per Kilobase Of Exon Per Million Fragments Mapped*) values ≥1, Log2FC ≤ –1 or ≥+1 and containing a status of 'OK' were considered. Expression levels were analysed in FPKM, being considered differentially expressed genes with values of $P \leq 0.05$.

Two sets of genes were obtained in the genotypes Oro and Tio Taka separately. Both sets were compared with reveal which genes were commonly or uniquely expressed in Oro and Tio Taka.

For a detailed discussion, those genes induced in Oro and repressed in Tio Taka were selected among the commonly

expressed genes in both genotypes. Among the genes induced in Oro, only those with Log2FC ≥2 were considered. For the detailed information on the function of these genes, they were aligned with Blastx against the following databases: RAP-DB (The Rice Annotation Project), MSU-Rice Genome Annotation Project, Tair (*Arabidopsis thaliana*), NCBI, CDD (NCBI-conserved domains) and Pfam (the Pfam protein families database).

The software Blast2GO was used for ontology analyses (function classes) and differential expressed genes. Other graph and analyses were performed using Microsoft Excel (Microsoft Corporation).

Phenotyping for cold tolerance

Seeds from both genotypes were sterilised in 70% ethanol for 30 s, with 5% sodium hypochlorite for 20 min and washed 6× with ddH_2O. Later, seeds were germinated in germitest paper rolls (Zhang *et al*. 2005), reaching 10 replicates containing 20 seeds per treatment, control (25°C/14 days) and cold (13°C/21 days). Only seeds presenting coleoptile and radicle were considered germinated (Cruz and Milach 2004).

A germination index (GI) was obtained for each genotype, being $GI = (N_{14} + N_{21}/2)/20 \times 100$, where N_{14} = number of germinated seeds 14 days after the beginning of the cold treatment; N_{21} = number of germinated seeds 21 days after the beginning of the cold treatment. The percentage of seeds with coleoptile superior to 5 mm (%Col >5) was also calculated: considering all the germinated seeds 21 days after the beginning of the cold treatment and by verifying the percentage that presented coleoptile length superior to 5 mm, according to the formula: %Col >5 = (number of seeds with coleoptile >5 mm) × 100/20. The relative values for shoot length were calculated, being RSL = ((SLcold/SLcontrol) × 100) and relative values for radicle length, being RRL = ((RLcold/RLcontrol) × 100), both measured at 21 days (Cruz and Milach 2004). A randomised experimental design was used. Statistical analyses, ANOVA and a Tukey's test were obtained using the SAS software (SAS Inc.).

Results

Phenotyping

Average phenotypic values for the genotypes Oro (tolerant) and Tio Taka (sensitive) under control and cold conditions are shown in Fig. 1. All variables were statistically different in ANOVA ($P \leq 0.05$) and Tukey's test.

For the GI, the genotype Oro presented higher values than the sensitive genotype Tio Taka, with values of 84.75% and 18.5%, respectively. For percentage of seeds with coleoptile >5 mm, Oro was also superior to Tio Taka (Fig. 1*a*). In Fig. 1*b*, the results for the variables shoot length and radicle length are shown. The relative value for shoot length (RSL) in the Oro genotype was high, indicating that shoot length under control and cold conditions were 95.24% similar, whereas for Tio Taka this value was only 16.70%. The relative radicle length (RRL) was greatly reduced on both genotypes; however, with better relative values for the tolerant genotype Oro.

Number of transcripts

Data was treated with Fastqc and Trimmotatic softwares, obtaining 241 million reads (96% alignment to the reference genome) and 244 million reads (88% alignment to the reference genome), for the genotypes Oro and Tio Taka respectively. In Fig. 2, the relationship between samples and treatments using MDS is shown. This software indicated robustness of the data, since the replications from each treatment (Oro-control, Oro-cold, Tio Taka-control and Tio Taka-cold) were very close, indicating a repeatability of samples. The results also indicated that Oro was less affected by the cold treatment than Tio Taka, since the samples Oro-control and Oro-cold were closer to each other than the replications when considered both treatments in the sensitive genotype (Tio Taka).

A total of 26 963 and 25 703 transcripts were obtained for each genotype. For Tio Taka (sensitive) and Oro (tolerant) 5579 (20.7%) and 259 (1.0%) genes with differential expression (DEGs) in response to cold tolerance were detected, respectively

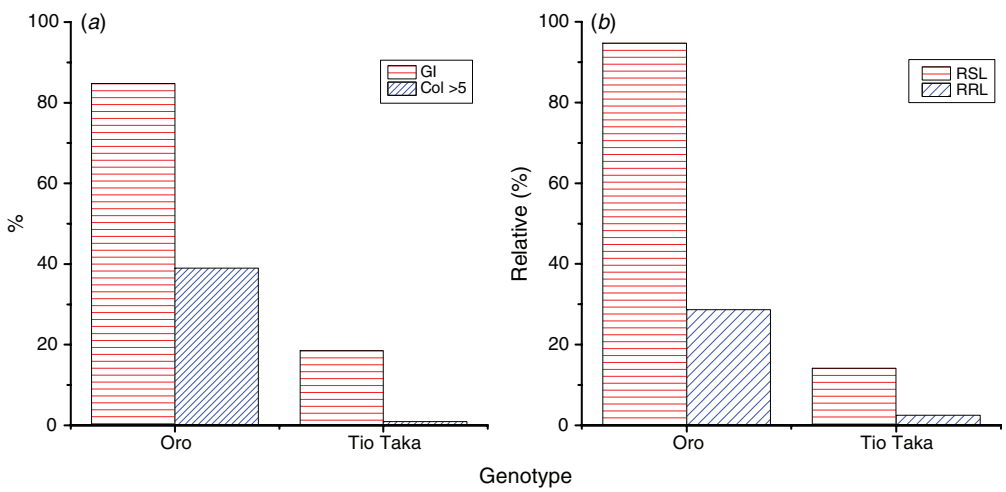

Fig. 1. Phenotypic means. (*a*) Germination index (GI, %); percentage of coleoptiles larger than 5 mm (Col >5, %), (*b*) relative shoot length (RSL, %); relative root length (RRL, %).

(Fig. 3*a*). In Tio Taka 53.9% of 5579 genes were upregulated, whereas in Oro all 259 genes were upregulated (Fig. 3*b*).

Among the 5579 and 259 transcripts differentially expressed in the genotypes Tio Taka and Oro, respectively, 118 were common to both genotypes, 141 unique to Oro and 5461 unique to Tio Taka (Fig. 4).

Gene ontology annotation

Considering the unique genes detected, an ontology based classification was obtained using Blast2GO. In general, in both genotypes, a large number of genes responsive to cold did not show an annotated ontology (not assigned). The genes with unpredicted function totalled 14.9% (Oro) and 29.8% (Tio Taka) for biological process and 38.3% (Oro) and 38.9% (Tio Taka) for molecular function (Fig. 5). For biological process, the majority of genes were: GO:0008152- metabolic process (27.66% and 26.39%), GO:0009987-celullar process (22.70% and 20.31%), GO:0044699-single organism process (10.64 and 6.59%), GO:0051179-localisation (7.80 and 4.67%) and GO:0065007-biological regulation (7.09 and 6.13%) for Oro and Tio Taka, respectively (Fig. 5*a*). In general, for all gene classifications, the highest percentage values were found in the Oro genotype (Fig. 5*a*). For molecular function, GO functions found were GO:0005488-binding (32.62% and 28.84%), GO:0003824-catalytic activity (19.15 and 24.61%), GO:0001071-nucleic acid binding transcription factor activity (4.26 and 1.92%) and GO:0005215-transporter activity (1.42 and 2.66%) for Oro and Tio Taka respectively (Fig. 5*b*).

The percentage of ontology classes for the 118 genes with significant differential expression, which are common between Oro and Tio Taka are presented (Fig. 6). The percentage of genes without annotated ontology was 6.78 and 25.42% for biological process and molecular function, respectively. Within biological process, the most represented classes were GO: 0008152-metabolic process (33.9%), GO:0009987-cellular process (25.42%) and GO:0044710-single organism process (8.47%) (Fig. 6*a*). For molecular function, the most common classes were: GO:0005488-binding (32.20%), GO:0003824-catalytic activity (31.36%) and GO:0001071-nucleic acid binding transcription factor activity (4.24%) (Fig. 6*b*). In general, the distribution patterns were similar when compared groups of unique genes (Fig. 5) with those expressed commonly in both cultivars (Fig. 6).

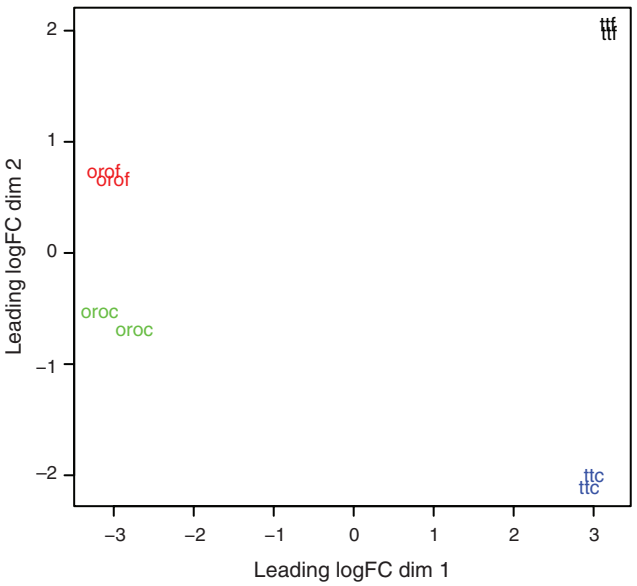

Fig. 2. Multidimensional scaling' (MDS), indicating the dispersion of treatments and replications, as a function of the general pattern of gene expression of each sample. Abbreviations: oroc, Oro-control; orof, Oro-cold; ttc, Tio Taka-control; ttf, Tio Taka-cold.

Differential expressed genes (DEGs) among all samples

The expression change values (Log2FC) observed in the sensitive genotype Tio Taka (blue bars) and tolerant Oro (red bars) are shown in Fig. 7. From a total of 118 genes commonly expressed in both genotypes, 92 genes had positive Log2FC in both genotypes, however, indicating higher expression values in the sensitive cultivar (blue bars) (see Table S1, available as Supplementary Material to this paper). Still in Fig. 7, a group of 26 genes showed reduction and increase in the Log2FC expression values, respectively, in the sensitive (blue bars) and tolerant (red bars) genotypes. Highly induced genes in the

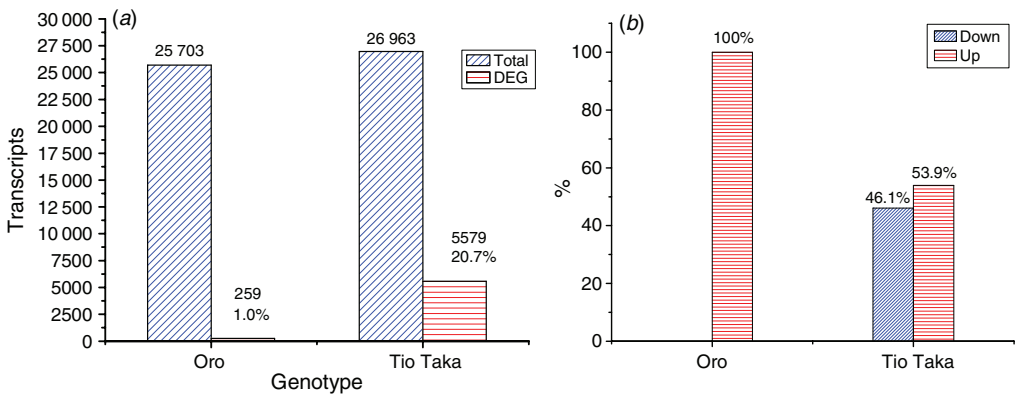

Fig. 3. (*a*) Total amount of expressed transcripts and those with differential expression in both genotypes. (*b*) Percentage of downregulated and upregulated genes.

sensitive genotype have lower probability of being related with the cold tolerance metabolism at germination stage. On the other hand, those 26 genes that had their expression reduced in the sensitive genotype and increased in the tolerant genotype, together with the 141 genes uniquely expressed in the tolerant genotype (Table S2), would have a higher probability of being involved in some important response mechanism to cold in the S3 stage. The 26 genes with positive response in the tolerant genotype Oro and negative in Tio Taka are displayed (Table 1). In Table 2, a set of 33 genes selected based on Log2FC $\geq$2 values, among the 141 genes uniquely expressed in Oro is shown.

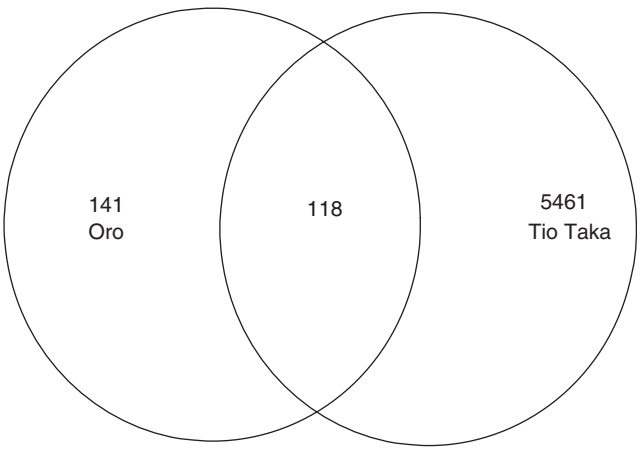

Fig. 4. Diagram indicating the number of differential expressed transcripts common to Oro and Tio Taka.

Discussion

Phenotyping – cold effect on seedling germination and growth

All variables analysed (RSL, RRL, GI and %Col >5) indicated a superior performance of the tolerant genotype Oro. The tolerant Oro was superior for germination index (GI) when compared with sensitive genotype Tio Taka, indicating which genotype had the higher number of seeds germinated at 14 and 21 days. A higher GI is associated to higher seed vigour and better performance of different genotypes, under cold conditions (Cruz and Milach 2004).

For the character percentage of seeds with coleoptile superior to 5 mm (%Col >5), the higher values were also observed in the tolerant genotype (Fig. 1*a*). The reduction of coleoptile growth under cold conditions during the initial phase of germination in rice is a limiting factor for the genotype establishment (Cruz and Milach 2004). The selection of genotypes with higher coleoptile size is efficient for cold tolerance during germination (Miura *et al*. 2002).

RSL and RRL values were higher in the tolerant genotype Oro. High RSL and RRL values indicate lower reductions in the performance of these variables in the genotype Oro under cold. Contrasting values for root and shoot length between cold tolerant and sensitive genotypes were previously reported (Zhang *et al*. 2005).

DEGs and GO

Next generation sequencing technologies have become an efficient technique to identify new genes and for the understanding of different expression patterns in gene

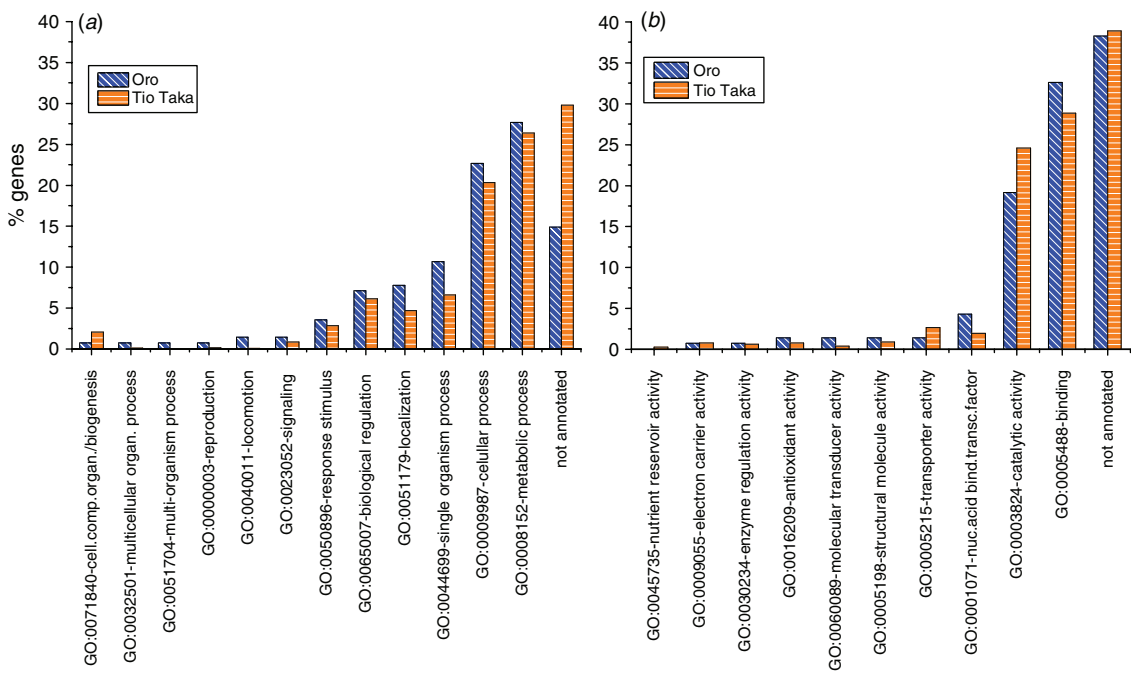

Fig. 5. Ontology classification (Blast2GO) of unique genes differential expressed in Oro (tolerant) and Tio Taka (sensitive). (*a*) Biological process and (*b*) molecular function.

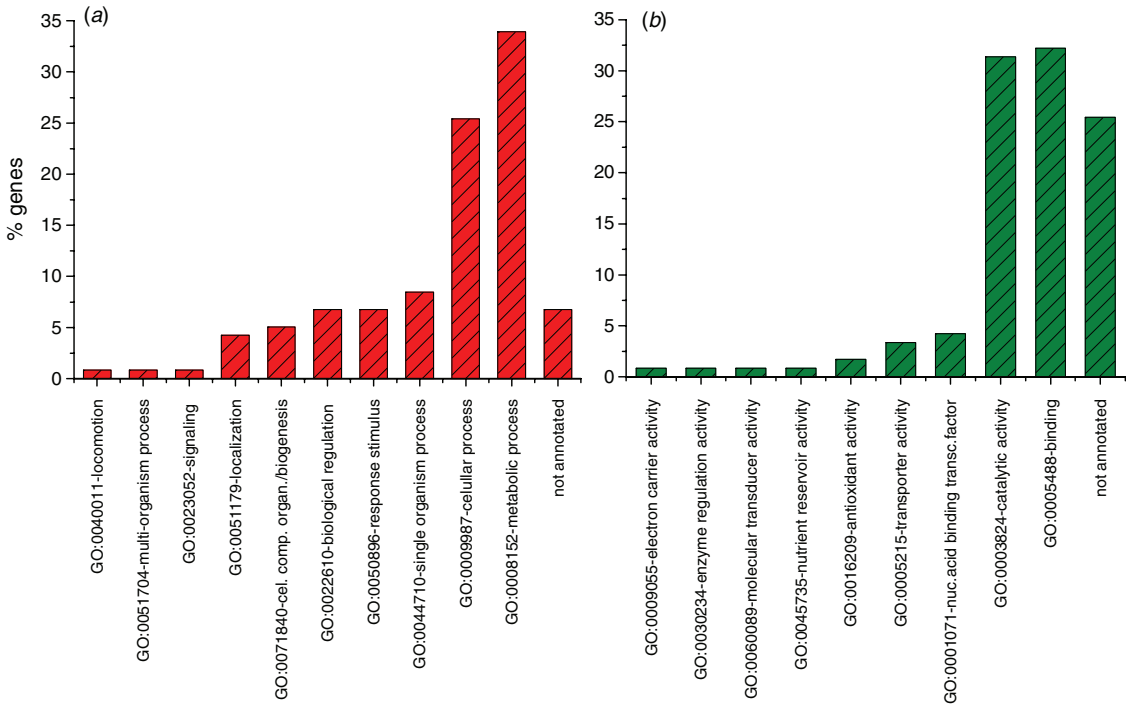

Fig. 6. Ontology classification (Blast2GO) of common genes differential expressed in Oro (tolerant) and Tio Taka (sensitive). (*a*) Biological process and (*b*) molecular function.

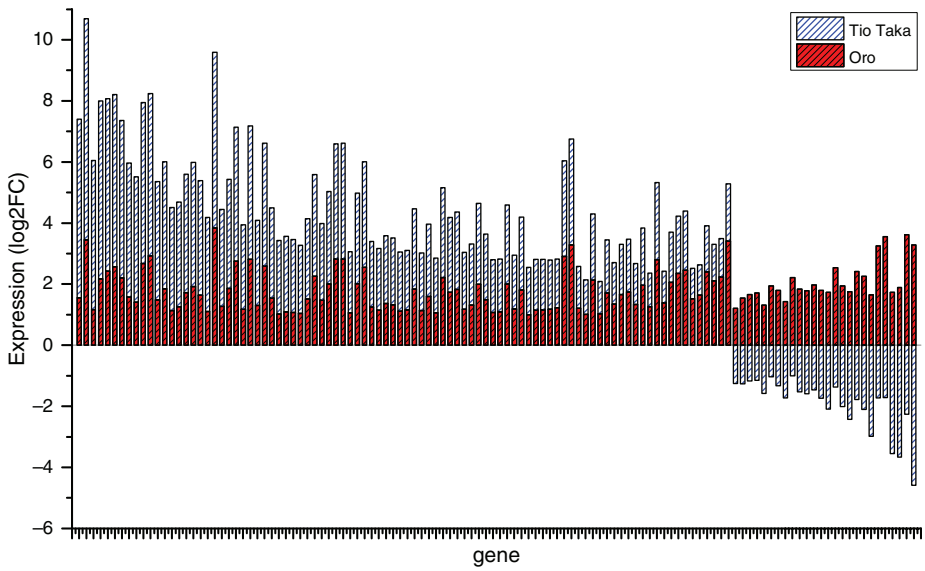

Fig. 7. Expression differences among the 118 genes commonly present in Oro and Tio Taka genotypes. Log2FC values for the sensitive cultivar Tio Taka (blue) and tolerant cultivar Oro (red).

networks modulated by specific conditions such as tissues, stage of development and stress conditions (Fujino and Matsuda 2010; Xu *et al*. 2012; Yang *et al*. 2015).

A total of 241 and 244 M of clean data resulted in 96% and 88% of aligned reads on the model reference genome of *Oryza sativa* cv. Nipponbare, for the genotypes Oro and Tio Taka, respectively, reaching 25 703 and 26 963 mapped genes in each genotype. A total of 141 and 5461 unique genes were identified in genotypes Oro and Tio Taka, respectively. Also, 118 genes common to both genotypes were differentially expressed. When analysing QTLs for cold tolerance at germination (*qLTG3*) in near-isogenic lines, 54 genes with increase in their expression in response to cold were detected (Fujino and Matsuda 2010), however, only one gene (*Os03 g0180800*) detected in the present

Table 1. List of 26 genes with positive and negative differential expression (Log2FC) in tolerant (Oro) and sensitive (Tio Taka) cultivars respectively

Log2FC TT	Log2FC Oro	Log2FC Oro-TT	Loci	Description
–1.25	1.21	2.46	Os01g0582600	DDE superfamily endonuclease
–1.27	1.54	2.81	Os01g0905200	Exo70 exocyst complex subunit family protein
–1.17	1.66	2.83	Os02g0703700	Cytochrome P450
–1.15	1.71	2.86	Os01g0972000	Zinc finger, Ring/FYVE/PHD-type
–1.58	1.32	2.90	Os01g0135700	EF-HAND 2, CALMODULIN-LIKE
–1.04	1.94	2.98	Os06g0683400	EF-hand Ca^{2+}-binding protein CCD1
–1.33	1.8	3.13	Os03g0301200	COBRA-like protein – cellulose deposition
–1.73	1.43	3.16	Os01g0124401	Similar to Bowman-Birk- protease inhibitors
–1.00	2.21	3.21	Os07g0566150	Hypothetical conserved gene
–1.53	1.84	3.37	Os06g0133500	Conserved hypothetical protein
–1.59	1.79	3.38	Os04g0684900	OsCAF1B-mRNA deadenylation activity
–1.46	1.97	3.43	Os02g0205700	3-KETOACYL-COA SYNTHASE 2, KCS2
–1.74	1.8	3.54	Os08g0482600	Cupredoxin domain, copper ion binding
–2.10	1.73	3.83	Os01g0186950	Similar to transposon protein CACTA
–1.37	2.54	3.91	Os05g0181700	Conserved hypothetical protein.
–2.01	1.94	3.95	Os03g0152000	Heavy metal transport/detoxification protein domain
–2.42	1.75	4.17	Os01g0186900	Similar to transposon protein CACTA
–1.78	2.41	4.19	Os03g0640000	Patatin-like protein
–2.11	2.26	4.37	Os01g0699500	Serine/threonine protein kinase domain – MAPKKK
–2.98	1.64	4.62	Os03g0180800	TIFY domain or Zim domain
–1.73	3.25	4.98	Os03g0734500	Conserved hypothetical protein
–1.71	3.56	5.27	Os01g0597600	Amino acid transporter, transmembrane domain
–3.55	1.73	5.28	Os04g0451700	Conserved hypothetical protein
–3.66	1.88	5.54	Os09g0522000	Similar to DREB 1B
–2.26	3.62	5.88	Os01g0699600	Serine/threonine protein kinase domain – MAPKKK
–4.59	3.28	7.87	Os02g0587000	Similar to glycine rich protein – GRP

work was previously detected in the QTL analysis of Fujino and Matsuda (Table 1).

In the present work, a strategy focusing on differential expressed genes that were unique to the tolerant genotype (Oro) and those showing opposite expression patterns between tolerant and sensitive genotypes was used. Genes differential expressed that were unique to the sensitive genotype were not discussed. Although one could not rule out that they could be negatively involved in tolerance, the odds are considered low and therefore they were not discussed.

For the unique in Oro (141) and Tio Taka (5461) and common sets of genes with opposite pattern in Oro/Tio Taka (118), between 14.9–29.8% of transcripts did not present annotated ontology (not assigned) for biological process and ~38% for molecular function. For the common genes between both genotypes 6.78 and 25.42% were not assigned for biological process and molecular function respectively. These results suggest that a large share of cold responsive genes are not yet characterised. For genes assigned to a given function, the major ontology classes under biological process were GO:0008152-metabolic process, GO:0009987-cellular process, GO:0044699-single organism process and under molecular function were GO:0005488-binding, GO:0003824-catalytic activity and GO:0001071-nucleic acid binding transcription factor activity.

Although the genes that compose the three sets of 141, 5461 and 118 genes were different, there was a consistency in the most abundant ontology classes. In a rice embryo transcriptome analysis using Blast2GO (Xu *et al*. 2012), 32.67% of transcripts did not have an assigned ontology and 3.12% fell into metabolic process, contrasting with the results presented here. However, similar results were obtained for binding (25.34%), as can be seen in Fig. 5*b*. Other reports on the analysis of the transcriptome of rice spikelets under cold show partial agreement with the present work (Yang *et al*. 2015).

Identified metabolic routes

In the initial phase, the analyses using genes with ± Log2FC 1 was considered in order to obtain an overview of the stress effect on both genotypes. For the selection of candidate genes, two groups were set up: in the first group, only genes with single transcripts in Oro and high changes (± Log2Fc 2) in expression levels were present (Table 2). The second group of genes are those with an increase in expression levels in Oro and reduction in Tio Taka (Table 1). The alignments on several databases enabled us to compare predictions using RAP-DB and other databases, resulting in differences for 10 transcripts (16.9%). However, no improvement in the annotation of 16 transcripts was observed (27.11%), which remained as hypothetical protein.

Signal transduction

Genes with functions associated to signal transduction were found in the gene sets considering positive response in Oro and negative in Tio Taka (Table 1) and genes unique to Oro (Table 2), such as calcium-binding EF-hand (CDPKs) (*Os01g0135700*, *Os06g0683400*, *Os05g0577500* and *Os01g0955100*), mitogen

Table 2. List of 33 genes with differential expression (Log2FC) in the tolerant cultivar (Oro)

Log2FC	Loci	Description
4.27	Os03g0820400	C2H2 zinc finger
3.92	Os06g0493100	Conserved hypothetical protein
3.14	Os03g0678800	Putative glycosyltransferase, family8protein
3.00	Os12g0628600	Putative thaumatin-like protein – TLP
2.82	Os06g0136650	Putative enolase
2.78	Os02g0618000	Non-protein coding transcript
2.78	Os08g0520700	Putative glycosyltransferase family 64
2.78	Os10g0491000	Plant basic secretory protein family protein
2.60	Os08g0402500	Protein of unknown function
2.58	Os02g0540700	Putative U-box domain
2.57	Os08g0395700	Conserved hypothetical protein
2.46	Os02g0193300	Conserved hypothetical protein
2.45	Os02g0756200	Putative PHI-1, PHOSPHATE-INDUCED 1
2.39	Os05g0545400	Serine/threonine protein kinase – MAPKKK
2.36	Os01g0955100	EF-hand, calcium binding motif
2.35	Os04g0572400	Putative DREB
2.27	Os10g0521900	Similar to membrane protein – RHOMBOID-like
2.21	Os03g0198200	Hypotheticalprotein.
2.21	Os04g0688200	Putative peroxidase (EC 1.11.1.7)
2.18	Os03g0197200	Putative sugar transporter
2.11	Os07g0103600	Putative gibberellin 2-β-dioxygenase
2.07	Os07g0680700	Hypothetical protein
2.07	Os10g0137300	Hypothetical protein
2.06	Os05g0552800	Conserved hypothetical protein
2.06	Os08g0386200	Putative WRKY transcription factor
2.06	Os10g0173000	Conserved hypothetical protein
2.06	Os12g0424700	Serine/threonine protein kinase – CDK
2.05	Os03g0302850	Hypotheticalprotein
2.03	Os04g0414500	Hypotheticalprotein
2.02	Os02g0647300	Putative leucine-rich repeat (LRR)
2.02	Os03g0302800	Hypotheticalprotein
2.01	Os05g0577500	EF-hand type domain containing protein
2.00	Os04g0571600	Putative MATE family protein

activated protein kinases (MAPKs) (*Os01g0699600*, *Os01g 0699500* and *Os05g0545400*), cyclin-dependent kinase (CDK) (*Os12g0424700*), C2H2-type zinc finger (*Os03g0820400 and Os01g0972000*), DREB (DEHYDRATION-RESPONSIVE ELEMENT-BINDING/COLD BINDING FACTOR) (*Os04g 0572400 and Os09g0522000*) and WRKY (*Os08g0386200*).

Calcium acts as secondary messenger in response to a wide range of biotic and abiotic stress signals, acting as sensors that activate responses concerning the regulation of the gene expression and phosphorylation in the signal transduction cascade. A transgenic rice overexpressing the gene *OsCDPK7* showed a significant gain in cold tolerance, salinity and dehydration (Wan *et al*. 2007).

MAP kinases are proteins involved in signal transduction in most known species. In plants, they have an important role in signalling for cold, salinity, UV radiation, ozone and remaining oxidative stresses (Rodriguez *et al*. 2010). In rice, the overexpression of a member of the C2H2-type zinc finger TFs increased the activity of antioxidant enzymes superoxide dismutase (SOD) and ascorbate peroxidase (APX). It also increased the drought tolerance and activated MAPKs (Zhang *et al*. 2014).

DREB proteins are transcription factors that regulate the expression of many ABA-independent genes and have important function in increasing plant abiotic stress tolerance and bind to genes that have DRE/CRT cis-elements in their promoter regions (Lata and Prasad 2011). *Arabidopsis* plants overexpressing *OsDREB1* showed increased tolerance to salinity and cold (Ito *et al*. 2006).

WRKY transcription factors are characterised by the presence of two or more WRKY and zinc finger-like motifs and have a response to different stress conditions. Gene expression analyses showed that 70% of *Arabidopsis* WRKY genes were differential regulated under stress induced by pathogens, salicylic acid (SA), dehydration, jasmonic acid and cold (Ross *et al*. 2007).

The transcript *Os03g0640000* (Table 1) found in the present analysis is homologous to PATATIN-like that contains a phospholipase that act as hydrolase in membrane lipids (Vancanneyt *et al*. 1989). Phospholipases are membrane proteins associated to stress recognition in plants (Chinnusamy *et al*. 2010). Modifications in cell membranes are important in the control of cold stress, being basically where the beginning of all the signalling process and signal cascade activation occurs. The majority of signalling is originated on conformation changes in membranes as a response to cold.

Phytohormones

In abiotic stress situations, one of the most important changes in plant metabolism concerns the production of phytohormones. During the stress, signal cascades modulate changes in gene expression, production and transport of phytohormones. This is particularly important regarding abscisic acid (ABA), which has a central effect on the signalling of gene clusters described as ABA-dependent and ABA-independent. Changes in ABA provoke other non-linear changes in the production of other phytohormones such as giberellic acid (GA) and salicylic and jasmonic acid. These changes can be stress as well as genotype dependent. This complex non-linear regulation between signalling and phytohormone production gene networks is called cross-talk. In the present transcriptome genes were detected which were involved in the production of JA, ABA and GA.

The gene *Os03g0180800* (Table 1) homologous to *JASMONATE ZIM-domain* (JAZ) found in this transcriptome codes for a protein involved in repressing the signalling of JA (Chung and Howe 2009).

For the transcript *Os02g0703700* (Table 1), an homology to Cytochrome P450 family (*CYP707A1*) protein, which codes for an ABA 8′-hydroxylase activity – ABA8ox, involved in ABA catabolism, was found. Mutants defective in this gene provoke changes in vigour and germination of seeds under cold conditions (Mega *et al*. 2015). The gene *Os02g0756200* (*PHI1-PHOSPHATE-INDUCED 1*) (Table 2) codes for a protein involved in modulating cellular phosphate interacting with signalling via ABA. Tobacco plants overexpressing this gene showed increased tolerance to osmotic stress (NaCl-, polyethyleneglycol and mannitol) and had a correlation with increase in chaperones (Sousa *et al*. 2014). There are evidences that the gene *Os07g0103600* (Table 2), homologous to gibberellin 2-β-dioxygenase (EC:1.14.11.13), which is associated to GA

production in the rice embryo and endosperm (Xue *et al*. 2012). In cassava, a homologue of this gene was also responsive to low temperatures (An *et al*. 2012).

Biotic stress

Several genes initially described as associated to biotic stress can also respond to abiotic stress stimuli. In the present transcriptome, a thaumatin-like protein (TLP) homologue was found (*Osg120628600*) (Table 2). These genes are normally associated to pathogen response, however TLP transcript increases were also reported during cold acclimatisation in wheat and ABA treatment (Kuwabara *et al*. 2002). Another gene associated to biotic stress response was *Os10 g0491000* (Table 2), which is homologous to basic secretory proteins (BSPs), which are described as associated to cell secretion and defence mechanisms (Basu *et al*. 2006).

ROS

The production of ROS is increased in plants when limiting conditions to CO_2 fixation are found, such as light, drought, salt, extreme temperatures and photorespiratory pathway disequilibrium stresses (Mittler *et al*. 2004). From O_2, derivative molecules such as singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), which are highly reactive and toxic to cells are produced. In *Arabidopsis*, a network of 152 genes is involved in maintaining cellular ROS levels. This network is highly dynamic and redundant and codes for different ROS-scavenging proteins. The major protein families involved in ROS detox system include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathion peroxidase (GPX) and peroxiredoxin (PRX) (Mittler *et al*. 2004).

Genes presenting differential expression belonging to the antioxidant system were found in the present work, such as *Os03g0152000* (Table 1), which contains a domain coding for copper linked proteins and has redox activity also present in enzymes such as laccases, SOD and multicopper oxidases, which catalyse many cellular processes. The gene *Os08g0482600* (Table 2) is homologous to cuprexin, which are proteins involved in the response to oxidative stress, responding to signals mediating oxidating electron transfer or homeostasis and protecting cell structures (Liu *et al*. 2012).

A homologue (*Os04g0688200*) (Table 2) to peroxidase proteins (EC 1.11.1.7) that are enzymes responsible for cleaning hydrogen peroxide, was identified. These enzymes are also described as Class III peroxidases, heme-dependent peroxidase or Horseradish peroxidase. The activities associated with these enzymes are oxidative stress protection under many biotic and abiotic stresses, such as pathogen attacks, dehydration, salinity and cold. Due to the large number of genes in this family, each copy can have a very specific function (Cosio and Dunand 2010).

Other potential candidates

Cold tolerance in rice is based on quantitative inheritance, since there are many genes and interactions, possibly epistatic, involved in defining the different tolerance levels among genotypes. Thus, one could expect that different metabolic routes and different genes be modulated in response to cold. Many genes with functions not clearly associated to cold response, known in the literature, were identified in the present transcriptome.

Genes with function similar to glycine rich protein-GRP (*Os02g0587000*) (Table 1), which compose a group of genes with response to several stresses, such as salt, cold and dehydration, were found (Mangeon *et al*. 2010). The genes *Os08g0520700* and *Os03g0678800* (Table 2) are similar to proteins containing glycosyl transferase family domains and *Os04g0571600* (Table 2) is similar to multidrug and toxic compound extrusion proteins (MATE efflux family), which are associated to plant toxic factor responses, such as herbicides and aluminum (Tiwari *et al*. 2014). For the energetic metabolism, a homologous phosphopyruvate hydratase, enolase (EC: 4.2.1.11), *Os06g0136650* (Table 2) was found. These genes have been shown to participate in the glycolysis.

The genes *Os02g0540700*, similar to *AT3G18710-U-box domain* (ubiquitin precursor) and *Os10g0521900* (regulated intramembrane proteolysis-RIP/RHOMBOID-like) code for different proteins with protease activity (Table 2).

Genes acting on cellular transport were also identified, such as *Os03g0197200* (Table 2), with homology to *Sugar transporter conserved site*. This function is associated to the transport of different carbohydrates, alcohol or acid through the membranes (Büttner and Sauer 2000). The gene *Os02g0647300* (Table 2) contains a leucine-rich repeat (LRR) protein homologue domain. This domain, is present in GTPase-activating, ribonuclease-inhibitor-like, spliceosomal protein, nuclear export protein and protein–protein interactions (Kobe and Kajava 2001). None of these have been reported to be associated to cold.

A transcript (*Os02g0205700*) similar to the gene described as *KETOACYL-COA SYNTHASE 2* (*KCS2*), involved in the long chain fatty acid metabolism formation, which are associated in larger/lower cuticular wax deposition in *Arabidopsis* (Lee and Suh 2015) was detected (Table 1).

Conclusion

RNAseq data indicated that the transcription response to cold is quite different when tolerant and sensitive genotypes are compared. In the sensitive genotypes, thousands of genes undergo changes in expression levels while only a few hundreds of genes are changed in the tolerant genotype. This indicates that cold induces changes in a higher number of metabolic routes than in the tolerant genotype.

The major ontology classes showing changes in expression levels due to cold stress are: metabolic process and celullar process for biological process and binding and catalytic activity for molecular function.

Two important sets of genes involved in cold response are present: those unique to the tolerant genotype (Oro) and those with opposite reaction in contrasting genotypes, i.e. increased and decreased expression levels in the tolerant and sensitive (Tio Taka) genotypes respectively.

The results indicate that many genes are differential expressed in the metabolic routes: signal transduction, phytohormone (ABA, GA and JA), biotic stress, antioxidant system and unknown function genes.

The dataset is useful for the identification of candidate genes for mapping and the validation of cold tolerance associated markers.

Our results increase the previous understanding of the molecular and physiological basis of cold response and provide tools for marker assisted selection approaches aiming for cold tolerance at germination stage.

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References

An D, Yang J, Zhang P (2012) Transcriptome profiling of low temperature-treated cassava apical shoots showed dynamic responses of tropical plant to cold stress. *BMC Genomics* **13**, 64. doi:10.1186/1471-2164-13-64

Andaya VC, Tai TH (2006) Fine mapping of the *qCTS12* locus, a major QTL for seedling cold tolerance in rice. *Theoretical and Applied Genetics* **113**, 467–475. doi:10.1007/s00122-006-0311-5

Anders S, McCarthy DJ, Chen Y, Okoniewski M, Smyth GK, Huber W, Robinson MD (2013) Count-based differential expression analysis of RNA sequencing data using R and Bioconductor. *Nature Protocols* **8**, 1765–1786. doi:10.1038/nprot.2013.099

Basu U, Francis JL, Whittal RM, Stephens JL, Wang Y, Zaiane OR, Goebel R, Muench DG, Good AG, Taylor GJ (2006) Extracellular proteomes of *Arabidopsis thaliana* and *Brassica napus* roots: analysis and comparison by MudPIT and LC-MS/MS. *Plant and Soil* **281**, 357–376. doi:10.1007/s11104-006-9048-9

Büttner M, Sauer N (2000) Monosaccharide transporters in plants: structure, function and physiology. *Biochimica et Biophysica Acta (BBA) – Biomembranes* **1465**, 263–274. doi:10.1016/S0005-2736(00)00143-7

Chinnusamy V, Zhu J-K, Sunkar R (2010) Gene regulation during cold stress acclimation in plants. *Methods in Molecular Biology* **639**, 39–55. doi:10.1007/978-1-60761-702-0_3

Chung HS, Howe GA (2009) A critical role for the TIFY motif in repression of jasmonate signaling by a stabilized splice variant of the JASMONATE ZIM-domain protein JAZ10 in *Arabidopsis*. *The Plant Cell* **21**, 131–145. doi:10.1105/tpc.108.064097

Cosio C, Dunand C (2010) Transcriptome analysis of various flower and silique development stages indicates a set of class III peroxidase genes potentially involved in pod shattering in *Arabidopsis thaliana*. *BMC Genomics* **11**, 528. doi:10.1186/1471-2164-11-528

Cruz RP, Milach SCK (2004) Cold tolerance at the germination stage of rice: methods of evaluation and characterization of genotypes. *Scientia Agrícola* **61**, 1–8. doi:10.1590/S0103-90162004000100001

do Amaral MN, Arge LWP, Benitez LC, Danielowski R, da Silveira Silveira SF, da Rosa Farias D, Deuner S, de Oliveira AC, Braga EJB, da Maia LC (2016) Differential expression of photosynthesis-related genes and quantification of gas exchange in rice plants under abiotic stress. *Acta Physiologiae Plantarum* **38**, 153. doi:10.1007/s11738-016-2176-9

Fujino K (2004) A major gene for low temperature germinability in rice (*Oryza sativa* L.). *Euphytica* **136**, 63–68. doi:10.1023/B:EUPH.0000019519.43951.67

Fujino K, Matsuda Y (2010) Genome-wide analysis of genes targeted by *qLTG3*-1 controlling low-temperature germinability in rice. *Plant Molecular Biology* **72**, 137–152. doi:10.1007/s11103-009-9559-x

Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant & Cell Physiology* **47**, 141–153. doi:10.1093/pcp/pci230

Kobe B, Kajava AV (2001) The leucine-rich repeat as a protein recognition motif. *Current Opinion in Structural Biology* **11**, 725–732. doi:10.1016/S0959-440X(01)00266-4

Kuwabara C, Takezawa D, Shimada T, Hamada T, Fujikawa S, Arakawa K (2002) Abscisic acid- and cold-induced thaumatin-like protein in winter wheat has an antifungal activity against snow mould, *Microdochium nivale*. *Physiologia Plantarum* **115**, 101–110. doi:10.1034/j.1399-3054.2002.1150112.x

Lata C, Prasad M (2011) Role of DREBs in regulation of abiotic stress responses in plants. *Journal of Experimental Botany* **62**, 4731–4748. doi:10.1093/jxb/err210

Lee SB, Suh MC (2015) Cuticular wax biosynthesis is up-regulated by the MYB94 transcription factor in *Arabidopsis*. *Plant & Cell Physiology* **56**, 48–60. doi:10.1093/pcp/pcu142

Lissarre M, Ohta M, Sato A, Miura K (2010) Cold-responsive gene regulation during cold acclimation in plants. *Plant Signaling & Behavior* **5**, 948–952. doi:10.4161/psb.5.8.12135

Liu Z, Kumari S, Zhang L, Zheng Y, Ware D (2012) Characterization of miRNAs in response to short-term waterlogging in three inbred lines of *Zea mays*. *PLoS One* **7**, e39786. doi:10.1371/journal.pone.0039786

Mackill DJ, Lei X (1997) Genetic variation for traits related to temperate adaptation of rice cultivars. *Crop Science* **37**, 1340–1346. doi:10.2135/cropsci1997.0011183X003700040051x

Mangeon A, Junqueira RM, Sachetto-Martins G (2010) Functional diversity of the plant glycine-rich proteins superfamily. *Plant Signaling & Behavior* **5**, 99–104. doi:10.4161/psb.5.2.10336

Mega R, Meguro-Maoka A, Endo A, Shimosaka E, Murayama S, Nambara E, Seo M, Kanno Y, Abrams SR, Sato Y (2015) Sustained low abscisic acid levels increase seedling vigor under cold stress in rice (*Oryza sativa* L.). *Scientific Reports* **5**, 13819. doi:10.1038/srep13819

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. *Trends in Plant Science* **9**, 490–498. doi:10.1016/j.tplants.2004.08.009

Miura K, Kuroki M, Shimizu H, Ando I (2002) Introduction of the long-coleoptile trait to improve the establishment of direct-seeded rice in submerged fields in cool climates. *Plant Production Science* **5**, 219–223. doi:10.1626/pps.5.219

Rodriguez MC, Petersen M, Mundy J (2010) Mitogen-activated protein kinase signaling in plants. *Annual Review of Plant Biology* **61**, 621–649. doi:10.1146/annurev-arplant-042809-112252

Ross CA, Liu Y, Shen QJ (2007) The *WRKY* gene family in rice (*Oryza sativa*). *Journal of Integrative Plant Biology* **49**, 827–842. doi:10.1111/j.1744-7909.2007.00504.x

Shinada H, Iwata N, Sato T, Fujino K (2013) Genetical and morphological characterization of cold tolerance at fertilization stage in rice. *Breeding Science* **63**, 197–204. doi:10.1270/jsbbs.63.197

Sousa AO, Assis ETCM, Pirovani CP, Alvim FC, Costa MGC (2014) *Phosphate-induced-1* gene from *Eucalyptus* (*EgPHI-1*) enhances osmotic stress tolerance in transgenic tobacco. *Genetics and Molecular Research* **13**, 1579–1588. doi:10.4238/2014.March.12.10

Tiwari M, Sharma D, Singh M, Tripathi RD, Trivedi PK (2014) Expression of OsMATE1 and OsMATE2 alters development, stress responses and pathogen susceptibility in *Arabidopsis*. *Scientific Reports* **4**, 763–768. doi:10.1038/srep03964

Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology* **28**, 511–515. doi:10.1038/nbt.1621

Vancanneyt G, Sonnewald U, Hofgen R, Willmitzer L (1989) Expression of a patatin-like protein in the anthers of potato and sweet pepper flowers. *The Plant Cell* **1**, 533–540. doi:10.1105/tpc.1.5.533

Wan B, Lin Y, Mou T (2007) Expression of rice Ca^{2+} -dependent protein kinases (CDPKs) genes under different environmental stresses. *FEBS Letters* **581**, 1179–1189. doi:10.1016/j.febslet.2007.02.030

Xu H, Gao Y, Wang J (2012) Transcriptomic analysis of rice (*Oryza sativa*) developing embryos using the RNA-Seq technique. *PLoS One* **7**, e30646. doi:10.1371/journal.pone.0030646

Xue L-J, Zhang J-J, Xue H-W (2012) Genome-wide analysis of the complex transcriptional networks of rice developing seeds. *PLoS One* **7**, e31081. doi:10.1371/journal.pone.0031081

Yang J, Chen X, Zhu C, Peng X, He X, Fu J, Ouyang L, Bian J, Hu L, Sun X, Xu J, He H (2015) RNA-seq reveals differentially expressed genes of rice (*Oryza sativa*) spikelet in response to temperature interacting with nitrogen at meiosis stage. *BMC Genomics* **16**, 959. doi:10.1186/s12864-015-2141-9

Zhang Z-H, Qu X-S, Wan S, Chen L-H, Zhu Y-G (2005) Comparison of QTL controlling seedling vigour under different temperature conditions using recombinant inbred lines in rice (*Oryza sativa*). *Annals of Botany* **95**, 423–429. doi:10.1093/aob/mci039

Zhang H, Liu Y, Wen F, Yao D, Wang L, Guo J, Ni L, Zhang A, Tan M, Jiang M (2014) A novel rice C2H2-type zinc finger protein, ZFP36, is a key player involved in abscisic acid-induced antioxidant defence and oxidative stress tolerance in rice. *Journal of Experimental Botany* **65**, 5795–5809. doi:10.1093/jxb/eru313