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Comparative Analysis of Expressed Sequence Tags in Wheat, Rice, and Barley under Cold Stress

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Abstract

Cold stress is an environmental factor limiting crop productivity and geographical distribution. To determine functional annotation and differential gene expressions of plants under cold stress, 3127, 1188, and 2292 expressed sequence tags (ESTs) from low temperature-treated rice, wheat, and barley seedlings, respectively, were analyzed. The ESTs from each library yielded 1995 (rice), 950 (wheat), and 1831 (barley) unigenes. BLASTX revealed 1458 (rice), 703 (wheat), and 1324 (barley) unigenes with important hits in the protein database of Arabidopsis. All the unigenes with significant hits were grouped with MapMan software. In the resulting three functional groups, photosynthesis, nucleotide metabolism, and signaling categories, a significant difference was observed between the transcripts of rice and barley under cold stress. We identified differentially expressed genes from the three plants under cold stress by assembling the ESTs, resulting in 1101 contigs. There were 12 genes identified that had significantly different expressions between the three libraries. Promoter analysis of a 1500-bp sequence upstream of the candidate genes' coding region showed various regulatory elements with different roles. The existence of elements involved in various stresses in the promoter regions of candidate genes confirmed the role of these genes in stress responses. The identified genes could be putative candidates for gene manipulation to improve the cold tolerance of valuable crop plants.

1. Introduction

The world's population is rapidly increasing and is expected to grow to around 9.8 billion people by 2050. Simultaneously, extreme climatic events due to climate changes threaten agricultural production, and this is a tremendous challenge for feeding the growing human population. Different abiotic stresses (e.g., cold, heat, drought, and salinity) disturb the environmental balance; among them, cold stress can be a major environmental factor that decreases agricultural products and the geographical distribution of staple food crops (Pirzadah et al., 2014).

Among grain crops, wheat has been adapted to grow in a wide range of environments

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(Sorahinobar et al., 2022). Although barley is resistant to cold, it has lower tolerance than wheat. In contrast, rice, one of the most essential foods, is a cold-sensitive plant (Sun et al., 2021). Rice seedlings are especially sensitive to cold in early spring, which causes slow growth, paleness, wilting, reduced tillering, and stunted growth (Ji et al., 2017; Edrisi, 2013).

The plant's responses to cold stress are associated with morphological, biochemical, and molecular changes (Ritonga et al., 2021). Understanding the molecular mechanisms of adaptation or tolerance to cold is particularly important. By comparing the differences in gene expressions of cold-resistant and sensitive plants, some of the major genes associated with cold stress can be identified and used for transgenic breeding crops with more cold tolerance.

At the molecular level, genes associated with the response to abiotic stresses are classified into three groups: those that encode signal transduction components, functional proteins, and transcription factors (Ciarmiello et al., 2011). It is well known that rapid responses and rapid systemic signaling pathways like ROS and Ca^{2+} could coordinate the systemic response of plants to a combination of different environmental conditions, enhancing the overall acclimation and ability of plants to withstand rapidly changing conditions within their environment (Kollist et al., 2019). However, identification of the genes involved in cold stress tolerance, including sensing, signaling, and creating physiological changes to cope with the effects of stress, is of great importance.

One of the functional genomics methods for identifying and analyzing gene expression is expressed sequence tag (EST) analysis, which provides the possibility of studying transcripts involved in metabolic and regulatory networks (Ali et al., 2011). EST is a short sub-sequence of a cDNA sequence that has been sequenced from the 5' or 3' ends (Kayesh et al., 2014). The quantitative expression of genes can be analyzed by EST sequencing to study the correlation of EST abundance with favorable traits of plants. EST analyses can also prepare a resource for novel gene detection, comparative analyses, targets for transgenesis, and genetic improvement of crop plants (Mondego et al., 2011).

In the present study, using bioinformatics methods, the EST library of rice, wheat, and barley as respectively sensitive, tolerant, and moderately tolerant to low-temperature species was investigated to identify new genes associated with cold stress and examine the difference in gene expressions between these plants.

2. Materials and Methods

2.1 Retrieving the data, trimming, and assemblage

The EST libraries of rice, wheat, and barley plants under cold stress were downloaded from the NCBI UniGene database. The raw ESTs were pre-processed using the online tool EGassembler (Masoudi-Nejad et al., 2006) for cleaning, repeat masking, vector trimming, organelle masking, clustering, and assembling (https://www.genome.jp/tools/egassembler/). The trimmed sequences with more than 4% or less than 100 bp ambiguous bases were discarded. To detect duplicate sequences repeatedly transcribed in each library, the EST sequences were clustered and assembled using EGassembler webserver with an overlap percent identity cut-off ≥95. Then, redundant and similar sequences were grouped into contigs. EST sequences that had fewer similarities were categorized into separate singleton groups.

2.2 Functional annotation analysis

The nucleotide sequences of the unigenes were blasted against the Arabidopsis proteins using BLASTX (E-value $\leq 1 \times 10^{-5}$). CLC-Main-Workbench software was used to create the protein codes required to dedicate functional categories. Then the functional classification of the EST sequences was performed using classify gene services from the Max Planck Institute of Molecular Plant Physiology (MapMan) (http://mapman.mpimp-

golm.mpg.de/general/ora/ora.shtml). The Audic-Claverie test ($\alpha = 0.05$ and 0.01), accessible in IDEG6 software, was used to detect different functional categories between the rice, wheat, and barley libraries.

2.3. In silico gene expression profiling

EST-sequences from each library were clustered and assembled with EGassembler in order to computationally detect differentially expressed genes from the three libraries. The contigs were then subjected to the Audic-Claverie test and the chi-squared test ($\alpha = 0.05$ and 0.01) using IDEG6 software to examine the significant differences in the number of ESTs between each library that contributed to each contig. The total number of ESTs in each library and the number of library-specific ESTs in each contig were the required inputs for these tests. To predict the putative functions of the significant differentially expressed contigs, they were searched against the nonredundant Arabidopsis protein (e-value $\leq 1 \times 10^{-5}$) using BLASTX from the CLC-Main-Workbench software.

2.4. Promoter analysis of candidate genes

The genes' protein-coding sequence (CDS) region was downloaded from NCBI to analyze the promoter of cold stress-responsive candidate genes, showing a statistically significant difference between the three libraries. We used BLASTN to search for candidate genes against genome sequences of A. thaliana in the Phytozome database (http://www.phytozome.net/). Additionally, a BLAT search was used for contigs with no hits among Arabidopsis protein sequences. The 1500-bp sequence upstream of the coding region of candidate genes was designated as the promoter sequence. Finally, cis-acting elements in the candidate gene promoters were analyzed using the Plant CARE program (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

3. Results and Discussion

Over the last two decades, many efforts have been made to improve cold tolerance in plants, especially in rice (Lee, 2001; Cruz et al., 2013; Jin et al., 2018; Yang et al., 2021). Cold tolerance is a complex trait controlled by multiple loci (Zhang et al., 2014; Zhang et al., 2017) that causes disturbances in metabolism and alters the physiological characteristics of the plants. These changes may be part of the cold sensor system that triggers the network of cold-responsive signaling (Zhang et al., 2014). Based on our analysis, more than 6500 (including 3114 from rice, 1184 from wheat, and 2288 from barely) high-quality ESTs were selected for clustering of non-redundant transcripts expressed in stem tissue under cold stress. These ESTs had average lengths of 478 bp in rice, 470 bp in wheat, and 500 bp in barley bp. EST assembly is summarized in Table 1.

Table 1. Summary statistics of ESTs generated from rice, wheat, and barley cDNA libraries.

Features Libraries	*Oryza sativa*	*Triticum aestivum*	*Hordeum vulgare*
Library code in dbEST	11160	5509	24104
Numbers of ESTs	3127	1188	2292
Number of high-quality ESTs	3108	1177	2283
Total length of ESTs in genome (bp)	1490954	556547	1145282
Average length of high-quality ESTs (bp)	478	470	500
Numbers of UniGenes	1995	950	1831
Number of Contigs	602	168	338
Number ESTs in Contigs	1715	395	790
Number of singletons	1393	782	1493

The BLASTX analysis revealed that 1458 rice unigenes (469 contigs and 989 singletons), 703 wheat unigenes (129 contigs and 574 singletons), and 1324 barley unigenes (263 contigs and 1061 singletons) had homology with putative or known functional genes. The 537 (rice), 247 (wheat), and 507 (barley) remaining unigenes did not show any significant match with the general protein databases. Unigenes with significant hits under cold stress were grouped into 33 functional categories (Fig 1). Audic-Claverie results of functional annotations are presented in Table 2. As can be seen, the photosynthesis category showed a significant difference between the rice and wheat transcriptomes. No functional categories showed significant differences between wheat and barley transcriptomes under cold stress (Table 2). In the misc functional group, there are large families of enzymes that are not assigned to any pathway, and several subgroups in the rice and barley library were different from each other.

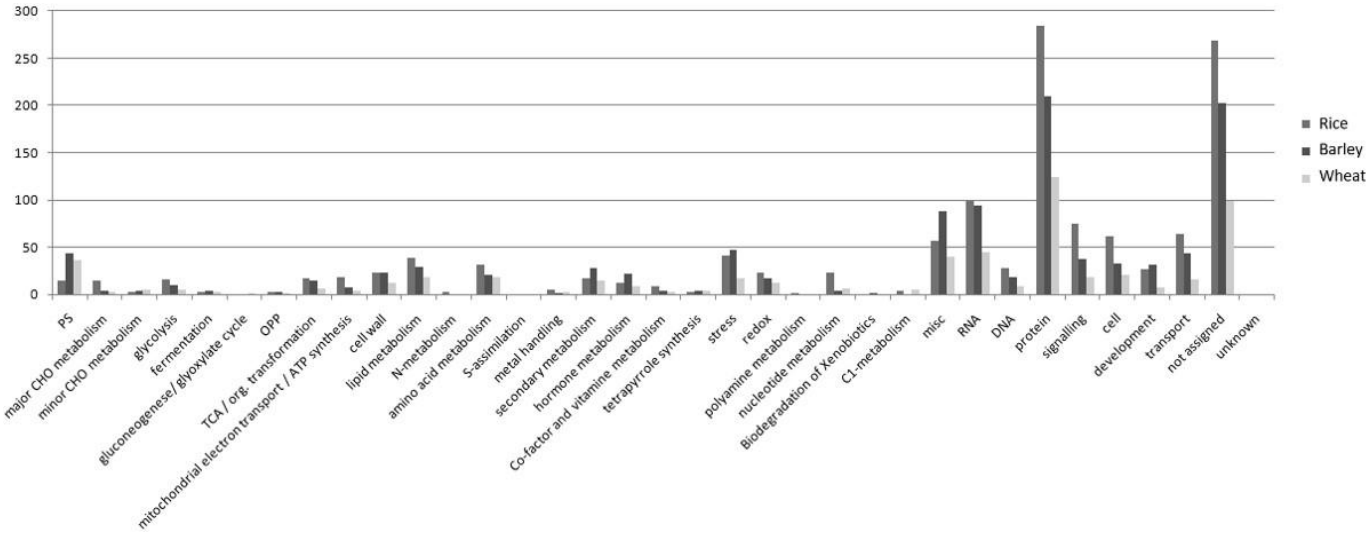

Figure 1: The gene expression categories in rice, wheat, and barley libraries under cold stress.

Mapman software analysis showed that Protein, RNA, signaling, transport and cell classes had the highest percentage of unigenes (Fig 1).

Regulation of photosynthesis under cold stress is one of the most important physiological processes leading to cold adaptation (Banerjee & Roychoudhury, 2019). Cold stress alters photosynthesis in rice by affecting chlorophyll content and fluorescence (Zhang et al., 2014). The decreased Fv/Fm (variable fluorescence to maximum fluorescence) ratio reported in response to cold stress in plants is another indication of photosynthetic apparatus damage (Zhao et al., 2020). Accordingly, a significant difference in the photosynthesis category between the rice and wheat transcriptomes under cold stress could be related to rice's sensitivity and wheat's tolerance against cold stress. On the other hand, an increased accumulation of ROS and Malondialdehyde (MDA) during cold stress in rice can cause oxidative damage, which in turn can affect plant metabolism (Han et al., 2017). In agreement with our findings, other studies have confirmed changes in nucleotide metabolism under cold stress (Sun et al., 2021; Jian et al., 2020). Researchers believe that the promotion of purine and pyrimidine nucleotide biosynthesis provides ATP energy under cold stress (Stasolla et al., 2003). Table 2 also shows signaling is the fourth functional group with statistically significant differences, at the 5% level between the rice and barley library under cold stress. Generally, the signaling pathway, including

antifreeze proteins, heat shock proteins (HSPs), chaperonins, pathogenesis-related (PR) proteins, and sugar, hormone, and Ca signaling pathways are engaged in cold stress responses (Jagodzik et al., 2018; Wen et al., 2002).

Table 2. Significantly different functional categories between rice, wheat, and barley libraries according to the Audic-Claverie test.

	Number of UniGene in rice library	Number of UniGene in barley library	Number of UniGene in wheat library	AC (rice, barley)	AC (rice, wheat)	AC (barley, wheat)
Photosynthesis	15	44	37	0.000008**	0**	0.007247
Nucleotide metabolism	24	5	7	0.00028*	0.054145	0.037123
misc	58	89	41	0.000247*	0.009903	0.036797
Signaling	76	38	19	0.000356*	0.001691	0.071186

* *Statistically significant at the 5 percent level and ** statistically significant at the 1 percent level.* Misc; enzymes that are not assigned to any pathway.

3.3. Gene expression profiling

ESTs collection from the three libraries led to the identification of 1101 different contigs between rice, wheat, and barley. After the BLASTX search against the Arabidopsis protein database, 742 contigs were functionally annotated. Analysis of the contigs using the chi-square and Audic-Claverie tests revealed differences in 12 genes that were statistically significant at α=0.05 between the three libraries (Table 3). Gene products induced by cold stress were classified into two major categories: 1) genes that encode proteins against cold stress and 2) genes that are activated by abiotic stresses to modulate the expression of genes involved in cold stress tolerance. One of the identified gene products was glyceraldehyde-3-phosphate dehydrogenase. This enzyme is pivotal in plant metabolism and is involved in stress response (Ghosh & Xu, 2014). Previous studies' results also indicated that this enzyme's expressions increased under cold stress (Cui et al., 2005; Hashimoto & Komatsu, 2007; Lee et al., 2009).

Low temperatures lead to a decrease in photosynthesis due to a general slowdown of the metabolic process and, consequently, an increased susceptibility of photosystem II (PSII) to photo-inhibition (Huang et al., 2018; Tikkanen et al., 2014; Murata et al., 2007). Similar to the results of other studies, our results confirmed PSII was expressed more in wheat and barley compared to rice under cold stress. In general, photosynthetic enzyme activity shows significantly more expression in the wheat and barley libraries than in the rice library. Additionally, studies on other plants confirmed that PSII is relatively sensitive to low temperatures (Feierabend et al., 1992; Liang et al., 2007; Huang et al., 2010). On the other hand, it is well- known is well- known that plant light-harvesting complex II (LHC-II) is phosphorylated by a specific kinase at a threonine residue which is conserved in Lhcb1 and b2 but not in Lhcb3. The main effect of LHC-II phosphorylation appears to be a partial dissociation of the complex from PS-II and a closer association with PS-I (Bennett et al., 1980; Wolman, 2001). Change in the expression level of these proteins under cold stress may be related to their role in funneling more energy into PS-I than into PS-II. Interestingly, consistent with this and our finding about photosystem II subunit QA, the redox state

of the plastoquinone pool controls the LHC-II kinase activity (Allen et al., 1981).

It seems that in addition to the enzymes involved in photoreactions, the expression level of the gene Rubisco, as the main enzyme effective in CO2 fixation, changed under low-temperature stress. According to our findings, an increase in the levels of rubisco subunits in response to cold stress was reported in Arabidopsis (Kawamura & Uemura, 2003).

Cold stress is known to produce reactive oxygen species (ROS) in plants, and ROS accumulation may play a role in regulating cell death (Skipsey et al., 1997). According to our results, the transcript levels of several proteins changed due to cold-stress-induced ROS production, which can lead to oxidative damage and oxidative stress. As seen in Table 3, the levels of the Glutathione S-transferase (GST) transcripts increased in the wheat under cold stress. Thus, similar to Li et al. (2023), it could be concluded that the GSTs act to limit oxidative damage caused by cold stress. Similarly, the change in the expression levels of metallothionein under cold stress may be related to its role in oxidative stress protection (Guo et al. 2008; Zhu et al. 2009).

NADP-isocitrate dehydrogenase (NADP-ICDH), which catalyzes the production of NADPH, is being recognized as an essential component of the antioxidative defense mechanisms in animals, yeast, and bacteria. Leterrier et al. (2007) showed that the transcripts level of NADP-ICDH is up-regulated by cold stress. They confirmed that this dehydrogenase could have a protective antioxidant role against certain environmental stresses in plants. General regulatory factor 7 (GRF-7), which is a 14–3-3 like protein, is reported to be induced under conditions of water deficit stress (Bray 2002). HSPs (including HSP70-1) are key components that facilitate the folding of de novo synthesized proteins, assist the translocation of precursor proteins into organelles, and are responsible for the degradation of damaged proteins under stress conditions. HSP70-1 is involved in protein targeting chloroplasts (Sung & Guy, 2003; Noel et al., 2007; Brkljacic, 2009); the protein also modulates stomatal aperture in response to various environmental conditions and physiological responses to the hormone abscisic acid (Clement et al., 2011).

The expression of circadian rhythm genes and RNA-binding proteins (RBPs), like CCR2 and CCR1, significantly increased in the wheat library compared to rice and barley libraries under cold stress. It is known that glycine-rich RBPs (GR-RBPs or GRPs) regulate circadian gene expression by controlling alternative polyadenylation (APA) (Liu et al., 2013). In agreement with our findings, it is confirmed that Arabidopsis GRP2 contributes to an increase in cold resistance and freezing tolerance, and it accelerates seed germination exposed to low temperatures (Kim et al., 2007).

3.4. Promoter analysis of candidate genes

This analysis aimed to identify cis-acting DNA sequences that may control the candidate gene's expression for cold tolerance. In this analysis, we found several putative cis-elements and a variety of regulatory elements with differences in the promoter region of the candidate genes (Table 4). The existence of elements involved in various stresses in the promoter regions of these candidate genes (Table 4). The existence of elements involved in various stresses in the promoter regions of these candidate genes affirmed the function of these genes in stress response. As seen in Table 5, regulatory elements are grouped into stress, hormone response, physiology, and cell development based on their roles. Analysis of candidate gene promoters indicated that the majority of the identified motifs act as light-responsive elements (LREs) (Table 4). In plants, light plays a vital role as an energy source for carbon fixation and photosynthesis in developmental pathways. Regulation of cold-induced photosynthetic processes is the most important physiological parameter leading to cold acclimation (Adam & Murthy, 2014; Banerjee &

Table 3. The list of significantly differentially expressed genes between rice, wheat, and barley libraries.

Gene name	Symbol	Accession number	No of UniGene in rice library	No of UniGene in barley library	No of UniGene in wheat library	AC (rice, barley)	AC (rice, wheat)	AC (barley, wheat)	Chi-square
Glyceraldehyde-3-phosphate dehydrogenase C subunit 1	GAPC	AT3G04120	18	0	0	0.000028	0.002193	.	0.000043*
Metallothionein 3	MT3	AT3G15353	37	0	0	0**	0.000005*	.	0**
Glutathione S-transferase family protein	GST30	AT1G10370	0	2	8	0.103411	0.000024	0.002355	0.000002**
Photosystem II light-harvesting complex protein B1B2	LHB1B2	AT2G34420	0	15	12	0.000001**	0**	0.054379	0.000002**
Ribulose bisphosphate carboxylase small chain 1A	RBCS1A	AT1G67090	0	37	0	0**	.	0**	0**
Cytosolic $NADP^+$-dependent isocitrate dehydrogenase	cICDH	AT1G65930	0	13	0	0.000008*	.	0.002913	0.000005**
General regulatory factor 7, NU	GRF7	AT3G02520	0	11	0	0.000045	.	0.006707	0.000032*
Heat shock cognate protein 70-1	HSC70-1	AT5G02500	0	0	6	.	0.000317	0.001036	0.000001**
Circadian rhythm and RNA binding 2	CCR2	AT2G21660	0	0	14	.	0**	0**	0**
Photosystem II subunit QA	PSBQ	AT4G21280	0	0	7	.	0.000087	0.000353	0**
Circadian rhythm and RNA binding 1	CCR1	AT4G39260	0	0	5	.	0.001149	0.003039	0.000011*
Contig41	-	-	37	0	0	0**	0.000005*	.	0**

* Statistically significant at the 5 percent level and ** statistically significant at the 1 percent level. AC means Audic-Claverie test and significant difference between 2 groups. 41 contigs represent ESTs that were not classified in any functional group.

Roychoudhury, 2019). In Arabidopsis, HY5, a bZIP transcription factor that plays a central role in light signaling, has been found to positively regulate cold-induced gene expression through the Z-box and other cis-acting elements (Catalá et

al., 2011). There is two-way communication between stress affects the expression of circadian clock genes, which in turn has a positive function in cold tolerance by regulating the CBF pathway (Hofmann, 2012; Maibam et al., 2013). The CBF/DREB is the most recognized pathway in plants and has a basic function in adapting to the cold (Guo et al., 2018; Ritonga & Chen, 2020). It is known that CBFs bind to the CRT/DRE in the promoter region of downstream genes.

Table 4. Promoter analyses of genes with different expressions in the 3 libraries using the PlantCARE database to identify specific motifs.

Gene	Function	Site name
GAPC	light-responsive element	ACE, ATC-motif, Box I, GATT-motif, Gap-box, GT1-motif, I-box, TCT-motif
	stress responsiveness	LTR, HSE, MBS, W box, Box-W1, ARE
	hormone-responsive element	TCA-element, TGACG -motif, CGTCA -motif, GARE-motif, ERE
	Physiology and cell development	GCN4_motif, Skn-1_motif, circadian
	other cis-acting regulatory element	TATA-box, CAAT-box, 5UTR Py-rich stretch, AT-rich element
MT3	light-responsive element	3-AF1 binding site, ATCC-motif, ATCT-motif, Box II, G-Box, G-box, GAG-motif, GATT-motif, GT1-motif, I-box, MRE, TCT-motif
	stress responsiveness	MBS, W box, TC-rich repeats, Box-W1, ARE
	hormone-responsive element	TCA-element, CGTCA-motif, TGACG-motif, P-box, TGA-element
	Physiology and cell development	CAT-box, Skn-1_motif, circadian
	other cis-acting regulatory element	TATA-box, CAAT-box, 5UTR Py-rich stretch,
GST30	light-responsive element	ACE, AE-box, ATCT-motif, Box I, Box II, CATT-motif, MRE, Sp1
	stress responsiveness	LTR, MBS, TC-rich repeats, ARE
	hormone-responsive element	CGTCA-motif, TGACG-motif, GARE-motif, P-box
	Physiology and cell development	CAT-box, Skn-1_motif, Circadian
	other cis-acting regulatory element	TATA-box, CAAT-box,O2-site
LHB1B2	light-responsive element	ACE, ATC-motif, ATCC-motif, ATCT-motif, Box 4, CATT-motif, G-Box, GAG-motif, GATA-motif, I-box, TCT-motif
	stress responsiveness	MBS, TC-rich repeats, ARE
	hormone-responsive element	CGTCA-motif, TGACG-motif,
	Physiology and cell development	CAT-box, Skn-1_motif, circadian
	other cis-acting regulatory elements	TATA-box, CAAT-box, 5UTR Py-rich stretch, Box III, O2-site
PSBQ	light-responsive element	3-AF1 binding site, ACE, CATT-motif, G-Box, G-box, GA-motif, GAG-motif, GT1-motif, I-box, LAMP-element, as-2-box
	stress responsiveness	LTR, HSE, MBS, W box, Box-W1
	hormone-responsive element	ABRE, CGTCA-motif, TGACG-motif, TATC-box
	Physiology and cell development	Skn-1_motif, circadian
	other cis-acting regulatory element	TATA-box, CAAT-box,
RBCS1A	light-responsive element	Box I, CATT-motif, G-Box, G-box, GA-motif, GAG-motif, I-box, MRE, Sp1,
	stress responsiveness	LTR, MBS, W box, TC-rich repeats, Box-W1, EIRE
	hormone-responsive element	ABRE, TCA-element, CGTCA-motif, TGACG-motif, GARE-motif, TGA-element, v
	Physiology and cell development	CCGTCC-box, Skn-1_motif , circadian
	other cis-acting regulatory element	TATA-box, CAAT-box, 5UTR Py-rich stretch, A-box, O2-site

Table 4. continued**:**

Gene	Function	Site name
CICDH	light responsive element	3-AF1 binding site, ACE, AE-box, ATC-motif, ATCT-motif, Box 4, G-Box, G-box, GTGGC-motif, I-box, L-box, MNF1, P-box, Sp1, TCT-motif, TCCC-motif, as-2-box,
	stress responsiveness	LTR, HSE, MBS, W box, TC-rich repeats, Box-W1, ARE
	hormone-responsive element	ABRE, TCA-element, CGTCA-motif, TGACG-motif
	Physiology and cell development	RY-element, circadian
	other cis-acting regulatory element	TATA-box, CAAT-box
GRF7	light-responsive element	ACE, G-Box, G-box, GAG-motif, GATA-motif, GT1-motif, I-box
	stress responsiveness	W box, TC-rich repeats, Box-W1, ARE
	hormone-responsive element	ABRE, TCA-element, GARE-motif
	Physiology and cell development	CAT-box, Skn-1_motif, GCN4_motif, circadian
	other cis-acting regulatory elements	TATA-box, CAAT-box, Box III, O2-site
HSC70-1	light responsive element	4cl-CMA2b, ACE, ATCT-motif, Box I, CCGTCC-box, G-Box, G-box, GAG-motif, Gap-box, L-box, P-box, TCT-motif,
	stress responsiveness	HSE, MBS, W box, TC-rich repeats, Box-W1, ARE
	hormone-responsive element	ABRE, TCA-element, GARE-motif, TATC-box, ERE
	Physiology and cell development	GCN4_motif, Skn-1_motif, circadian
	other cis-acting regulatory element	TATA-box, CAAT-box , 5UTR Py-rich stretch , A-box
CCR1	light responsive element	AAAC-motif, AE-box, Box I, G-Box, G-box, GA-motif, GT1-motif, TCT-motif, TCCC-motif
	stress responsiveness	W box, TC-rich repeats, Box-W1, ELI-box3, ARE
	hormone-responsive element	CGTCA-motif, GARE-motif, TGA-element, TGACG-motif
	Physiology and cell development	CAT-box, , Skn-1_motif, circadian
	other cis-acting regulatory element	TATA-box, CAAT-box, 5UTR Py-rich stretch, ATGCAAAT motif , O2-site
CCR2	light-responsive element	3-AF1 binding site, ATCT-motif, Box 4, Box I, G-Box, G-box, GAG-motif, GT1-motif, Sp1, as-2-box, I-box
	stress responsiveness	LTR, HSE, MBS, ARE
	hormone-responsive element	ABRE, CGTCA-motif, ERE, TGACG-motif
	Physiology and cell development	CAT-box, , Skn-1_motif, RY-element, circadian
	other cis-acting regulatory element	TATA-box, CAAT-box, O2-site
Contig41	light-responsive element	ATC-motif, ATCT-motif, GAG-motif, I-box, TCT-motif, CHS-Unit 1 m1, Sp1
	stress responsiveness	MBS, W box, Box-W1, ARE
	hormone-responsive element	AuxRR-core, CGTCA-motif, TGACG-motif, GARE-motif
	Physiology and cell development	GCN4_motif, Skn-1_motif, circadian
	other cis-acting regulatory element	TATA-box, CAAT-box, OBP-1 site

Table 5. The function of *cis*-acting regulatory elements in the promoter region of candidate genes

Function	Motif
core promoter element around -30 of the transcription start	TATA-box
cis-acting element in promoter and enhancer regions	CAAT-box, 5UTR Py-rich stretch
cis-acting regulatory element	A-box, ATGCAAAT motif, OBP-1 site

Table 5. continued**:**

Function	Motif
Protein binding site	Box III, AT-rich element
Light responsive element	3-AF1 binding site, 4cl-CMA2b, AAAC-motif, ACE, AE-box, ATC-motif, ATCC-motif, ATCT-motif, Box 4, Box I, Box II, CATT-motif, G-Box, G-box, GA-motif, GAG-motif, GATA-motif, GTGGC-motif, GATT-motif, Gap-box, GT1-motif, I-box, LAMP-element, L-box, MRE, MNF1, Sp1, TCT-motif, TCCC-motif, ‹as-2-box, chs-Unit 1 m1
Low-temperature responsiveness	LTR
Heat stress responsiveness	HSE
Drought stress responsiveness	MBS, W box
Element involved in defense and stress responsiveness	TC-rich repeats
Elicitor-responsive element	Box-W1, EIRE, ELI-box3
An element essential for the anaerobic induction hormone-responsive element	ARE
Abscisic acid responsiveness	ABRE
Salicylic acid responsiveness	TCA-element
Methyl jasmonate responsiveness	CGTCA-motif, TGACG-motif
Gibberellins responsiveness	GARE-motif, P-box, TATC-box
Auxin responsiveness	AuxRR-core, TGA-element
Ethylene responsiveness	ERE
Element related to meristem expression	CAT-box, CCGTCC-box
Element involved in endosperm expression	GCN4_motif, Skn-1_motif
Element involved in seed-specific regulation	RY-element
Element involved in circadian control	Circadian
Element involved in zein metabolism regulation	O2-site

4. Conclusion

ESTs from cold stress-treated rice, wheat, and barley seedlings were analyzed and compared to determine functional annotation and differential gene expressions of plants under cold stress. The plants were selected because they show a different tolerance level to cold stress. Functional annotation of ESTs confirmed a significant difference between the transcripts of these plants under cold stress, especially in photosynthesis, nucleotide metabolism, and signaling categories. Based on our analysis, 12 genes were recognized with significantly different expressions between rice, wheat, and barley libraries under cold stress. The genes were related to photosynthesis, metabolism, ROS detoxification, and signaling pathways. Promoter analysis of a 1500-bp sequence upstream of the coding region of the identified genes *showed* a variety of regulatory elements in which the majority of the identified motifs act as light-responsive elements. Therefore, the importance of photosynthesis and light in plant response to cold stress was observed at three levels (functional annotation, identification of candidate genes, and analysis of promoters). Taken together, it seems that the response to light has a crucial role in plant reaction to cold stress.

Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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