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Full Length Article

Overexpression of *OsETR4*, a Putative Ethylene Receptor Increases Cold Tolerance in Rice

Wenzhu Jiang†, Zhao Li†, Miaomiao Wan, Tao Wu, Mingdi Bian, Kai Huang, Xue Yang, Hongjia Zhang, Ziming Ma, Shanshan Ju, Liping Guo, Lin Du, Xunming Zhang and Xinglin Du*

Jilin Province Engineering Laboratory of Plant Genetic Improvement, College of Plant Science, Jilin University, Changchun, China

*For correspondence: xldu@jlu.edu.cn; yslizhao@163.com

†Contributed equally to this work and are co-first authors

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Abstract

With origin from tropical and subtropical areas, rice (*Oryza sativa* L.) is sensitive to low temperature stress, which is a major limiting factor for rice production and geographical distribution. As an important gaseous plant hormone, ethylene plays important roles in many biological processes. In this study, the functions of an ethylene receptor, *OsETR4*, in the cold stress response of rice were investigated. Overexpression of *OsETR4* could increase the seedling survival rates under cold stress. Transcriptomic analysis by RNA-sequencing (RNA-seq) showed significant difference in *OsETR4-OE* lines compared to wild type (WT) in response to cold. We identified 258 DEGs (differential expressed genes) that were only differentially expressed between WT and *OsETR4-OE* after cold treatment. A total of 400 genes showed significant difference between the WT and *OsETR4-OE* after cold treatment. GO terms related to "oxidation reduction", "carbohydrate metabolic process", "lipid metabolic process" and "protein phosphorylation" were identified as cold-responsive GO terms. "Protein phosphorylation" was also significantly enriched in DEGs that were only differentially expressed between WT and *OsETR4-OE* after cold treatment, indicating altered kinase activities may explain the differential cold stress tolerance. In addition, the expression profiles of genes involved in ethylene synthesis or signaling showed significant difference. DEGs involved in ethylene signaling were identified and validated by quantitative real time PCR (qRT-PCR). These results indicate that genetic engineering of ethylene receptor *OsETR4* could contribute to the cold climate tolerance of rice. © 2020 Friends Science Publishers

Keywords: Rice; Ethylene receptor 4; Cold stress tolerance; RNA-seq analysis

Introduction

During the geographical distribution and production of crops in temperate and high-altitude environments, low temperature stress often constitutes one of the major limiting factors (Zhu *et al.* 2007). Rice (*Oryza sativa* L.) is one of the most important staple crops worldwide, feeding around half of the world population (Wing *et al.* 2018). However, among the major cereal crops, rice is one of the most sensitive to cold stress (Zhang *et al.* 2014). Therefore, improving cold tolerance in rice has always been a primary goal in both breeding and genetic research. Based on the genetic diversity of different rice cultivars, many QTLs have been identified including *qLTG3-1*, *Ctb1*, *COLD1*, *CTB4a* and *bZIP73* (Fujino *et al.* 2008; Saito *et al.* 2010; Ma *et al.* 2015; Schläppi *et al.* 2017; Zhang *et al.* 2017; Liu *et al.* 2018).

Low temperature signals, a response by plants to cold climates, can be perceived through the changes in membrane fluidity, molecular conformation, and metabolite concentration (Chinnusamy *et al.* 2007; Arshad *et al.* 2017). Based on the studies in the model plant *Arabidopsis*, a major low temperature response signaling pathway has been identified, including the transcriptional regulation cascades CBFs (C-repeat/dehydration-responsive element binding factors) (Medina *et al.* 2011). CBFs can bind to the C-repeat/dehydration-responsive element (CRT/DRE), a common *cis*-element in cold-responsive genes (Wang *et al.* 2008). Many key components have been identified in the signaling cascade upstream of CBFs, including transcription factors ICE1 (Inducer of CBF Expression 1), *ICE2*, *CAMTAs*, and *MYB15* (Chinnusamy *et al.* 2003; Agarwal *et al.* 2006; Doherty *et al.* 2009; Fursova *et al.* 2009). Meanwhile, the key transcription factor ICE1 is also under regulation at post-transcriptional levels by *SIZ1*, *HOS1* and *SnRK2.6/OST1* (Dong *et al.* 2006; Miura *et al.* 2007; Ding

et al. 2015).

As a gaseous plant hormone, ethylene plays important roles in plant growth, development, and responses to biotic and abiotic stresses (Yang and Hoffman 1984; Morgan and Drew 1997; Alexander and Grierson 2002; Anderson *et al.* 2004; Dubois *et al.* 2018). Ethylene induces the accumulation of antifreeze proteins (AFPs) in winter rye (*Secale cereale*) and the expression of *LeCBF1* in tomato (*Lycopersicon esculentum*) (Yu *et al.* 2001; Zhao *et al.* 2009). It has been shown that increasing the ethylene synthesis can promote the chilling stress resistance in tomato (Ciardi *et al.* 1997; Zhang and Huang 2010). In addition, genes involved in ethylene signaling could be induced rapidly under low temperature in *Arabidopsis* (Fowler and Thomashow 2002).

The major components in the ethylene signaling pathway have been identified in *Arabidopsis*. Membrane localized (especially ER-localized) receptors can sense ethylene and negatively regulate the ethylene signaling pathway. In *Arabidopsis*, ethylene receptors can be classified into two subfamilies, subfamily I (*AtETR1* and *AtERS1*) and subfamily II (*AtETR2*, *AtERS2* and *AtEIN4*) (Light *et al.* 2016). Five ethylene receptor genes have been cloned in rice (Yau *et al.* 2004). It has been reported that *OsETR2*, one ethylene receptor can negatively regulate ethylene sensitivity and the transition from the vegetative to the reproductive stage (Wuriyanghan *et al.* 2009). In addition, *OsETR2* also functions in starch metabolism (Wuriyanghan *et al.* 2009). The differentially localized *OsERS1* and *OsETR2* are involved in submergence tolerance with differential functions (Yu *et al.* 2017). In addition, ethylene receptor genes can also respond to various abiotic stresses at transcriptional level (Zhang *et al.* 2001). However, the function of ethylene receptors in response to low temperature stress remains elusive. In this study, we identified that the transgenic rice seedling overexpressing *OsETR4* (*OsETR4-OE* lines) showed higher cold resistance than wild types. Transcriptomic analysis revealed significant differences in cold response between *OsETR4-OE* and *WT*.

Materials and Methods

Generation of *OsETR4* overexpression transgenic rice

The full-length CDS of *OsETR4* was cloned and fused to overexpressing vector driven by maize *Ubiquitin* promoter to generated *pUbi::OsETR4* construct. *Agrobacterium*-mediated transformation was adopted to produce transgenic lines in the Kitaake (*Oryza sativa* L. sspp. *japonica*) calli (Hiei *et al.* 1994). The primers used were given in Table 1.

Growth conditions and cold tolerance analysis

Seeds of *OsETR4-OE* transgenic rice and *WT* were incubated in distilled water under 30°C for two days. Then germinated seeds with uniform plumule length were planted in pots with nutrient soil and then incubated in growth chamber of 12-h light (28°C, 80Lx)/12-h dark (25°C) photoperiod. For cold treatment, the seedlings of 3-leaf stage were transferred to a growth chamber of 8°C 12-h light/12-dark for two weeks and then recovered at 28°C for one week. Then the seedling survival rates were counted. Three independent replicates with 100 seedlings of each line were performed at one time. For RNA-seq analysis, rice seedlings were sampled as control first, and the rest were transferred to 8°C for 3 h and sampled as cold treatment.

RNA extraction, cDNA preparation and RNA-seq analysis

Total RNA was extracted using the RNA extraction kit (TianGen, Beijing). The following construction of cDNA library and sequencing were conducted by Novelbio (Shanghai). Three independent samples were collected for RNA extraction and Next-generation sequencing by ABI IonProton (2 × 101 bp). Raw sequence data were obtained, and the sequence reads were submitted to NCBI SRA database under accession number PRJNA524199. FASTQ_Quality_Filter tool was used to generate clean data for further analysis. The reads were mapped to the rice reference genome (MSU RefGen_v7) using a spliced aligner Tophat. The software Cufflinks was adopted to generate transcript fragments (Trapnell *et al.* 2010). Cuffdiff program was used for the differential expression analysis between four samples (Trapnell *et al.* 2013).

Quantitative real-time PCR

The cDNA was synthesized using One-step gDNA Removal and cDNA Synthesis Super Mix (TRansScript, Beijing). qRT-PCR was performed with SYBR Green mix (TOYOBO, Japan) using Chromo4 real-time PCR detection system (BIO-RAD, CFX96). The rice *Actin1* gene was used as the internal control. The primers used for qRT-PCR was given in Table 1.

Results

OsETR4 is involved in cold stress response in rice

The expression of *OsETR4* was significantly up-regulated in OsETR4-overexpression (OE) lines compared with Kitaake (wild-type) under the normal growth conditions (Fig. 1A). After cold treatment, seedlings of the *OsETR4* overexpression lines showed a significantly higher survival rates compared to Kitaake seedlings (Fig. 1B, D). The seedling survival rates of two *OsETR4-OE* lines *ETR4-1* and *ETR4-2* were 45 and 47% respectively after low temperature treatment, and only 18% of the Kitaake (Fig. 1B).

Table 1: The primers used in this study

Vector construction	Forward	Reverse
ETR4	TTTGGAGAGGACAGGGTACCATGGCGATGGTGACGGCGCG	CGACTCTAGAGGATCCGTTGTCCTGGAGCACTCGGC
qRT-PCR	Forward	Reverse
Actin	CCTGGCAGTATGAAGGTAGTTG	GAAGCACTTCATGTGGACGAT
ETR4	AGGATCAGGGAGCTCAGGAA	GACCAGCCCGTTGATCCC
LOC_Os02g52670	GAAGGACCAGGAGATCTG	CATGGCATGGACCAGAAG
LOC_Os05g41780	TGGATCTGGCACCATCAC	GCGGACAGTTCAGATCAAGA
LOC_Os01g49830	GGTACTCGTACTGGAACA	GAGACCCTTCTCCTTGAC
LOC_Os04g41570	AAGGATAAGATTGGTGAGAG	TGAAGAACAGAAGCAGTG
LOC_Os01g64790	CTCCATGACCAATCCCTA	GGGAAAGAGTAGTAGGAG
LOC_Os03g08490	CGGCGTTTGAGAGATTCG	CTTCTTCCCAACCACCAC
LOC_Os03g51920	TCCCTCGTCACCTCTCTC	GCTTCTTCTTGAGCTTGTACTAG

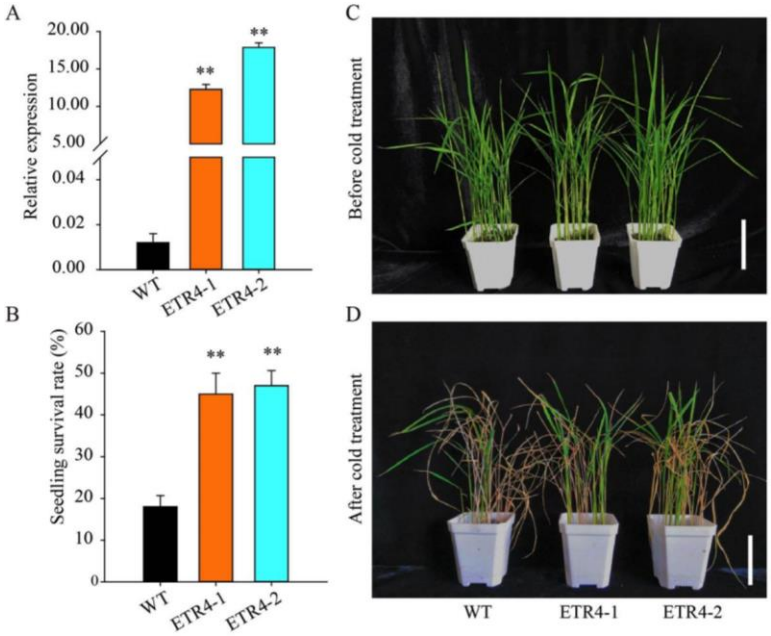

Fig. 1: Overexpression of *OsETR4* increased cold tolerance of rice seedlings
(**A**) Expression level of *OsETR4* in wild type (WT) and *OsETR4-OE* lines (*ETR4-1* and *ETR4-2*) by qRT-PCR. (**B**) The seedling survival rates of the *OsETR4-OE* lines and *WT* (Kitaake). (**C**, **D**) Representative phenotype of *OsETR4-OE* lines and *WT* before (**C**) and after (**D**) cold treatment. Bar = 10 cm

Transcriptomic analysis of the *OsETR4-OE* and *WT* lines

To further investigate the functions of *OsETR4* in the cold stress responses in rice, we performed RNA-seq analysis of the *OsETR4-OE1* and *WT* before and after cold treatment. Three independent samples were collected for RNA extraction and Next-generation sequencing by ABI IonProton. A total of 192,044,538 reads (93.3%) were mapped to the rice genome. The detailed mapped reads of each samples were listed in Table 2. Cluster analysis of the 12 samples was performed with R software Cluster package. The samples before and after cold treatment were separately clustered, indicating significant transcriptome changes after cold treatment (Fig. 2A). In addition, after cold treatment, overexpressed line (*ETR4*) and wildtype (Kitaake) were separated clearly. Principle component analysis (PCA) was also performed, and the result was in consistent with the cluster analysis (Fig. 2B). Therefore, the transcriptomic responses of *OsETR4-OE1* and *WT* were different and also consistent with the different seedlings' survival rates. In conclusion, the transcriptome data showed high quality results and could be used for subsequent analysis.

Transcriptomic responses to cold treatment of WT and OsETR4-OE

We identified a total of 33958 annotated transcripts in the four treatments by Cufflinks (Trapnell *et al.* 2010). A total of 25844 genes (76%) were represented in all treatments. Before low temperature stress, 28987 (85.3%) and 28880 (85.0%) of the genes were expressed in wildtype (Kitaake) and *ETR4* overexpressed lines respectively. After low temperature stress, 88.1% (29915) and 86.9% (29501) were expressed in wildtype (Kitaake) and ETR4 overexpressed lines, respectively (Fig. 3).

Table 2: RNA-seq quality control

Samples	All reads	Mapped reads	Mapped rate	Unique mapped reads	Unique Mapped rate
WTck-1	16178423	15301867	0.946	14922684	0.922
WTck-2	17450414	16434862	0.942	16061031	0.92
WTck-3	19697319	18398817	0.934	17921457	0.91
WTcold-1	19598087	18408826	0.939	17879129	0.912
WTcold-2	22032388	20467877	0.929	19674060	0.893
WTcold-3	20818966	19353748	0.93	18664732	0.897
ETR4ck-1	12813972	11760748	0.918	11435127	0.892
ETR4ck-2	13036904	12066714	0.926	11771001	0.903
ETR4ck-3	13081902	12112308	0.926	11791192	0.901
ETR4cold-1	16092949	15044371	0.935	14654613	0.911
ETR4cold-2	17369630	16225798	0.934	15783432	0.909
ETR4cold-3	17587772	16468602	0.936	16027628	0.911
Sum	205758726	192044538	0.933	186586086	0.907

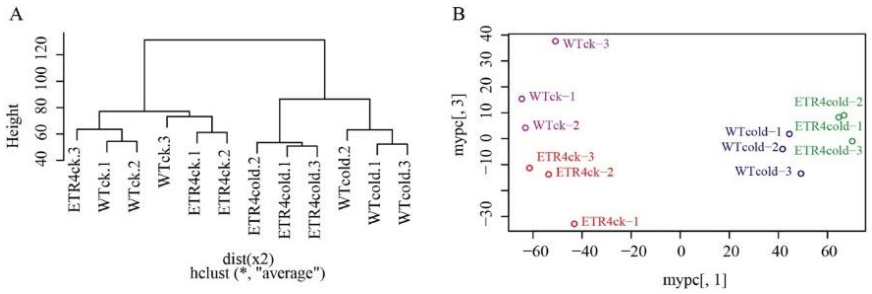

Fig. 2: Cluster (**A**) and PCA (**B**) diagram of 12 rice RNA-seq samples
WTck and WTcold indicate samples of *WT* before and after cold treatment. ETR4ck and ETR4cold indicate samples of *OsETR4-OE1* before and after cold treatment. Numbers indicate independent biological replicates

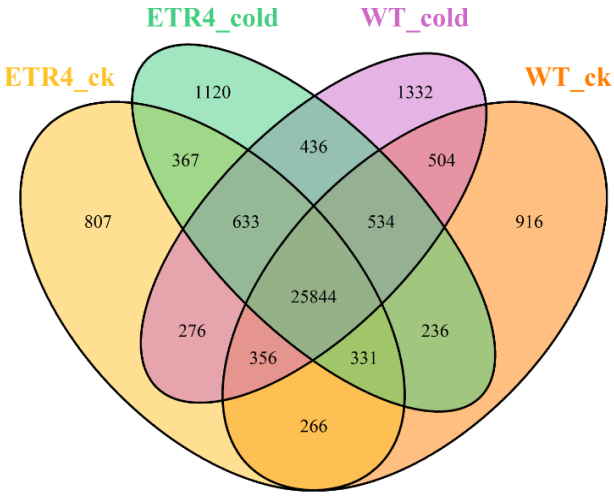

Fig. 3: Profile of gene expression of *WT* and *OsETR4-OE* line before and after low temperature treatment

Differential expression analysis of WT and OsETR4-OE

To further explore the differential transcriptomic responses between *WT* and *OsETR4-OE*, the differential expressed genes (DEGs) of four groups were analyzed: WTck_WTcold, WTck_ETR4ck, WTcold_ETR4cold, and ETR4ck_ETR4cold. We used the fold change ≥ 2 and FDR value ≤ 0.05 as a screening criterion. A total of 4199 genes were differentially expressed in WT before and after cold treatment, among which 2399 genes were up-regulated and 1800 were down-regulated after cold treatment (Fig. 4A). In the *OsETR4-OE* line, 2550 genes were up-regulated and 2654 were down-regulated after cold treatment. Before cold treatment, it was found that 91 DEGs between *WT* and *OsETR4-OE*. Among those, 75 genes had higher expression levels in *OsETR4-OE* and 16 genes had higher expression levels in *WT*. After cold treatment, 875 genes were differentially expressed between *WT* and *OsETR4-OE*: the

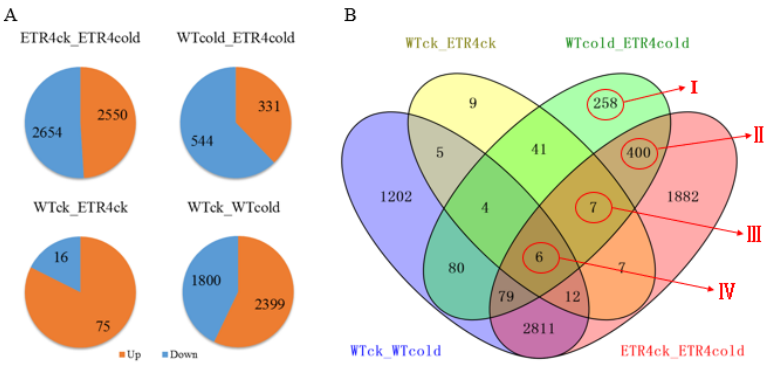

Fig. 4: Profile of differentially expressed genes (DEGs)
(**A**) The number of DEGs in the four groups of comparison, *i.e.*, WTck_WTcold, WTck_ETR4ck, WTcold_ETR4cold and ETR4ck_ETR4cold. The number of up-regulated DEGs and down-regulated DEGs were also shown. (**B**) Venn diagram illustrating the relationship between the four groups of DEGs

expression level of 331 genes was higher in OsETR4-OE and 544 genes were higher in WT.

A Venn diagram including the four different comparisons was drawn in order to elucidate the relationships of the four groups of DEGs (Fig. 4B). The numbers in the different parts of the diagram indicate the DEGs specifically shared between different comparison groups. In addition, some parts of genes (I, II, III, and IV) possessed specific biological meanings and Gene Ontology (GO) analysis of these genes using AgriGO was performed (Tian *et al.* 2017).

Gene Ontology analysis of differentially responsive DEGs

Part I represents DEGs that were only differentially expressed between *WT* and *OsETR4-OE* after cold treatment (WTcold_ETR4cold). This group of genes showed little response to cold treatment in *WT* and *OsETR4-OE* and showed no significant difference before cold treatment between *WT* and *OsETR4-OE*. However, after cold treatment, these genes showed significant difference between *WT* and *OsETR4-OE*. A total of 258 genes were identified. The GO terms related to protein phosphorylation were significantly enriched in the biological process (P) (Fig. 5A). GO terms related to ATP binding and kinase activity were significantly enriched in the molecular function (F). Three GO terms were significantly enriched in the cellular component (C), *i.e.*, "cell wall", "external encapsulating structure" and "membrane".

Part II represents DEGs specifically shared between WTcold_ETR4cold and ETR4ck_ETR4cold. This group of genes showed significant difference between *WT* and *OsETR4-OE* after cold treatment and also respond to the cold treatment in the *OsETR4-OE*. A total of 400 genes were identified and GO analysis were also performed. The GO terms related to "oxidation reduction", "carbohydrate metabolic process", "lipid metabolic process" and "protein phosphorylation" were significantly enriched in the biological process (Fig. 5B). In the molecular function, GO terms related to "oxidoreductase activity", "hydrolase activity" and "protein kinase activity" were significantly enriched, consistent with the biological process identified (Fig. 5C). Three GO terms were significantly enriched, including "cell wall", "apoplast" and "external encapsulating structure".

Specific DEGs shared between WTck_ETR4ck, WTcold_ETR4cold and ETR4ck_ETR4cold

In part III, a total of 7 genes were identified as DEGs specifically shared between WTck_ETR4ck, WTcold_ETR4cold and ETR4ck_ETR4cold. This group of genes showed no significant response to cold treatment in the WT, but responded to cold treatment in *OsETR4-OE* and showed significant difference between *WT* and *OsETR4-OE* before and after cold treatment. Therefore, the *OsETR4* overexpression may have changed the cold responses of rice seedlings (Table 3). After cold treatment, 3 genes showed induced expression and 4 genes showed repressed expression in *OsETR4-OE*. *LOC_Os11g17954* may be involved in glutathione-mediated detoxification. *LOC_Os03g60000* and *LOC_Os02g06290* encode proteins involved in the mitochondrial functions. *LOC_Os06g06490* encodes a U-box domain-containing protein kinase and may be involved in stress response. *LOC_Os12g33194* belongs to the trichome birefringence-like gene family and its homologues in *Arabidopsis* have been shown to be involved in the synthesis and deposition of secondary wall cellulose (Bischoff *et al.* 2010). *LOC_Os04g09604* encodes an O-methyltransferase and its homologue in *Arabidopsis* has been shown to be involved in lignin biosynthetic process (Goujon *et al.* 2003).

Common freezing responsive genes with different expression levels

Part IV represents DEGs shared between all four groups of comparison. Besides being cold responsive both in *WT* and *OsETR4-OE*, these 6 genes were also differentially expressed between *WT* and *OsETR4-OE*, both before and

Table 3: Differentially expressed genes in part III of Fig. 4B

Gene ID	WTck	WTcold	ETR4ck	ETR4cold	Description
LOC_Os11g17954	0.012	0.079	1.867	3.692	Homeodomain-like containing protein
LOC_Os03g60000	0.760	0.421	2.645	5.798	Similar to Mitochondrial prohibition complex protein 2.
LOC_Os06g06490	2.988	2.666	6.051	17.389	U-box domain containing heat shock protein
LOC_Os02g06290	0.747	0.497	10.326	4.523	rhodanese-like domain containing protein
LOC_Os04g09604	2.816	1.245	15.545	3.221	Similar to O-methyltransferase
LOC_Os04g18770	0.009	0.012	0.690	0.207	retrotransposon protein
LOC_Os12g33194	3.737	2.049	23.201	9.159	trichome birefringence-like 33

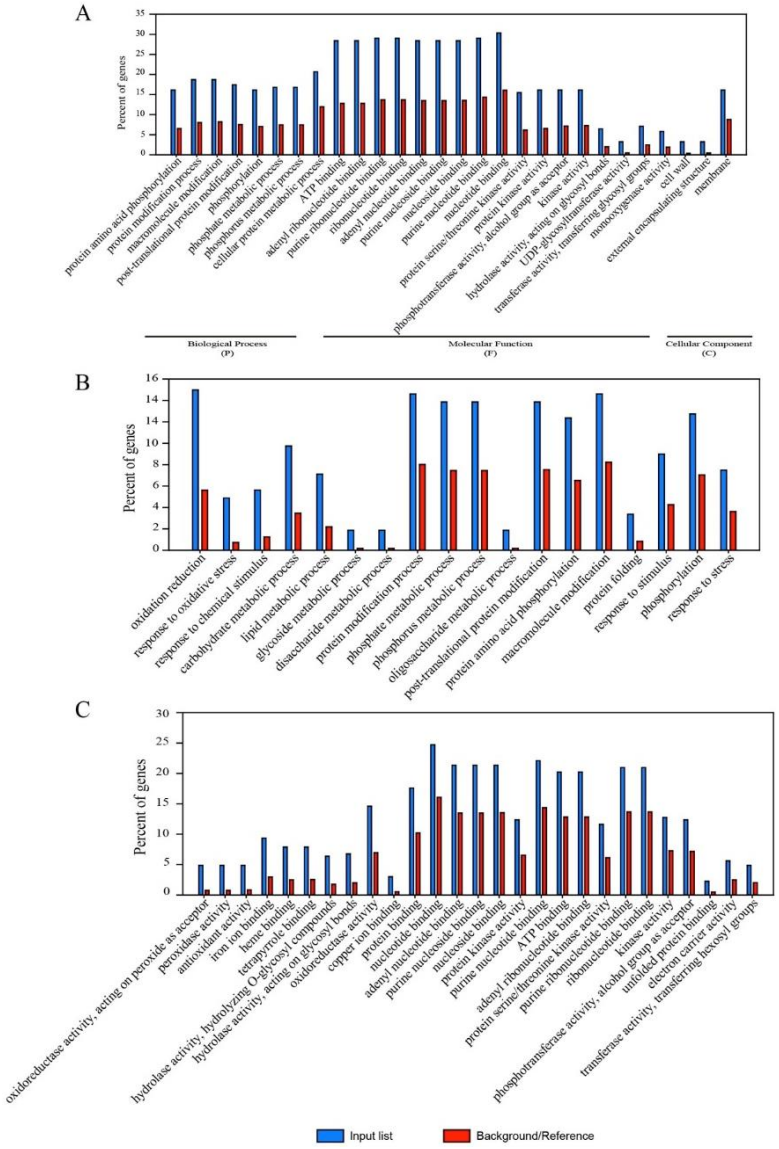

Fig. 5: Gene Ontology analysis of differentially expressed genes
(**A**) GO analysis of the 258 DEGs. (**B**) Significantly enriched GO terms belonging to biological process of 400 DEGs. (**C**) Significantly enriched GO terms belonging to molecular function of 400 DEGs

after cold treatment. This group of genes was cold-responsive in both *WT* and *OsETR4-OE*, but they were differentially expressed between *WT* and *OsETR4-OE* before and after cold treatment (Table 4). Therefore, the different expression level of these genes may contribute to the different seedling survival rates.

All 6 genes are repressed after cold treatment in *WT* and *OsETR4-OE*. Among these, the expression levels of 2 genes were consistently higher in WT than *OsETR4-OE*. *LOC_Os01g09700* is a member of 1-aminocyclopropane-1-

Table 4: Differentially expressed genes in part IV of Fig. 4B

Gene ID	WTck	WTcold	ETR4ck	ETR4cold	Description
LOC_Os01g09700	38.641	1.800	5.256	0.301	1-aminocyclopropane-1-carboxylate synthase
LOC_Os01g23710	7.165	0.809	1.289	0.140	NAC 30
LOC_Os04g17660	0.906	0.233	11.686	2.398	Arsenate reductase, Sulfurtransferase/rhodanese-like protein
LOC_Os07g23494	0.936	0.092	3.851	0.667	Galactosyl transferase domain containing protein.
LOC_Os07g44250	8.865	1.283	21.185	5.568	Disease resistance responsive (dirigent like protein) family protein
LOC_Os08g03020	3.462	1.435	9.454	3.684	lectin-like receptor kinase

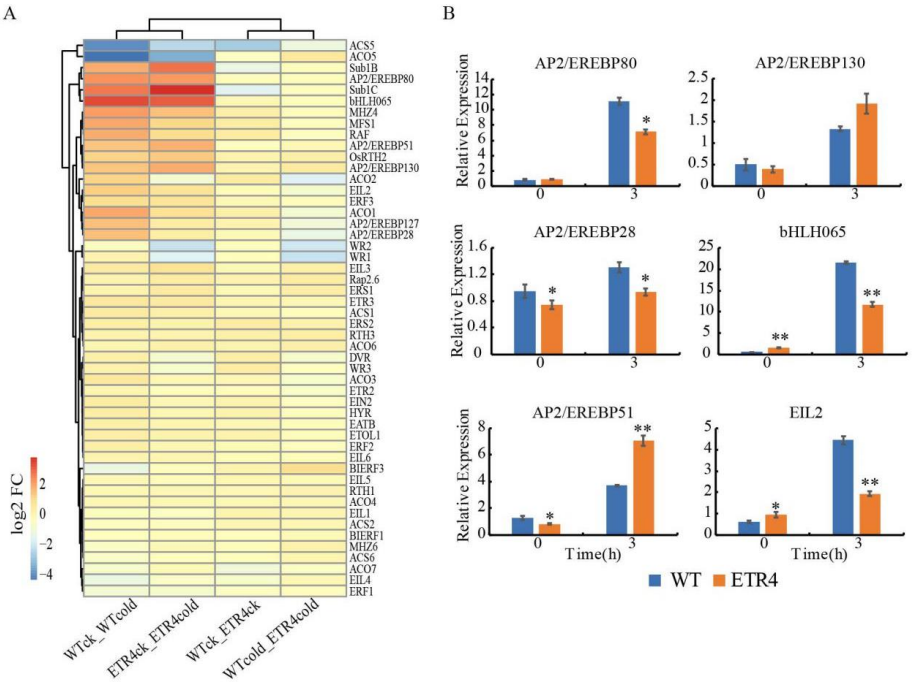

Fig. 6: Expression levels of ethylene-related genes
(**A**) The expression profile of 45 ethylene-related genes in *WT* and *OsETR4-OE* before and after cold treatment. (**B**) Validation of the expression levels of 6 genes at 0 h and 3 h after cold treatment by qRT-PCR. Values are means± SD (n = 3). **: $P < 0.01$, *: $P < 0.05$

carboxylate synthase (ACS) gene family, also known as *OsACS5*. *LOC_Os01g23710* encodes a NAC family transcription factor and may participate in signal transduction in the cold response. The other 4 genes all showed higher expression levels in the *OsETR4-OE* than WT, indicating that the high expression of these genes may contribute to the cold tolerance of *OsETR4-OE*. *LOC_Os04g17660* encodes an arsenate reductase, *OsHAC1;2* and overexpression of *OsHAC1;2* can enhance the tolerance to arsenate (Shi *et al.* 2016). No reference literatures were found in the cases of *LOC_Os07g44250* and *LOC_Os08g03020*.

Different expression patterns of ethylene signaling related genes

To further investigate the role of ethylene signaling in the cold response of rice seedlings, the expression profile of 45 genes involved in the rice ethylene biosynthesis and signaling were analyzed. Many ethylene-related genes were differentially expressed, either between *WT* and *ETR4-OE*, or between control and cold treatment (Fig. 6A). The detailed expression value and gene information were listed in Table 5. *LOC_Os05g05680*, also known as *OsACO5*, was both repressed in *WT* and *ETR4-OE* after cold treatment, consistent with the *OsACS5* identified in Part IV of Fig. 4b. The expression of *OsACO1*, *OsACO3*, and *OsACS1* were upregulated after cold treatment both in *WT* and *ETR4-OE*. *OsACO2* was induced by cold treatment in *WT* but repressed in *ETR4-OE*. The expression of the other members of *OsACS* and *OsACO* family showed no significant differences. Most of the ethylene response factors investigated here were upregulated both upregulated in *WT* and *ETR4-OE* after cold treatment, including *LOC_Os09g11480* and *LOC_Os09g11460*. *LOC_Os09g11480* and *LOC_Os09g11460* are also known as *Sub1B* and *Sub1C* and participate in the submergence response of rice (Xu *et al.* 2006). Two ethylene response factor gene, *OsWR1* and *OsWR2*, both showed opposite response patterns between *WT* and *ETR4-OE*, which were induced in *WT* but repressed in *ETR4-OE*. Therefore, the ethylene signaling was different between *WT* and *ETR4-OE* under cold treatment, which may contribute to the

Table 5: Expression information of ethylene related genes

Gene ID	MSU_Locus	ETR4-CK	ETR4-cold	WT-CK	WT-cold
OsEIL3	LOC_Os09g31400	18.28	34.45	19.50	28.30
OsBIERF1	LOC_Os09g26420	1176.10	1031.74	1413.92	1129.46
Sub1C	LOC_Os09g11480	1.35	30.09	5.67	39.18
Sub1B	LOC_Os09g11460	3.34	27.42	8.66	33.02
OsEIL4	LOC_Os08g39830	6.37	4.16	10.03	4.16
OsEIL2	LOC_Os07g48630	45.17	76.87	51.17	123.43
OsEIN2; MHZ7	LOC_Os07g06130	3.86	3.84	3.52	5.05
OsWR2	LOC_Os06g40150	8.23	1.26	10.30	7.61
OsRTH2	LOC_Os05g46240	10.81	28.86	10.01	23.35
MFS1	LOC_Os05g41760	3.71	8.94	2.38	11.55
ERS2; Os-ERS2	LOC_Os05g06320	25.84	28.20	18.31	23.89
OsERF1	LOC_Os04g46220	34.75	21.14	46.65	27.53
OsRap2.6	LOC_Os04g32620	0.67	1.29	0.54	0.69
ETR2; OS-ETR2	LOC_Os04g08740	27.08	22.02	30.53	31.39
OsRTH3	LOC_Os03g58520	19.17	20.83	15.33	16.37
Os-ERS1; OsERS1	LOC_Os03g49500	71.47	102.26	76.25	80.90
OsDVR	LOC_Os03g22780	12.47	7.61	9.35	11.73
MHZ6; OsEIL1	LOC_Os03g20790	115.29	105.44	129.96	90.12
OsEIL1	LOC_Os03g20780	76.81	55.58	71.73	55.15
OsETOL1	LOC_Os03g18360	2.63	2.85	2.40	3.32
OsRAF	LOC_Os03g08470	12.82	27.21	8.94	34.89
HYR	LOC_Os03g02650	0.32	0.26	0.21	0.57
ETR3; OS-ETR3	LOC_Os02g57530	23.79	33.78	23.24	35.00
OsBIERF3	LOC_Os02g43790	5.91	4.57	5.40	1.89
OsEIL5	LOC_Os02g36510	0.25	0.07	0.21	0.15
OsWR1	LOC_Os02g10760	8.86	2.29	11.22	12.66
OsERF3; OsAP37	LOC_Os01g58420	50.68	86.68	51.26	100.99
OsRTH1	LOC_Os01g51430	15.14	14.89	15.13	13.85
MHZ4	LOC_Os01g03750	5.48	17.35	4.05	19.85
AP2/EREBP127	LOC_Os01g49830	36.23	49.77	32.87	92.92
AP2/EREBP80	LOC_Os01g64790	0.82	7.00	1.06	8.80
AP2/EREBP130	LOC_Os02g52670	0.19	3.25	0.09	2.00
AP2/EREBP28	LOC_Os03g08490	89.03	117.88	95.27	268.80
OsbHLH065	LOC_Os04g41570	16.21	143.79	16.80	171.54
AP2/EREBP51	LOC_Os05g41780	8.42	29.66	10.83	30.93
OsACO7	LOC_Os01g39860	7.91	7.37	16.10	10.36
OsACS2	LOC_Os04g48850	0.69	0.29	0.75	0.33
OsACS1	LOC_Os03g51740	0.00	0.22	0.03	0.49
OsACS5	LOC_Os01g09700	5.26	0.30	38.64	1.80
OsACS6	LOC_Os06g03990	91.90	72.93	104.57	72.28
OsACO3	LOC_Os02g53180	40.65	40.24	40.68	62.86
OsACO6	LOC_Os05g05600	22.84	26.68	21.10	21.47
OsACO1	LOC_Os09g27750	16.01	29.74	13.27	52.55
OsACO2	LOC_Os09g27820	19.149	10.637	14.170	34.150
OsACO5	LOC_Os05g05680	61.512	5.463	84.335	3.143

differences in cold tolerance. The expression of 6 ethylene-related genes was further validated using qRT-PCR and the expression patterns were in agreement with our RNA-seq results (Fig. 6B).

Discussion

In *Arabidopsis*, genes involved in ethylene signaling can be rapidly up-regulated after exposure to low temperatures (Fowler and Thomashow 2002). In addition, it has been reported that ethylene synthesis is positively associated with cold tolerance in tomato plants (Ciardi *et al.* 1997; Zhang and Huang 2010). Therefore, ethylene has been shown to have positive effects on plant cold tolerance. However, ethylene receptors usually play negative roles in the ethylene response pathway. *OsACS5*, which participates in the ethylene synthesis, can be induced abiotic and biotic stress (Straeten *et al.* 2001; Iwai *et al.* 2006; Hu *et al.* 2011). Among 6 common freezing responsive genes with different expression levels, *OsACS5* was repressed both in WT and OsETR4-OE after cold stress. More importantly, the expression level of *OsACS5* was consistently lower in *OsETR4-OE* than *WT*. Two ethylene response factor genes, *OsWR1* and *OsWR2* can be induced by drought and their over-expression can enhance the drought tolerance in rice (Wang *et al.* 2012; Zhou *et al.* 2014). Interestingly, *OsWR1* and *OsWR2* both showed opposite response patterns between *WT* and *OsETR4-OE*. Therefore, the differential ethylene signaling response between *WT* and *OsETR4-OE* under cold treatment may contribute to their different cold tolerance.

In addition, *OsACO5*, responsible for encoding an aminocyclopropane-1-carboxylate oxidase which is

involved in ethylene biosynthesis (Iwai *et al.* 2006), was both repressed in *WT* and *ETR4-OE* after cold treatment. Most of the ethylene response factors investigated were both upregulated in *WT* and *ETR4-OE* after cold treatment, indicating that cold stress indeed induced the ethylene signaling response in rice.

Although *OsETR4* also expresses in young seedlings and anthers of rice, its expression level is quite low (Yau *et al.* 2004), which is also in agreement with our RNA-seq results. Among the five reported ethylene receptors in rice, *OsERS1* had the highest expression level and was constitutively expressed in different tissues, while *OsETR2* and *OsERS2* had lower expression level (Yau *et al.* 2004). The expression of *OsETR2* and *OsERS1* can be induced by exogenous stimulus such as IAA or ethylene while *OsERS2* was repressed (Yau *et al.* 2004). In our RNA-seq results, ethylene receptors barely respond to the cold treatment both in *WT* and *OsETR4-OE*, suggesting that ethylene receptors may not respond to cold stress in rice and the *OsETR4-OE* had no effects on the other ethylene receptors.

The ethylene receptors also have kinase activities: AtETR1 has histidine kinase activity, whereas the others mainly have Ser/Thr kinase activities (Gamble *et al.* 1998; Xie *et al.* 2003; Moussatche and Klee 2004). The Gene Ontology term "protein phosphorylation" is significantly enriched in DEGs that are only differentially expressed between *WT* and *OsETR4-OE* after cold treatment. This means that after cold treatment, genes involved in protein phosphorylation are differentially expressed between *WT* and *OsETR4-OE* and these genes may contribute to the cold tolerance of *OsETR4-OE*. Overexpressing *OsETR4* protein may help the rice seedlings in response to cold treatment through enhancing its kinase activities. However, the detailed protein activity assay needs to be further studied.

Conclusion

Overexpression of an ethylene receptor *OsETR4* could increase the seedling survival rates of rice under cold stress. Transcriptomic analysis by RNA-sequencing (RNA-seq) showed significant difference in OsETR4-OE line compared to wild type (WT) in response to cold. The Gene Ontology term "protein phosphorylation" is significantly enriched in DEGs that are only differentially expressed between *WT* and *OsETR4-OE* after cold treatment, indicating that the enhanced kinase activities by *OsETR4* overexpression may contribute to its cold tolerance. In addition, genes involved in the rice ethylene biosynthesis and signaling were differentially expressed, either between *WT* and *ETR4-OE*, or between control and cold treatment. Therefore, genetic engineering of ethylene receptor *OsETR4* could contribute to the cold climate tolerance of rice in the future.

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Author Contributions

XD and WJ designed the experiments; ZL, MW, KH, XY, HZ, ZM, SJ, LG, LD and XZ performed the experiments; ZL analyzed the RNA-seq data, WJ and ZL wrote the manuscript, XD, TW and MB modified the manuscript; all the authors agreed on the final submission and posted no conflicting interest.

References

Agarwal M, Y Hao, A Kapoor, CH Dong, H Fujii, X Zheng, JK Zhu (2006). A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. *J Biol Chem* 281:37636–37645

Alexander L, D Grierson (2002). Ethylene biosynthesis and action in tomato: A model for climacteric fruit ripening. *J Exp Bot* 53:2039–2055

Anderson JP, E Badruzsaufari, PM Schenk, JM Manners, OJ Desmond, C Ehlert, DJ Maclean, PR Ebert, K Kazan (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. *Plant Cell* 16:3460–3479

Arshad MS, M Farooq, F Asch, JSV Krishna, PVV Prasad, KHM Siddique (2017) Thermal stress impacts reproductive development and grain yield in rice. *Plant Physiol Biochem* 115:57–72

Bischoff V, S Nita, L Neumetzler, D Schindelasch, A Urbain, R Eshed, S Persson, D Delmer, WR Scheible (2010). *TRICHOME BIREFRINGENCE* and its homolog *At5g01360* encode plant-specific DUF231 proteins required for cellulose biosynthesis in *Arabidopsis*. *Plant Physiol* 153:590–602

Chinnusamy V, J Zhu, JK Zhu (2007). Cold stress regulation of gene expression in plants. *Trends Plant Sci* 12:444–451

Chinnusamy V, M Ohta, S Kanrar, BH Lee, X Hong, M Agarwal, JK Zhu (2003). ICE1: A regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev* 17:1043–1054

Ciardi JA, J Deikman, MD Orzolek (1997). Increased ethylene synthesis enhances chilling tolerance in tomato. *Physiol Plantarum* 101:333–340

Ding Y, H Li, X Zhang, Q Xie, Z Gong, S Yang (2015). OST1 kinase modulates freezing tolerance by enhancing ICE1 stability in *Arabidopsis*. *Dev Cell* 32:278–289

Doherty CJ, HAV Buskirk, SJ Myers, MF Thomashow (2009). Roles for *Arabidopsis* CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. *Plant Cell* 21:972–984

Dong CH, M Agarwal, Y Zhang, Q Xie, JK Zhu (2006). The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. *Proc Natl Acad Sci USA* 103:8281–8286

Dubois M, LVD Broeck, D Inzé (2018). The pivotal role of ethylene in plant growth. *Trends Plant Sci* 23:311–323

Fowler S, MF Thomashow (2002). *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell* 14:1675–1690

Fujino K, H Sekiguchi, Y Matsuda, K Sugimoto, K Ono, M Yano (2008). Molecular identification of a major quantitative trait locus, *qLTG3-1*, controlling low-temperature germinability in rice. *Proc Natl Acad Sci USA* 105:12623–12628

Fursova OV, GV Pogorelko, VA Tarasov (2009). Identification of ICE2, a gene involved in cold acclimation which determines freezing tolerance in *Arabidopsis thaliana*. *Gene* 429:98–103

Gamble RL, ML Coonfield, GE Schaller (1998). Histidine kinase activity of the ETR1 ethylene receptor from *Arabidopsis*. *Proc Natl Acad Sci USA* 95:7825–7829

Goujon T, R Sibout, B Pollet, B Maba, L Nussaume, N Bechtold, F Lu, J Ralph, I Mila, Y Barrière, C Lapierre, L Jouanin (2003). A new *Arabidopsis thaliana* mutant deficient in the expression of O-methyltransferase impacts lignins and sinapoyl esters. *Plant Mol Biol* 51:973–989

Hiei Y, S Ohta, T Komari, T Kumashiro (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* 6:271–282

Hu J, J Zhou, X Peng, H Xu, C Liu, B Du, H Yuan, L Zhu, G He (2011). The *Bphi008a* gene interacts with the ethylene pathway and transcriptionally regulates *MAPK* genes in the response of rice to brown planthopper feeding. *Plant Physiol* 156:856–872

Iwai T, A Miyasaka, S Seo, Y Ohashi (2006). Contribution of ethylene biosynthesis for resistance to blast fungus infection in young rice plants. *Plant Physiol* 142:1202–1215

Light KM, JA Wisniewski, WA Vinyard, MT Kieber-Emmons (2016). Perception of the plant hormone ethylene: Known-knowns and known-unknowns. *JBIC J Biol Inorg Chem* 21:715–728

Liu C, S Ou, B Mao, J Tang, W Wang, H Wang, S Cao, MR Schläppi, B Zhao, G Xiao, X Wang, C Chu (2018). Early selection of *bZIP73* facilitated adaptation of *japonica* rice to cold climates. *Nat Commun* 9; Article 3302

Ma Y, X Dai, Y Xu, W Luo, X Zheng, D Zeng, Y Pan, X Lin, H Liu, D Zhang, J Xiao, X Guo, S Xu, Y Niu, J Jin, H Zhang, X Xu, L Li, W Wang, Q Qian, S Ge, K Chong (2015). *COLD1* confers chilling tolerance in rice. *Cell* 160:1209–1221

Medina J, R Catala, J Salinas (2011). The CBFs: Three *Arabidopsis* transcription factors to cold acclimate. *Plant Sci* 180:3–11

Miura K, JB Jin, J Lee, CY Yoo, V Stirm, T Miura, EN Ashworth, RA Bressan, DJ Yun, PM Hasegawa (2007). SIZ1-mediated sumoylation of ICE1 controls *CBF3/DREB1A* Expression and freezing tolerance in *Arabidopsis*. *Plant Cell* 19:1403–1414

Morgan PW, MC Drew (1997). Ethylene and plant responses to stress. *Physiol Plantarum* 100:620–630

Moussatche P, HJ Klee (2004). Autophosphorylation activity of the *Arabidopsis* ethylene receptor multigene family. *J Biol Chem* 279:48734–48741

Saito K, Y Hayano-Saito, M Kuroki, Y Sato (2010). Map-based cloning of the rice cold tolerance gene *Ctb1*. *Plant Sci* 179:97–102

Schläppi MR, AK Jackson, GC Eizenga, A Wang, C Chu, Y Shi, N Shimoyama, DL Boykin (2017). Assessment of five chilling tolerance traits and GWAS mapping in rice using the USDA Mini-Core collection. *Front Plant Sci* 8; Article 957

Shi S, T Wang, Z Chen, Z Tang, Z Wu, DE Salt, DY Chao, FJ Zhao (2016). *OsHAC1;1* and *OsHAC1;2* function as arsenate reductases and regulate arsenic accumulation. *Plant Physiol* 172:1708–1719

Straeten DVD, Z Zhou, E Prinsen, HAV Onckelen, MCV Montagu (2001). A comparative molecular-physiological study of submergence response in lowland and deep water rice. *Plant Physiol* 125:955–968

Tian T, Y Liu, H Yan, Q You, X Yi, Z Du, W Xu, Z Su (2017). agriGO v2.0: A GO analysis toolkit for the agricultural community, 2017 update. *Nucl Acids Res* 45:122–129

Trapnell C, DG Hendrickson, M Sauvageau, L Goff, JL Rinn, L Pachter (2013). Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat Biotechnol* 31:46–53

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

Wang Q, Y Guan, Y Wu, H Chen, F Chen, C Chu (2008). Overexpression of a rice *OsDREB1F* gene increases salt, drought, and low temperature tolerance in both *Arabidopsis* and rice. *Plant Mol Biol* 67:589–602

Wang Y, L Wan, L Zhang, Z Zhang, H Zhang, R Quan, S Zhou, R Huang (2012). An ethylene response factor *OsWR1* responsive to drought stress transcriptionally activates wax synthesis related genes and increases wax production in rice. *Plant Mol Biol* 78:275–288

Wing RA, MD Purugganan, Q Zhang (2018). The rice genome revolution: From an ancient grain to Green Super Rice. *Nat Rev Genet* 19:505–517

Wuriyanghan H, B Zhang, WH Cao, BA Ma, G Lei, YF Liu, W Wei, HJ Wu, LJ Chen, HW Chen, YR Cao, SJ He, WK Zhang, XJ Wang, SY Chen, JS Zhang (2009). The ethylene receptor *ETR2* delays floral transition and affects starch accumulation in rice. *Plant Cell* 21:1473–1494

Xie C, JS Zhang, HL Zhou, J Li, ZG Zhang, DW Wang, SY Chen (2003). Serine/threonine kinase activity in the putative histidine kinase-like ethylene receptor *NTHK1* from tobacco. *Plant J* 33:385–393

Xu K, X Xu, T Fukao, P Canlas, R Maghirang-Rodriguez, S Heuer, AM Ismail, J Bailey-Serres, PC Ronald, DJ Mackill (2006). *Sub1A* is an ethylene-response-factor-like gene that confers submergence tolerance to rice. *Nature* 442:705–708

Yang SF, NE Hoffman (1984). Ethylene biosynthesis and its regulation in higher plants. *Ann Rev Plant Physiol* 35:155–189

Yau CP, LM Wang, SY Zee, WK Yip (2004). Differential expression of three genes encoding an ethylene receptor in rice during development, and in response to indole-3-acetic acid and silver ions. *J Exp Bot* 55:547–556

Yu M, CP Yau, WK Yip (2017). Differentially localized rice ethylene receptors OsERS1 and OsETR2 and their potential role during submergence. *Plant Signal Behav* 12; Article e1356532

Yu XM, M Griffith, SB Wiseman (2001). Ethylene induces antifreeze activity in winter rye leaves. *Plant Physiol* 126:1232–1240

Zhang JS, C Xie, YG Shen, SY Chen (2001). A two-component gene (*NTHK1*) encoding a putative ethylene-receptor homolog is both developmentally and stress regulated in tobacco. *Theor Appl Genet* 102:815–824

Zhang Q, Q Chen, S Wang, Y Hong, Z Wang (2014). Rice and cold stress: Methods for its evaluation and summary of cold tolerance-related quantitative trait loci. *Rice* 7:24–35

Zhang Z, R Huang (2010). Enhanced tolerance to freezing in tobacco and tomato overexpressing transcription factor *TERF2/LeERF2* is modulated by ethylene biosynthesis. *Plant Mol Biol* 73:241–249

Zhang Z, J Li, Y Pan, J Li, L Zhou, H Shi, Y Zeng, H Guo, S Yang, W Zheng, J Yu, X Sun, G Li, Y Ding, L Ma, S Shen, L Dai, H Zhang, S Yang, Y Guo, Z Li (2017). Natural variation in *CTB4a* enhances rice adaptation to cold habitats. *Nat Commun* 8; Article 14788

Zhao D, L Shen, B Fan, M Yu, Y Zheng, S Lv, J Sheng (2009). Ethylene and cold participate in the regulation of *LeCBF1* gene expression in postharvest tomato fruits. *FEBS Lett* 583:3329–3334

Zhou X, MA Jenks, J Liu, A Liu, X Zhang, J Xiang, J Zou, Y Peng, X Chen (2014). Overexpression of transcription factor *OsWR2* regulates wax and cutin biosynthesis in rice and enhances its tolerance to water deficit. *Plant Mol Biol Rep* 32:719–731

Zhu J, CH Dong, JK Zhu (2007). Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. *Curr Opin Plant Biol* 10:290–295