Analysis of Stress-Responsive Gene Expression in Cultivated and Weedy Rice Differing in Cold Stress Tolerance

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Abstract

Rice (*Oryza sativa* L.) cultivars show impairment of growth in response to environmental stresses such as cold at the early seedling stage. Locally adapted weedy rice is able to survive under adverse environmental conditions, and can emerge in fields from greater soil depth. Cold-tolerant weedy rice can be a good genetic source for developing cold-tolerant, weed-competitive rice cultivars. An in-depth analysis is presented here of diverse *indica* and *japonica* rice genotypes, mostly weedy rice, for cold stress response to provide an understanding of different stress adaptive mechanisms towards improvement of the rice crop performance in the field. We have tested a collection of weedy rice genotypes to: 1) classify the subspecies (ssp.) grouping (*japonica* or *indica*) of 21 accessions; 2) evaluate their sensitivity to cold stress; and 3) analyze the expression of stress-responsive genes under cold stress and a combination of cold and depth stress. Seeds were germinated at 25°C at 1.5- and 10-cm sowing depth for 10d. Seedlings were then exposed to cold stress at 10°C for 6, 24 and 96h, and the expression of cold-, anoxia-, and submergence-inducible genes was analyzed. Control plants were seeded at 1.5cm depth and kept at 25°C. The analysis revealed that cold stress signaling in *indica* genotypes is more complex than that of *japonica* as it operates via both the CBF-dependent and CBF-independent pathways, implicated through induction of transcription factors including *OsNAC2*, *OsMYB46* and *OsF-BOX28*. When plants were exposed to cold + sowing depth stress, a complex signaling network was induced that involved cross talk between stresses mediated by CBF-dependent and CBF-independent pathways to circumvent the detrimental effects of stresses. The experiments revealed the importance of the CBF regulon for tolerance to both stresses in *japonica* and *indica* ssp. The mechanisms for cold tolerance differed among weedy *indica* genotypes and also between weedy *indica* and cultivated *japonica* ssp. as indicated by the up/downregulation of various stress-responsive pathways identified from

gene expression analysis. The cold-stress response is described in relation to the stress signaling pathways, showing complex adaptive mechanisms in different genotypes.

Introduction

About two-thirds of the global land area undergo freezing cycles, or is in permafrost, and 42% of agricultural land can experience freezing temperatures up to -20°C [1]. To survive under these conditions, plants have developed specialized mechanisms involving adaptive morphological, physiological, and biochemical changes. Low temperature stress impairs crop growth and development, causing crop yield losses [2]. Plants exhibit differential responses to chilling (0–15°C) or freezing (< 0°C) temperatures. Plants in temperate regions acclimate to cold temperatures [3] through various biochemical and physiological changes [4, 5], enabled by increased expression of cold-responsive genes that eventually leads to alteration in lipid composition of membranes and accumulation of osmolytes [6]. In contrast, tropical and subtropical plants lack cold-adaptation mechanisms and are highly sensitive to cold stress.

Survival under cold stress is a highly complex process involving various metabolic pathways and cellular compartments [7]. Conventional breeding methods have produced some cold-tolerant cultivars, but progress is slow owing to the complexity of stress tolerance traits, low genetic variance of yield components under stress conditions, and absence of suitable selection criteria. Locally adapted cold-tolerant cultivars are therefore needed. Exploring genomic resources to develop cold-tolerant crops is necessary.

Rice (*Oryza sativa* L.) feeds more than half of the global population [8], second only to wheat (http://www.idrc.ca/EN/Resources/Publications/Pages/ArticleDetails.aspx?PublicationID=565). Rice is grown in a wide range of environments (tropical, sub-tropical, temperate) but it is a tropical C3 crop [9], which yields best under warm temperatures and high solar radiation while still remaining inefficient [10]. Therefore, in non-tropical environments (and in high altitudes), low temperature stress during crop establishment and reproductive stage causes significant yield losses. Cold stress around planting time impairs rice emergence and hampers early seedling growth and development [11]. This makes rice vulnerable to competition from weeds, especially weedy rice. A competitive variety is a strong component of an integrated weed management program [12–13], and work on varietal improvement for competitive ability needs to be pursued.

When cold temperature occurs at the reproductive stage, it causes spikelet sterility [14–15] and reduces spikelet number and overall panicle volume [16]. In the rice-producing region of northern Japan, rice yield can decline by up to 60% in a cold year [17]. In the USA, cold stress is a major constraint to the establishment and growth of early-planted rice and is detrimental to yield of late-planted rice. There is good potential for varietal improvement in cold tolerance because of the observed genetic diversity in rice germplasm with respect to this trait [18]. About 40 quantitative trait loci (QTLs), in various combinations, impart cold tolerance in various rice varieties [19], with the *japonica* ssp. generally more cold-tolerant than *indica* ssp. [20–21].

Exposure of plants to cold temperatures disrupts the expression of its full genetic potential owing to direct inhibition of cellular metabolism, or indirectly via cold-induced oxidative and other abiotic stresses. Acclimation to cold is achieved by chronic exposure to cold temperatures [22] through accumulation of cold-adaptive genes with time. One such gene is *FRO1* (*FROST-BITE 1*). The *Arabidopsis fro1* (*frostbite1*) mutant exhibits susceptibility to chilling and freezing

stress through impaired expression of cold responsive (COR) genes and a substantial accumulation of reactive oxygen species (ROS) [23]. Thus, cold stress signaling in plants is significantly affected by ROS accumulation and ABA, which are secondary cellular messengers. However, some weedy rice ecotypes may have a better cold adaptation mechanism because weedy rice seedlings grow faster than cultivated rice even during early planting in temperate climate when cold stress often occurs (Burgos, NR pers. observation). This warrants investigation of cold tolerance or cold adaptation mechanisms in weedy rice.

Difference in cold tolerance is a result of centuries of adaptation in cold, rice-growing regions [24]. Cold tolerance is a complex trait, involving many genes, and is highly genotype-dependent. For example, the transcription factor *MYBS3* mediates cold stress signaling and cold tolerance in rice via a *MYBS3*-dependent pathway that allows long-term cold adaptation, and acts in conjunction with the short-term cold stress signaling pathway mediated by *DREB1/CBF* [25]. The rice *FATTY ACID DESATURASE 2* (*OsFAD2*) also enhances cold tolerance by maintaining membrane fluidity during low temperature stress [26]. Other cold-tolerance mechanisms have also been reported.

Outside of Asia, Brazil and the USA are among the highest rice-producing countries (http://globalriceproduction.com/). Although the USA primarily grows *japonica* cultivars, yield depression by cold stress still occurs periodically. The attempt to avoid cold stress narrows the rice planting window and creates constraints on farming resources. Brazil, on the other hand, grows primarily *indica* rice, which is generally cold-sensitive; and farmers thus have to contend with problems such as reduced or variable rice emergence and reduced yield due to cold stress [27–30]. Rice growers in regions like these need to plant rice early to synchronize the onset of reproductive stage with the highest solar radiation period and obtain the highest possible yield. Weedy rice may grow faster than a cold-sensitive cultivar at early planting, making the weed even more adaptive and competitive than the crop. Thus, improving cold tolerance in rice seedlings could boost rice production tremendously. Cold-tolerant *japonica* rice lines [31] are excellent sources of cold-tolerance genes that could be accumulated in desirable high yielding cultivars; sourcing these traits from *japonica* lines to improve cold tolerance in *indica* varieties has been challenging because of high sterility among *indica* x *japonica* hybrids. An alternative is to source cold tolerance traits for *indica* cultivars from different *indica* lines. The majority (>90%) of weedy rice in the USA are *indica* [32–33] and some *indica* weedy ecotypes may be cold-tolerant.

Seeding depth exacerbates the effect of cold stress on rice establishment, as it affects seedling vigor. Rice cultivars, having been selected for uniform and quick emergence, do not emerge from deep in the soil profile. The weedy ecotypes, however, exhibit a wide diversity in ability to emerge from a greater depth [34–35].]. The US weedy ecotypes that could emerge from deep placement are most likely *indica* because of the dominance of weedy *indica* ssp. in the region; such ecotypes have high seedling vigor and may be able to withstand cold stress at the seedling stage [34]. Therefore, screening of rice germplasm for seedling cold tolerance is an important first step in finding resources for cold tolerance improvement. The present study investigates diverse weedy rice genotypes and rice cultivars for tolerance to cold stress, and makes a comparative analysis of the response mechanisms between weedy and cultivated rice in the *indica* and *japonica* subspecies. To dissect the cold tolerance mechanisms, we conducted real time quantitative-PCR (qPCR) analysis of gene expression in response to cold stress and a combination of cold and depth stress compared to normal temperature, of *indica* and *japonica* genotypes differing in cold sensitivity. Data from this experiment will be a resource in improving cold stress tolerance in rice and its competitive ability with weeds in early planting in temperate and sub-tropical regions.

Methods

Plant Materials and Subspecies Identification

Weedy rice seed samples, comprised of 21 weedy red rice accessions and rice cultivars for sub-species (ssp.) identification, were collected from rice fields in Arkansas, the largest rice-growing state in the southern USA, and from Rio Grande do Sul, Brazil. Weedy rice seed collection was done primarily in collaboration with Extension Agents with permission from growers and, occasionally, with the growers themselves. Some rice cultivars were obtained from rice breeders, but the majority were requested from the US Germplasm Resources Information Network-National Plant Germplasm System (GRIN-NPGS) (http://www.ars-grin.gov/npgs/aboutgrin.html). No endangered species were involved nor impacted by this activity. The phenol test [32] was conducted to determine the ssp. genotype (*japonica* or *indica*) of the materials to be used in subsequent experiments. The phenol test is based on the color change of rice hulls or endosperm upon exposure to phenol as a result of polyphenol oxidase (PPO) activity, where *japonica* varieties show no color change (a negative response), while *indica* varieties and wild *Oryza* species take on a dark brown or black coloration [32]. Intact grains soaked in 1.5% non-buffered aqueous phenol for 48h were compared with nontreated seeds from the same seed lot. A positive reaction to phenol is indicated by color change of the grain, from light to dark (S1 Fig). The *indica* genotype has positive reaction to phenol. The experiment was conducted with one positive control (1602) and one negative control, 'Spring'. Samples showing negative phenol reaction were tested twice for verification.

Plant Materials and Growth Conditions for Stress Tolerance Phenotyping

A subset of samples used in the phenol test (31) was phenotyped for cold- and seeding-depth stress tolerance. To overcome dormancy, the seeds were soaked in 1.5% NaOCl for 24h. Seeds were rinsed with deionized water, surface sterilized with 0.1% $HgCl_2$ for 20min then planted at different depths in pots, filled with vermiculite.

For cold stress only, the seeds were planted 1.5cm deep and placed in a growth chamber in the dark for 14d. Control plants were germinated at 25°C; cold-stressed plants were grown at alternating temperatures of 18°C/13°C, 16/8h cycle, in the dark. For depth stress only, the seeds were planted at 1.5-, 5-, 10-, and 15-cm depth and germinated at 25°C in the dark for 14d. For the combined stress treatments (depth + cold), seeds were planted at different depths (5, 10, and 15cm); the cold-stressed plants were germinated at 18°C/13°C, 16/8-h cycle, in the dark, while the controls were kept at 25°C. At 14d, the shoot lengths of five seedlings per growth condition per accession were measured. The experiments were repeated. Shoot length reduction (%) was then calculated relative to the control plants of each accession and used as a measure of sensitivity to cold and seeding depth stress [36–38]. Accessions showing <50% reduction in shoot length under cold or depth stress, relative to their corresponding non-stressed plants, were deemed cold- or depth-tolerant. The cold-tolerant and selected sensitive accessions were used in the gene expression analysis experiment. Phenotyping was done at 18°C/13°C to allow plants to germinate from deep seeding and identify a broader spectrum of cold stress-tolerant genotypes. We learned in preliminary experiments that at 10°C, few accessions could germinate at normal seeding depth and none could germinate when planted deeper.

For gene expression analysis in response to cold stress only, pregerminated seeds were planted at 1.5-cm depth and allowed to grow for 10d at 25°C. Thereafter, cold stress was initiated by transferring plants to 10°C [39–40] while the control plants were kept at 25°C. Leaves of stressed and non-stressed plants were harvested at 6, 24, and 96h of incubation in cold

Table 1. Summary of genes analyzed.

Gene	Trigger	Result	Reference
OsNAC2	Salt, Drought, Cold	Abiotic Stress Tolerance	[80]
OsDREB2C	Salt, Mannitol and Cold	Thermotolerance in *Arabidopsis thaliana* (*AtDREB2C*)	[93–94]
OSMYB46	Drought	Overexpression led to ectopic deposition of secondary cell wall in nonsclerenchymatous cells	[95–96]
OsFBOX28	Cold	None	[97]
OVP1	Cold	Improves Cold tolerance in rice	[69]
Rab 16A	Salt, Drought, Cold	Improves Salt tolerance in rice	[42,98]
GLP	ABA, Cold, H_2O_2	None	[99]
GDH	Salt, Cold, Drought	None	[64,100]
APX1	Salt, Cold	Overexpression in rice showed enhanced tolerance to cold stress at booting stage	[101–102]
ADH1	Anoxia, Cold	*ADH1* overexpression in *Arabidopsis* had no effect on flooding tolerance but was essential for survival under anoxic conditions	[89,92,103]
EXPA 7	Anoxia, Submergence	None	[88,104]
EXPA 12	Anoxia, Submergence	None	[89]
RAMY3D	Anoxia, Cold	None	[90]
ERF 70	Anoxia, Salt, Drought	Overexpression in *Arabidopsis thaliana* gives tolerance to osmotic stress	[89,104,105]
ERF 68	Anoxia, Drought, Salt	None	[89,106,107]
SUB1b	ABA, Drought, Submergence	None	[89,108]

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temperature. Each treatment had three replicates. The same procedure was followed for the cold + seeding-depth stress experiment, except that the pregerminated seeds were planted at a depth of 10cm, grown at 25°C for 10d, then incubated at 10°C for 6, 24, and 96hr before harvesting leaf tissues. Control plants were kept at 25°C. Cold stress-induced gene expression was studied at 10°C to capture genes most strongly involved in adaptation to chilling stress.

Gene Expression Analysis

Total RNA was isolated using Trizol reagent (Invitrogen) from stress-tolerant and-sensitive genotypes identified from the phenotyping experiment. cDNA synthesis was conducted using 2μg total DNAse-treated RNA using GoScript Reverse Transcription System (Promega). The qRT-PCR experiments were conducted using GoTaq qPCR Master Mix (Promega), gene-specific primers (Table 1 and S1 Table), and ubiquitin as standard with three biological replicates and each replicate divided into two technical replicates in a CFX-96 Bio-Rad thermocycler (Bio-Rad). Increasing temperature (0.5°C 10 s^{-1}) from 55°C to 95°C was used for melt curve analysis. Un-transcribed RNA was also run as negative control. The relative difference in expression for each sample in individual experiments was determined by normalizing the Ct value for each gene against the Ct value of ubiquitin and was calculated relative to the respective control samples as calibrator using the equation 2-ΔΔCt [41]. The data from qPCR analysis was imported into TM4 microarray software suite, normalized using GC-RMA algorithm to generate the heat map. The average of three biological replicates was used to obtain each expression value [42].

Results and Discussion

Classification of Weedy and Cultivated Rice Subspecies

Weedy red rice has diverse phenotypic characteristics, possessing traits that are common to both wild and cultivated rice. A diverse set of rice genetic resources was used here to examine

Table 2. Weedy and cultivated rice (*Oryza sativa* L.) genotypes subspecies grouping by phenol test following the method of Gross et al., 2009.

Accession code	Hull color	Origin	Rice type	Subspecies	GPS Coordinates
ARK-5-SH	Strawhull	Arkansas, AR, USA	weedy red rice	*Indica*	91°22.246' W, 34°07.220' N
CHI08-C-SH	Strawhull	Chicot, AR, USA	weedy red rice	*Indica*	91°03.680' W 35°25.958' N
CON-1-BH	Blackhull	Conway, AR, USA	weedy red rice	*Indica*	92°11.326' W, 34°71.388' N
CRA08-D-SH	Strawhull	Craighead, AR, USA	weedy red rice	*Indica*	90°58.976' W, 35°45.791' N
DES-1-SH	Strawhull	Desha, AR, USA	weedy red rice	*Indica*	91°27.695' W, 33°46.151' N
GRE08-D-SH	Strawhull	Greene, AR, USA	weedy red rice	*Indica*	90°46.677' W, 36°01.764' N
LEE08-C-SH	Strawhull	Lee, AR, USA	weedy red rice	*Indica*	90°02.237' W, 34°45.791' N
LIN08-B-BH	Blackhull	Lincoln, AR, USA	weedy red rice	*Indica*	91°42.018' W, 33°54.839' N
LIN08-B-SH	Strawhull	Lincoln, AR, USA	weedy red rice	*Indica*	91°42.008' W, 33°54.854' N
LIN08-C-SH	Strawhull	Lincoln, AR, USA	weedy red rice	*Indica*	91°42.529' W, 33°57.653' N
LON-3-SH	Strawhull	Lonoke, AR, USA	weedy red rice	*Indica*	91°52.475' W, 34°52.258' N
1200	Strawhull	Pelotas, RS-Brazil	weedy red rice	*Indica*	52°26.483' W, 31°49.024' S
1602	Strawhull	Pelotas, RS-Brazil	weedy red rice	*Indica*	52°25.761' W, 31°48.758' S
RAN-2-SH	Strawhull	Randolph, AR, USA	weedy red rice	*Indica*	90°56.703' W, 36°12.510' N
LAW08-B-SH	Strawhull	Lawrence, AR, USA	weedy red rice	*Indica*	90°54.981' W, 35°58.380' N
PRA08-D-BH	Blackhull	Prairie, AR, USA	weedy red rice	*Japonica*	91°26.955' W, 34°44.770' N
WOO-5-BH	Strawhull	Woodruff, AR, USA	weedy red rice	*Japonica*	91°17.156' W, 35°00.315' N
3011(Diamante)	Strawhull	Chile	rice cultivar	*Japonica*	Not available
Oro	Strawhull	Chile	rice cultivar	*Japonica*	Not available
Hayayuki	Strawhull	Japan	rice cultivar	*Japonica*	Not available
Spring	Strawhull	USA	rice cultivar	*Japonica*	91°25.134' W, 37°28.567' N

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the diversity of tolerance to abiotic stress, and the molecular basis of such traits, in this weedy relative of rice. Previous research classified the US weedy rice strains as either wild- or crop-like, but the adaptive phenotypic variation of weedy rice has not been fully characterized. Rice is classified into two major subspecies *indica* and *japonica*, which differ in a suite of classical diagnostic traits, i) seedling reaction to $KClO_3$, ii) seedling survival in cold temperatures, iii) grain apiculus hair length, and iv) phenol reaction. The phenol test used to distinguish the rice cultivars and weedy accessions separated the 21 tested genotypes into 6 *japonica* and 15 *indica* subspecies (Table 2). The four rice cultivars were all *japonica*. Of the 15 Arkansas weedy rice accessions, 13 (87%) were *indica*. Previous studies also have shown that *japonica* weedy rice is rare in the USA. Analysis of weedy rice accessions from the southern USA ricebelt (Arkansas, Mississippi, Louisiana, Texas) using simple sequence length polymorphic (SSLP) markers, showed that 19 of 21 accessions (90%) belonged to the *indica* ssp. group [33]. More recently, the phenol reaction test on 108 weedy rice accessions from southern USA showed that 96% were *indica* [32]. Thus, there are abundant *indica* genotypes that are potential sources of desirable traits for crop improvement, especially of *indica* cultivars.

Phenotyping for Cold Tolerance

The rice plants growing in cold temperatures show various symptoms of stress-induced damage such as reduced leaf expansion, chlorosis, and impaired growth. Reduction in seedling growth is a good indicator for cold tolerance during seed germination and seedling emergence. Tolerance to cold stress at this period is invaluable in achieving faster crop establishment and better competitive ability with weeds. The three rice *japonica* cultivars (Hayayuki, Spring, and 3011) had mean shoot lengths of 105, 98, and 41mm, respectively, when planted at normal depth (1.5cm) under normal temperature (25°C) for 14d (Table 3). 'Hayayuki' from Japan

Table 3. Seedling growth of weedy and cultivated rice (*Oryza sativa* L.) genotypes in response to cold stress under normal seeding depth of 1.5cm.

Accession code	Origin	Rice type	Subspecies	Shoot length at 25°C (mm)[a]		Shoot length reduction under cold stress (%)[b]	Cold tolerance[c]
CHI08-C-SH	Chicot, AR, USA	weedy red rice	*Indica*	122	b	94	sensitive
CRA08-D-SH	Craighead, AR, USA	weedy red rice	*Indica*	162	a	95	sensitive
GRE08-D-SH	Greene, AR, USA	weedy red rice	*Indica*	103	b	29	tolerant
LEE08-C-SH	Lee, AR, USA	weedy red rice	*Indica*	173	a	92	sensitive
LIN08-B-BH	Lincoln, AR, USA	weedy red rice	*Indica*	55	c	92	sensitive
LIN08-B-SH	Lincoln, AR, USA	weedy red rice	*Indica*	94	b	91	sensitive
LIN08-C-SH	Lincoln, AR, USA	weedy red rice	*Indica*	138	a	95	sensitive
1200	Pelotas, RS-Brazil	weedy red rice	*Indica*	186	a	95	sensitive
1602	Pelotas, RS-Brazil	weedy red rice	*Indica*	76	b	31	tolerant
PRA08-D-BH	Prairie, AR, USA	weedy red rice	*Japonica*	191	a	95	sensitive
3011(Diamante)	Chile	rice cultivar	*Japonica*	41	c	82	sensitive
Hayayuki	Japan	rice cultivar	*Japonica*	105	b	30	tolerant
Spring	USA	rice cultivar	*Japonica*	98	b	37	tolerant

[a] Means followed by the same letter differ by Scoot-Knott test at 5% level, using square root data transformation in the form: y + 0.5-sqrt (y + 0.5). Shoot length was the average of five seedlings measured per replication per genotype, at 14d of incubation.

[b] Seeds were germinated in the dark at 18/13°C, 16/8h cycle temperature fluctuation. Shoot length reduction was calculated relative to control seedlings grown at 25°C; reduction = [(control—cold stress)/control]*100.

[c] Accessions showing <50% reduction in shoot length under cold stress were deemed cold-tolerant. Means were compared using t-test at $P \leq 0.05$

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[43], has been used as cold-tolerant reference in other experiments and so was included here together with the more recently commercialized cold-tolerant, tropical *japonica* 'Spring' from Arkansas and another putative cold-tolerant *japonica* cultivar from Chile '3011'. The 10 weedy rice accessions tested had shoot lengths ranging from 55 to 191mm, with PRA08-D-BH as the fastest growing ecotype under normal temperature. When subjected to cold stress, at the normal 1.5-cm seeding depth, shoot length was reduced 30% to 95% relative to the non-stressed controls (Table 3). The cultivars and weedy rice accessions showing <50% reduction in shoot length under cold stress were classified as cold-tolerant. With this criterion, only two *indica* weedy rice (GRE08-D-SH and 1602) and one *japonica* cultivar (Spring) were deemed tolerant to cold stress at the seedling stage. The cold-tolerant 3011 (from Chile) turned out to be sensitive to the level of cold stress implemented (18°C/13°C day/night) at this growth stage. It should be noted that rice can be tolerant to cold stress at later growth stages, but sensitive at the seedling stage. It should also be noted that the tolerant genotypes were not totally immune to cold, showing 29% to 37% shoot length reduction relative to the non-stressed plants, with Spring (reference cold-tolerant genotype) showing 37% shoot growth reduction. The most cold-sensitive ones, such as PRA08-D-BH, had 95% reduction in shoot length. This indicates that the cold tolerance trait is not common among *indica* genotypes, which makes the discovery of tolerant *indica* genotypes of greater importance for the development of cold-tolerant *indica* cultivars.

Tolerance to Seeding Depth

Planting depth is a key factor that influences seedling vigor, and eventually crop yield [44]. Rapid and uniform emergence of seedlings provides crops with temporal and spatial

advantages to compete with weeds, and is therefore necessary for maximizing yield [45]. It has been shown that sowing depth influences the ability of soybean to emerge from the soil and establish a uniform stand [46]. Weedy rice has the versatility to emerge from greater depth, over a prolonged period than the crop, and thus becomes a big problem. Under normal temperature, the weedy rice accessions and cultivars generally were not affected by seeding at 5-cm depth (S2 Fig). However, 60% (6 of 10) of the accessions tested showed at least 50% reduction in shoot length when planted at 10-cm depth. In general, shoot growth declined further when seeds were planted at 15-cm depth. Altogether, 7 of 10 genotypes tested were classified tolerant to deep sowing; of these, the most depth-tolerant were the weedy rice accessions LEE08-C, CRA08-D, and LIN08-B3. However, when planted deep (10cm) and subjected to cold stress (18°C/13°C, 16/8-h temperature fluctuation cycle, dark-incubated), all accessions lost any tolerance to deep sowing (S3 Fig).

Genetic Mechanisms for Cold Stress Acclimation in Cultivated and Weedy rice

Low temperature causes cell dehydration, resulting in damage to the plasma membrane, which is the most detrimental effect of chilling stress [47]. Low temperature induces secondary stress messengers like abscisic acid and ROS that in turn induce calcium signatures, effecting cold stress signaling in plants. The existence of some cold-tolerant ecotypes here and elsewhere [48], suggests some diversity in stress adaptation mechanism among weedy genotypes, which might be useful to incorporate in rice cultivars.

The exposure of plants to harsh environmental conditions triggers a wide variety of genes that are responsible for modulation of stress-induced signal transduction cascade. The stress-induced genes are classified into two groups [49–51]: i) those that encode proteins that endow stress tolerance including antifreeze proteins, detoxification enzymes, heat-shock proteins or osmotins, among others; and ii) transcription factors that regulate the expression of other genes and induce tolerance. A genetic-based approach uses natural variation among rice cultivars and weedy rice, and the analysis of differentially expressed genes in response to stress. The greatest challenge in post-genomic research is to determine the function of candidate genes that are identified by analysis of their expression profiles upon exposure to cold stress.

When temperature approaches freezing, osmotically active water typically moves out of the cells, and the osmotic potential of the remaining unfrozen intracellular and intercellular fluid increases. Severe chilling stress causes multiple forms of membrane damage as a consequence of freeze-induced cellular dehydration including expansion-induced lysis and cross talk between stresses [47]. To gain insight into the stress tolerance mechanism in *indica* and *japonica* rice genotypes we studied the expression of key stress-regulated genes *RAB16A* (Lea group of proteins), H^+*PYROPHOSPHATASE* (*OVP1*), *GERMIN*-like proteins (*GLP*s), *GLUTAMATE DEHYDROGENASE* (*GDH*) and *ASCORBATE PEROXIDASE* (*APX1*) (Figs 1B and 2B). *GLP*s have been reported previously to be activated by abiotic stresses like salinity and high temperature [52–54]. *GLP*s are stress-responsive genes that are known to encode proteins with SOD activity, thereby helping in scavenging superoxide radicals [53–54]. In barley, six subfamilies of *GLP*s have been well characterized and are found to encode proteins with oxalate oxidase or superoxide dismutase activity [53–54]. Moreover, GLPs have also been reported to build a defensive barrier during barley emergence [55]. Among the plant materials studied, the tolerant *japonica* cultivar Spring showed induction of *GLP*s at 6h, induced maximally at 24h and reduced completely at 96h, while the sensitive *japonica* weedy genotype from the USA [PRA08-D-BH] showed induction only at 24h and maintained its level at 96h (Fig 1B). The cold-sensitive and tolerant weedy *indica* CHI08-C-SH and GRE08-D-SH showed induction at
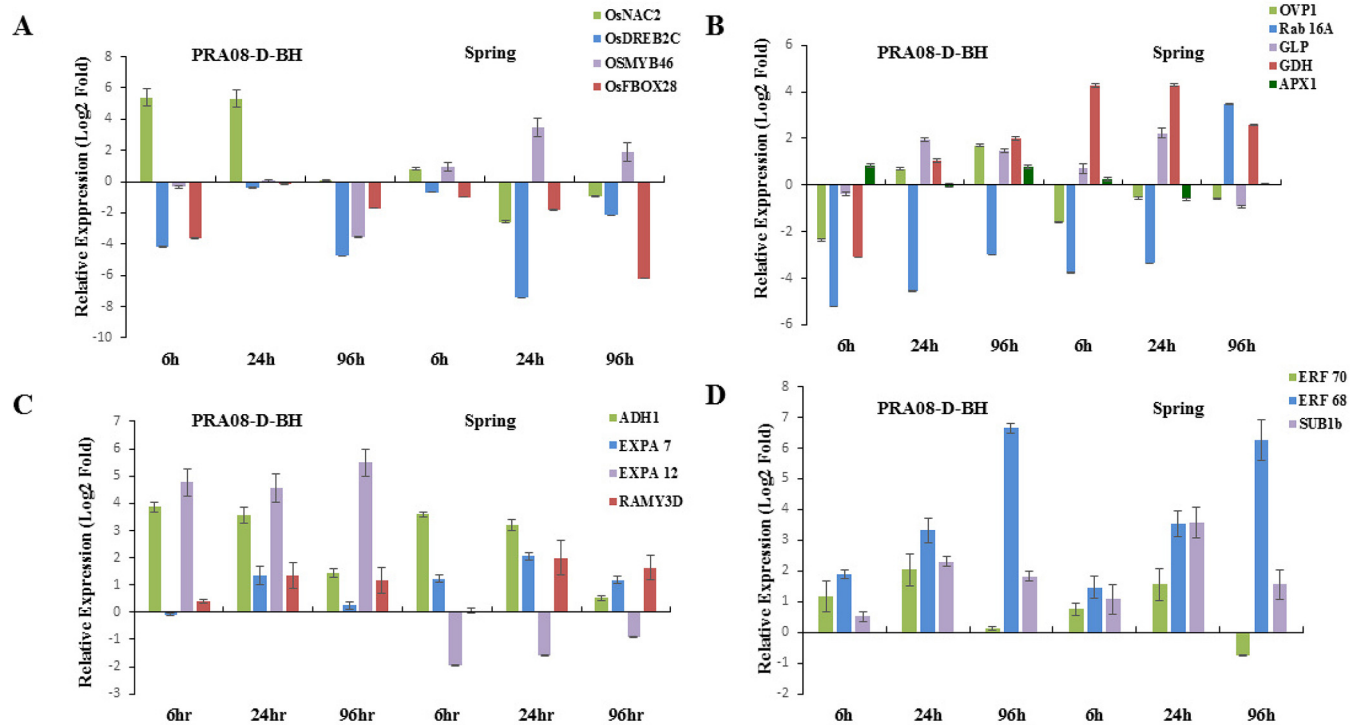
Fig 1. Relative gene expression under cold stress (10°C) in 10-d-old seedlings of sensitive (PRA08-D-BH) and tolerant (Spring) *japonica* rice. The data are means of three replicates ± SE. A) Response of transcription factors: *OsNAC2*, *OsDREB2C*, *OsMYB46* and *OsF-BOX28*. B) Transcript accumulation of defense- and metabolism-related genes: *OVP1*, *Rab16A*, *APX1*, *GLP*, *GDH*. C) Genes related to cell expansion and carbohydrate metabolism: *ADH1*, *EXPA7*, *EXPA12 and RAMY3D*. D) Pattern of expression of ERFs: *ERF70*, *ERF68* and *SUB1B*.

6h and maximizing at 24h and 96h, respectively. Another tolerant weedy *indica* rice (1602) showed induction only at 24h ([Fig 2B]).

The immediate response of plants to stress is accumulation of ROS, which creates ionic imbalance within the cell. To characterize the involvement of ROS scavenging enzymes we studied the expression of *APX1*. The expression of this cytosolic enzyme is governed by H_2O_2 accumulation, ABA level, and leaf water status. *APX1* is highly upregulated in roots and shoots in response to salinity, osmotic, and heat stress [56]. Ascorbate peroxidases are involved in stress tolerance, being central components of the hydrogen peroxide scavenging networks in plants. *Arabidopsis* plants deficient in APX1 are highly susceptible to oxidative stress [57–58], suggesting that APX1 is linked to ROS signaling or scavenging. Our research showed that the cold-sensitive *japonica* (PRA08-D-BH) has early induction of *APX1* ([Fig 1B]) which declined at 24h and increased again at 96h. On the other hand, the tolerant *japonica* (Spring) had a barely perceptible induction of *APX1* at 6h of cold stress, and disappeared at 24h, suggesting the presence of a strong defense mechanism. The sensitive *indica* (CHI08-C-SH) showed the same expression pattern of *APX1* under cold stress as the sensitive *japonica* PRA08-D-BH ([Fig 2B]). The tolerant *indica* genotypes from Brazil and the USA showed different expression patterns of *APX1*. The tolerant *indica* weedy rice from USA, GRE08-D-SH, showed high induction of *APX1* at 6h, with no detectable expression at 24h and induced maximally again at 96h. On the contrary, the tolerant *indica* weedy rice from Brazil (1602) had no detectable expression. The maximal induction of *APX1* at 96h suggests that cold stress also induces secondary stress responses such as anoxia and cold-induced dehydration due to ROS generation. The role of APX1 in cold stress tolerance differed between *japonica* and *indica* and between *indica*
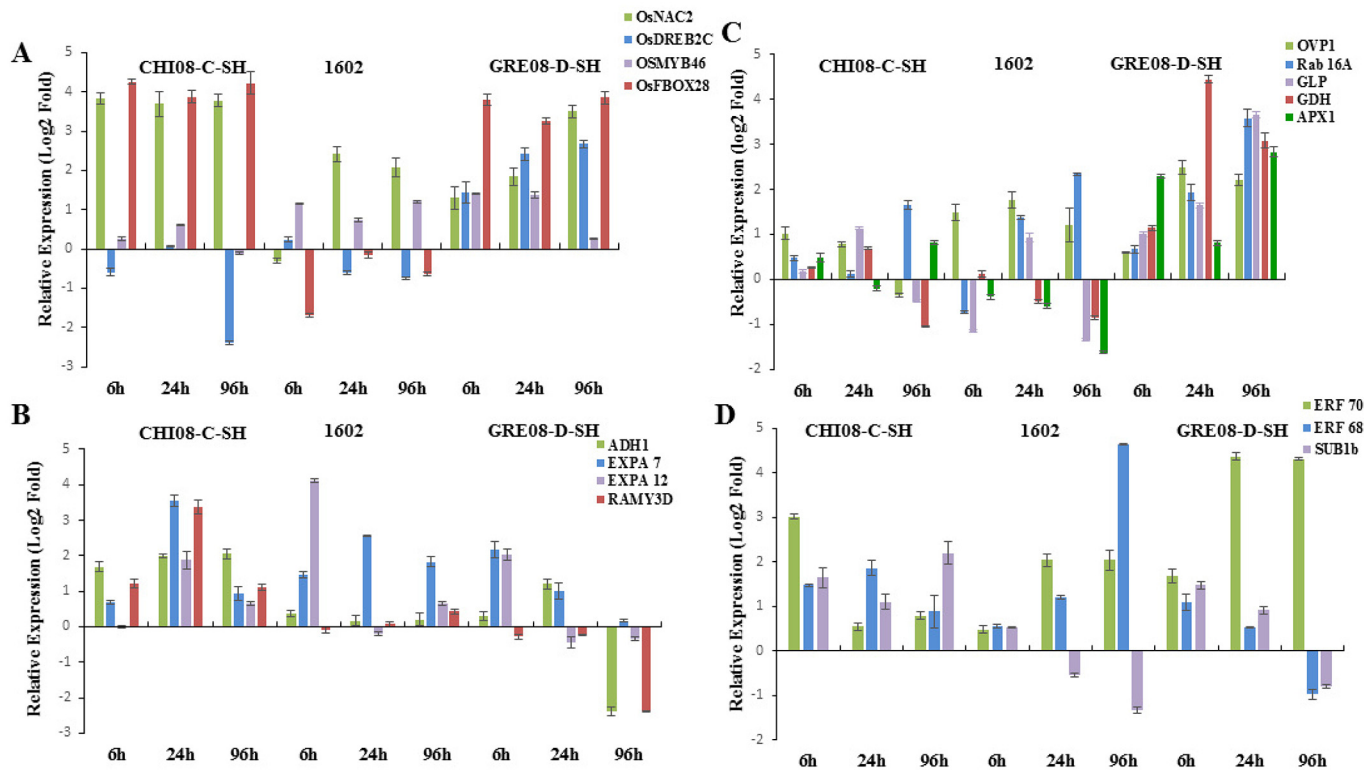
Fig 2. Relative expression of genes under cold stress (10°C) in 10-d-old seedlings of sensitive (CHI08-C-SH) and tolerant (1602 and GRE08-D-SH) *indica* **rice.** The data are means of three replicates ± SE. A. Response of transcription factors: *OsNAC2*, *OsDREB2C*, *OsMYB46* and *OsF-BOX28*. B. Transcript accumulation of defense- and metabolism-related genes: *OVP1*, *RAB16A*, *APX1*, *GLP*, *GDH*. C. Genes related to cell expansion and carbohydrate metabolism: *ADH1*, *EXPA7*, *EXPA12 and RAMY3D*. D. Expression patterns of ERFs: *ERF70*, *ERF68* and *SUB1B*.

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genotypes from Brazil and the USA suggesting the presence of different ROS scavenging machinery involving APX1 and other antioxidant enzymes that reflects localized adaptations.

The role of phytohormone ABA-responsive gene expression in response to cold stress was studied through the expression of the *RAB16A* gene, a well-characterized member of the *LEA* gene family [59]. *RAB16A* is induced by abiotic stresses such as salinity and ABA treatment [60–61], which cause dehydration, and plays a crucial role in stabilizing the cell membrane and preventing denaturation of proteins; thus, endowing stress tolerance [62]. RAB16A also binds to iron and acts as antioxidant under abiotic stress [61]. The tolerant *indica* genotype; GRE08-D-SH showed early induction of *RAB16A* (6h) with maximum transcript accumulation at 96h of cold incubation while another tolerant genotype, 1602, showed induction at 24h and increased maximally at 96h (Fig 2B). The sensitive *indica* genotype had lower level of *RAB16A* transcript than the tolerant ones, which was downregulated at 96h. The expression of *RAB16A* was not induced in the tolerant *japonica* genotype until 96h of incubation and had no detectable expression, at any time, in cold-sensitive *japonica* (Fig 1B). These results suggest the activity of an ABA-dependent tolerance pathway in the *indica* genotypes, which might not be functional in the *japonica* ssp.

Glutamate dehydrogenase (GDH) (EC 1.4.1.2) is a key enzyme involved in amino acid biosynthesis catalyzing the reductive amination of 2-oxoglutarate (2OG) and the oxidative deamination of Glu *in vitro* under nitrogen-limiting conditions and is upregulated under abiotic stresses [63–64]. ROS plays a critical role in the signaling pathway for *GDH* expression and protease activation that also contributes to intracellular hyper-ammonia. GDH also has a

crucial role in germinating seeds and senescing leaves, converting amino acids into transport compounds with a low C/N ratio [65]. The expression of *GDH* is induced upon exposure to salinity stress; it is likely involved in ammonia detoxification and protease inhibition by activating protein synthesis [66]. The *GDH* gene was strongly induced at 6h of cold stress in the cold-tolerant *japonica* cultivar (Spring) with a slight decrease at 96h, while the sensitive weedy *japonica* (PRA08-D-BH) showed induction only at 24h. The sensitive weedy *indica* (CHI08-C-SH) showed lower transcript accumulation of *GDH* at 6 and 24h compared with the tolerant weedy *indica* (GRE08-D-SH), which maintained higher transcript accumulation and reaching maximum at 24h. However, the cold-tolerant weedy *indica* rice (1602) from Brazil had barely detectable transcript accumulation at 6h, which then subsided at 24h. Our observation supports a stress-protective role of GDH along with its physiological role and the aminating activity of the anionic isozymes of GDH. Our data also indicate that the involvement of GDH in cold stress adaptation differs between genotypes and between regions.

Plants have two distinct proton pump systems in the vacuolar membrane H$^+$-ATPase (V-ATPase, EC 3.6.1.3) and H$^+$- translocating inorganic pyrophosphatase (V-PPase EC 3.6.1.1) [67]. The proton pump creates an electrochemical potential gradient across the membrane, thereby helping in translocation of solutes across the membrane. Earlier it has been shown that overexpression of H$^+$-*TRANSLOCATING INORGANIC PYROPHOSPHATASE* (*OVP1*) in rice imparts tolerance to cold stress [67–69]. Here, we observed that the induction of *OVP1* expression under cold stress occurred only in *indica* weedy rice, with stronger expression in tolerant genotypes (Fig 2B). The maximum expression was observed at 24h with slight reduction at 96h in GRE08-D-SH; in 1602, the maximum induction was observed at 24h and was completely downregulated at 96h. The sensitive weedy *japonica* (PRA08-D-BH) showed late induction at 24h with no detectable transcript accumulation in the tolerant *japonica* cultivar (Spring). These observations suggest a role for *OVP1* in maintaining the integrity of the proton pump machinery and oxidative phosphorylation. Our observations suggest the presence of a different cold tolerance mechanism in *japonica* than in *indica* rice.

Role of Transcription Factors in Stress Tolerance

Cold stress-activated ROS accumulation induces several transcription factors (TFs) that operate via the ABA-dependent and ABA-independent pathways, eliciting cold tolerance. Cold stress induces the accumulation of CBFs (C-repeat binding factors, also known as DEHYDRATION-RESPONSIVE ELEMENT-BINDING protein or DREBs), which bind to the *cis*-elements in the promoters of cold responsive genes and activate their expression [68,70], consequently inducing the expression of genes involved in ROS detoxification, membrane transport, osmolyte biosynthesis, phosphoinositide metabolism [49,71–72]. The DREB2C protein binds to the C-repeat/dehydration response element (CRE) in vitro and possesses transcriptional activity. It has also been shown that *Arabidopsis* plants overexpressing *DREB2C* are cold- or thermo- tolerant [73]. The expression of *DREB2C* gene in tolerant *indica* US weedy rice was induced early and at high levels in a time-dependent manner, but no detectable expression was observed in the other tolerant *indica* rice (1602) nor in the sensitive weedy *indica* wherein a low transcript accumulation was observed only at 6h and then disappeared (Fig 2A). *DREB2C* also was not detected in cold-stressed *japonica* genotypes (Fig 1A). Thus, our data support previous research, which showed that *DREB2C* overexpression endowed tolerance to freezing and heat stress [74] with only transient induction by other abiotic stresses [75].

Another important class of TFs that play a role in cold-induced gene expression are members of the MYB family. Previous reports have shown that *hos10-1* (R2R3-type MYB) mutant *Arabidopsis* plants are susceptible to cold stress, even though they have induced expression of

*CBF*s. Moreover, abiotic stresses like cold, salinity and drought induce *OsMYB3R-2* (an R1R2R3 MYB) that positively regulates stress tolerance via a CBF-independent pathway [76]. A characteristic feature of cold stress tolerance is the maintenance of cell wall integrity, as cells lose water owing to freezing-induced dehydration. Previously it has been shown that plants reduce the expression of cell wall-modifying enzymes like XTHs (Xyloglucan Endotransgluco-sylase/Hydrolases) in *Vigna radiata*, deduced from reduced hypocotyl elongation [77]. *OsMYB46* is a unique TF directly regulating the biosynthetic genes for major components of the secondary wall, as well as TFs in the biosynthesis pathway [78]. Both the tolerant weedy *indica* genotypes showed early and high *OsMYB46* transcript accumulation up to 24h with a slight reduction observed at 96h in GRE08-D-SH in comparison to the sensitive one (Fig 2A). The tolerant *japonica* cultivar showed early induction of *OsMYB46* and sustained expression for 96h of cold stress, while no induction was observed in the sensitive weedy *japonica* geno-type (Fig 1A). The elevated level of *OsMYB46* upon stress imposition suggests that the tolerant genotypes attempt to alleviate the stress and minimize stress impact by fortifying the cell wall.

NAC transcription factors are an important group of transcriptional regulators in plants whose members play diverse roles in stress tolerance [79]. In previous reports *SNAC1*, *SNAC2*, *OsNAC45* genes were shown to be induced by salinity, drought, and cold stress, and overex-pression of these conferred stress tolerance [80–82]. In this study, although the tolerant *indica* weedy rice (GRE08-D-SH) showed a progressive increase in *OsNAC2* transcript accumulation and reaching maximum at 96h (at 24h for 1602), the transcripts generally were at lower levels than the sensitive *indica* weedy rice (CHI08-C-SH) (Fig 2A). The sensitive weedy *japonica* (PRA08-D-BH) also maintained a high transcript level up to 24h of cold treatment. In contrast, the tolerant *japonica* cultivar (Spring) showed low accumulation of *OsNAC2* transcripts at 6h that disappeared thereafter (Fig 1A).

Overexpression of F-box proteins confers cold tolerance to plants. The tolerant and sensitive USA *indica* weedy rice maintained a high *OsFBOX28* transcript level over the stress period (Fig 2A), while no detectable transcript accumulation was observed in the *japonica* subspecies or the tolerant *indica* weedy rice from Brazil (Fig 1A). This suggests that genes like *OsNAC2* and *OsFBOX28* are responsive to cold stress in some genotypes, but such response is not always observed in a cold-tolerant phenotype.

Overall, we observed that tolerance to cold stress in *indica* rice occurs via the ABA-depen-dent as well as the ABA-independent pathway (CBF-mediated) while cold tolerance in *japonica* occurs primarily via the ABA-dependent pathway. The cold stress tolerance mechanisms are different between the weedy rice ecotypes from Brazil and USA origins and between *japonica* and *indica* genotypes.

Cross-talk Between Stresses

Cold stress induces accumulation of reactive oxygen species represented by peroxide, peroxyl radicals, and others. Due to the lack of efficient ROS scavenging machinery in the cell, plants continue accumulating the negatively charged radicals that consequently leads to oxygen defi-ciency, giving rise to an anoxic condition. Expansins are proteins that are responsible for shoot elongation under anoxic conditions [83–84]. Previously it has been shown that the rice *EXPANSIN* genes *EXPA7* and *EXPA12* are transiently upregulated under anoxia, presumably facilitating shoot elongation [85]. The *japonica* ssp. genotypes (PRA08-D-BH, Spring) showed high transcript accumulation of *EXPA7*, which declined with time (Fig 1C). Transcript accu-mulation of *EXPA12* was higher earlier in the sensitive than the tolerant *japonica* and declined with time. The *indica* ssp. genotypes (CHI08-C-SH, sensitive; 1602, tolerant) showed high transcript accumulation of *EXPA7* at 24h and declining at 96h of cold stress, whereas

GRE08-D-SH (tolerant) showed high transcript accumulation at 6h then declining thereafter (Fig 2C). Thus, *EXPA12* was highly induced early and downregulated at 24h or later in tolerant weedy *indica* rice, while upregulation was delayed (at 24h) in the sensitive weedy *indica* rice. These data show that transient upregulation of *EXPANSINS* also occur in cold stress (as in anoxia) and that the cold-tolerant genotypes have a different mechanism of controlling the expression of cell wall-modifying proteins than the sensitive ones.

Anoxia induces the level of several *AP2/ERF* genes in *Arabidopsis* [85]. Anoxic condition induced by submergence also triggers the expression of *SUB1A*, *SUB1B*, and *SUB1C*, respectively belonging to the B-2 subgroup of the ERF proteins [86]. To elucidate the role of ERFs in cold stress-induced anoxia we analyzed the expression of *ERF70*, *ERF68* and *SUB1B*. *ERF70* was generally highly induced in all genotypes while *ERF68* showed the opposite pattern of expression. (Figs 1D and 2D). In the sensitive *indica* genotype *ERF70* and *ERF68* were induced maximally at 6h and 24h, respectively. In the tolerant genotype (1602) both TFs were induced with time while the tolerant GRE08-D-SH, showed maximum induction of *ERF70* and *ERF68* at 24h and 6h, respectively. *SUB1B* was induced early in the sensitive and tolerant *japonica* ecotypes then declined with time (Fig 1D). The same pattern was observed in both tolerant *indica* weedy rice, but induction was sustained with time in the sensitive *indica* (Fig 2D). This group of genes does not seem to contribute to cold tolerance in *japonica* cultivar, but may contribute to cold tolerance in *indica* weedy rice.
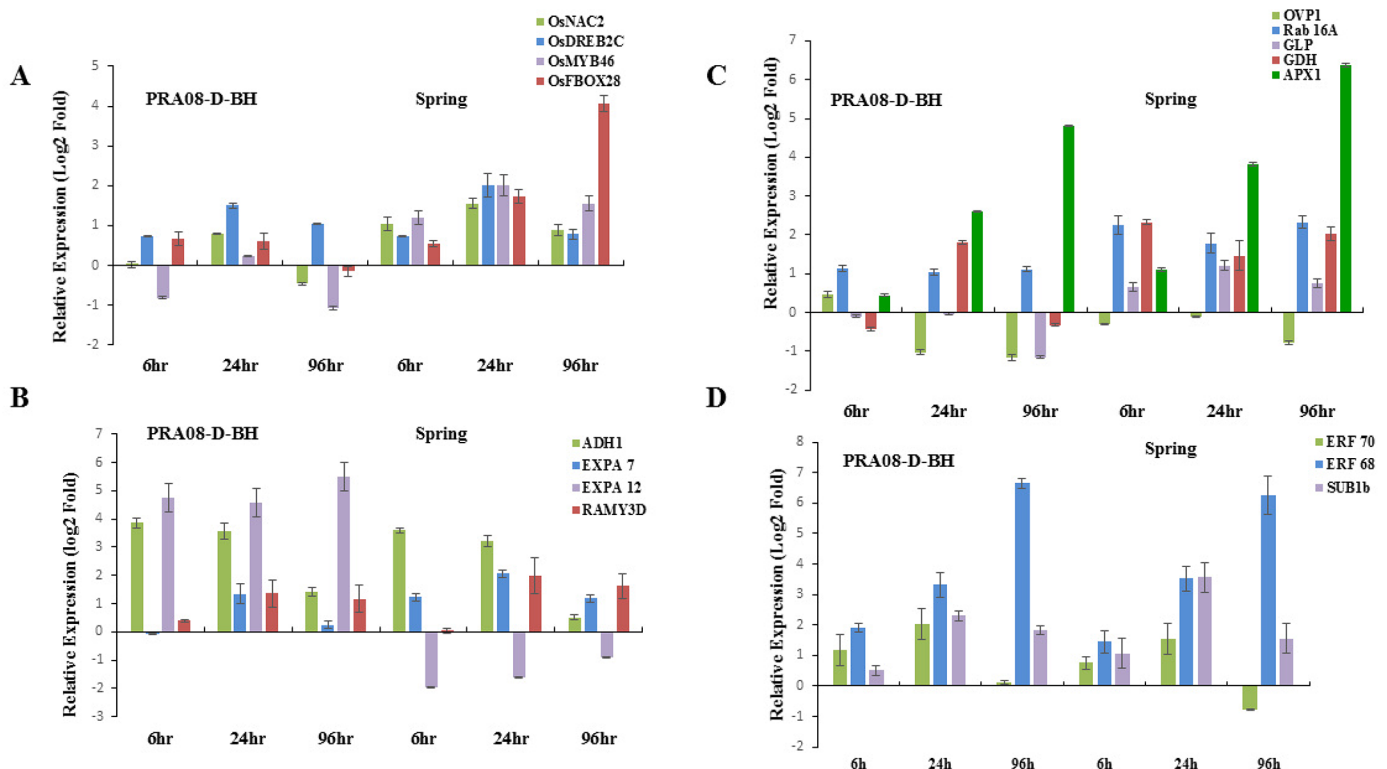

Fig 3. Relative expression of genes in response to combined cold stress (10°C) and seeding depth stress (10cm) in 10-d-old seedlings of sensitive (PRA08-D-BH) and tolerant (Spring) *japonica* rice. The data are means of three replicates ± SE. A. Response of transcription factors: *OsNAC2*, *OsDREB2C*, *OsMYB46* and *OsF-BOX28*. B. Transcript accumulation of defense- and metabolism-related genes: *OVP1*, *RAB16A*, *APX1*, *GLP*, *GDH*. C. Genes related to cell expansion and carbohydrate metabolism: *ADH1*, *EXPA7*, *EXPA12 and RAMY3D*. D. Expression patterns of ERFs: *ERF70*, *ERF68* and *SUB1B*.

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Role of Alpha Amylase and Alcohol Dehydrogenase

Alpha amylase (RAMY3D) is a key enzyme involved in the hydrolysis of starch into metabolizable sugars, providing energy for shoot elongation [87] and is known to be a gene induced by starvation [85,88]. *RAMY3D* has been reported to be induced by cold stress as well as anoxia in hyacinth and rice shoots [89–90]. Rapid degradation of starch in the anoxic rice shoots suggests a significant role for this gene in starch metabolism [89]. *RAMY3D* was induced early at 6h of cold stress in both sensitive and tolerant *japonica* and approached basal level at 96h (Fig 1C). *RAMY3D* was induced at all time-points in the sensitive *indica*, but generally was down-regulated in tolerant *indica* genotypes (Fig 2C). This gene does not contribute to cold tolerance in the *japonica* cultivar, but suppression of amylase activity may contribute to cold tolerance in *indica* weedy rice by conserving energy. ADH is a key enzyme triggered under low oxygen conditions, involved in redirecting the switch from aerobic to anaerobic fermentation [91]. Previous reports have shown that *ADH* is highly induced by cold stress and anoxia in maize and rice seedlings [89, 92]. The increased expression of *ADH* probably helps in the regeneration of NAD+ by reducing acetaldehyde to ethanol thereby providing an alternative pathway to glycolysis and in turn preventing cellular acidosis. We observed that *ADH* expression follows a similar pattern as that of *RAMY3D*. This suggests that cold stress induces an anoxic condition where plants respond by increasing sugar metabolism and switching to anaerobic respiration.
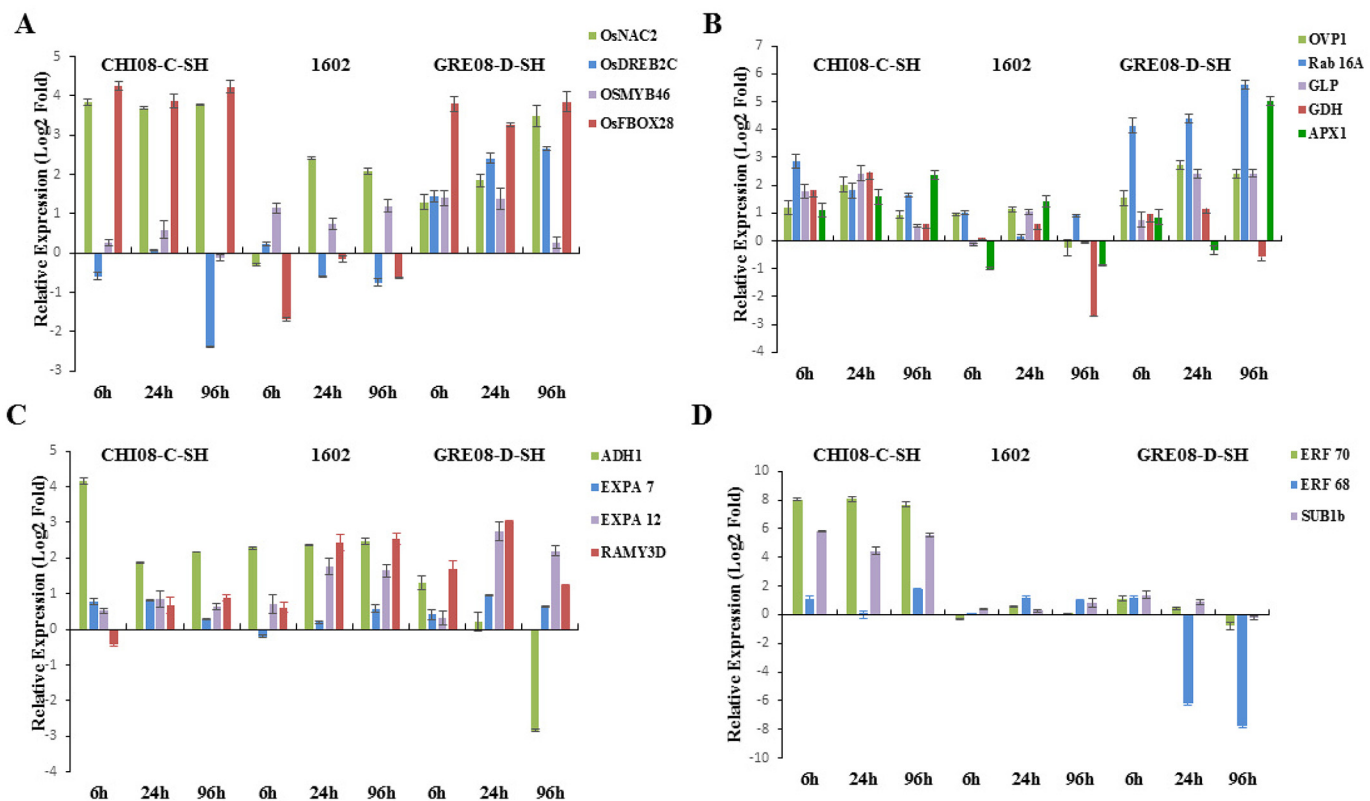

Fig 4. Relative expression of genes in response to combined cold stress (10°C) and depth stress (10cm) of sensitive (CHI08-C-SH) and tolerant (GRE08-D-SH and 1602) *indica* rice A. Response of transcription factors: *OsNAC2*, *OsDREB2C*, *OsMYB46* and *OsF-BOX28*. B. Response of defense- and metabolism-related genes: *OVP1*, *RAB16A*, *APX1*, *GLP*, *GDH*. C. Genes related to cell expansion and carbohydrate metabolism: *ADH1*, *EXPA7*, *EXPA12* and *RAMY3D*. D. Expression patterns of ERFs: *ERF70*, *ERF68* and *SUB1B*. The data are means of three replicates ± SE.

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Combined Effects of Cold and Deep Sowing Stress on Stress-Responsive Gene Expression

Rice genotypes are not known to emerge well from greater soil depth, but weedy rice ecotypes primarily belonging to the *indica* ssp. can [33–34]. Planting, or being covered, at a greater depth in the soil allows seeds to avoid temperature extremes, but there is an optimum seedling emergence depth beyond which the seedling will incur loss of vigor or perish. We studied the effect of combined stress of sowing depth (10cm) and cold, on the expression of the stress responsive genes in the rice genotypes. The cold-tolerant *japonica* cultivar Spring showed an early and generally higher transcript accumulation of all TFs (*OsDREB2C*, Os*MYB46*, *OsNAC2* and *OsF-BOX28*) than the sensitive weedy *japonica* (Fig 3A). Similarly, all TFs were highly induced in the tolerant USA weedy *indica* GRE08-D-SH whereas only *OsNAC2* and *OsF-BOX28* were highly upregulated in sensitive *indica* CHI08-C-SH (Fig 4A). Only *OsMYB46* appeared to play a role in cold + sowing depth response of the tolerant Brazilian weedy *indica* 1602.

The severity of stress was also marked by increased transcript accumulation of ROS scavenging genes *APX1* and *GLPS*. In addition, both the *indica* and *japonica* weedy rice showed enhanced transcript levels of *RAB16A* and *GDH* in contrast to $H^+PYROPHOSPHATASE$ (*OVP1*), which was not detectable in the *japonica* ssp. (Figs 3B and 4B). These results suggest that the stress-tolerant *indica* and *japonica* genotypes activated a tolerance mechanism operating via the CBF-dependent and -independent pathways. Moreover, this indicates that the
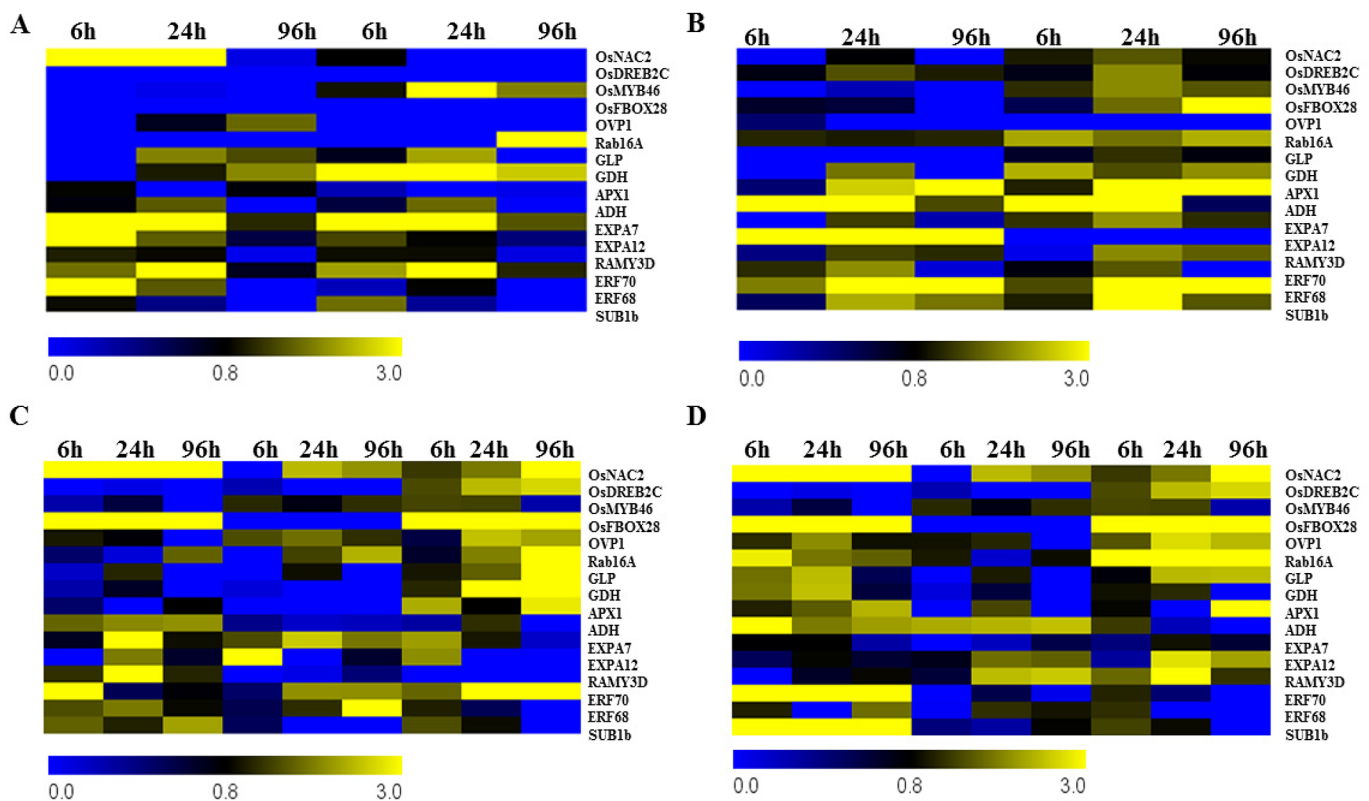

Fig 5. Heat Map showing the differential expression of genes in response to cold stress and combined cold + seeding depth stress in rice. Expression profile of genes in response to cold stress in: A. susceptible (PRA08-D-BH) and tolerant (Spring) *japonica* rice and B. sensitive (CHI08-C-SH) and tolerant (1602 and GRE08-D-SH) *indica* rice. Expression profile of genes in response to combinatorial stress in: C. sensitive (PRA08-D-BH) and tolerant (Spring) *japonica* rice and D. sensitive (CHI08-C-SH) and tolerant (1602 and GRE08-D-SH) *indica* rice.
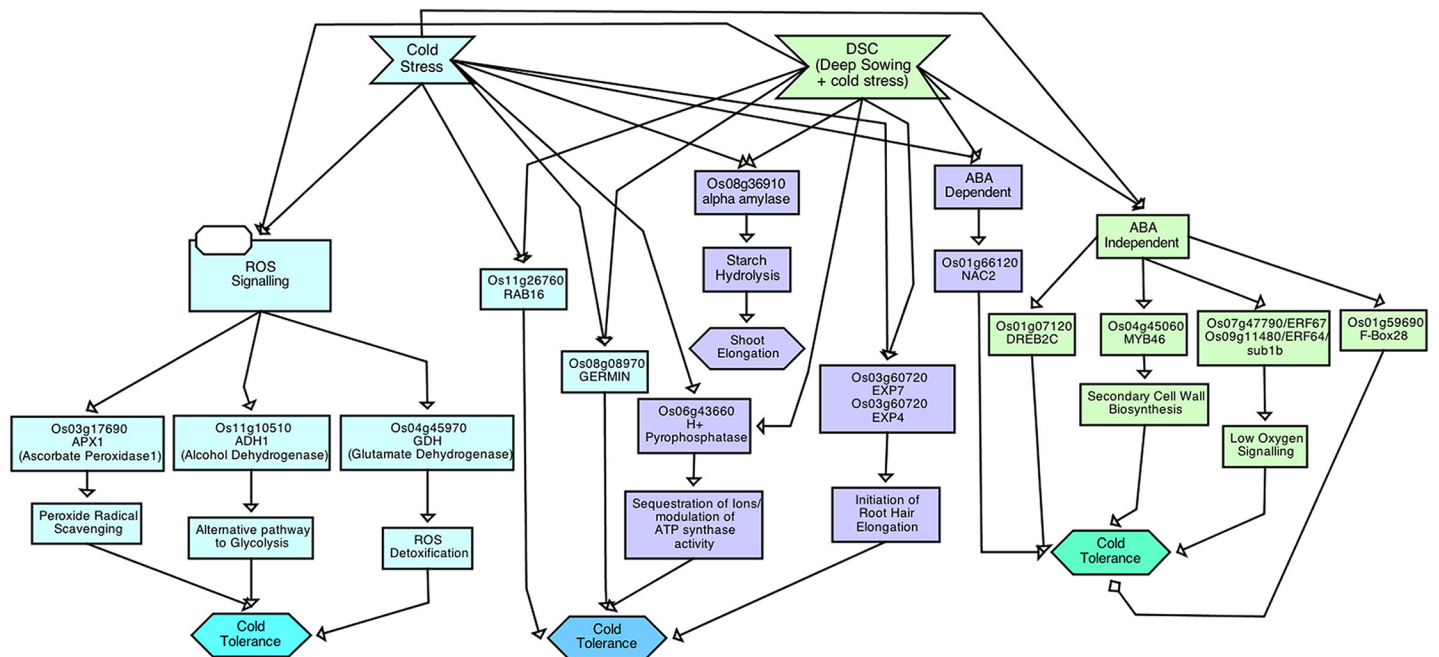
doi:10.1371/journal.pone.0132100.g005

Fig 6. Overview of the regulatory pathway of cold stress and combination of cold and sowing depth stress response in weedy rice (*Oryza sativa*
L.). The data represent the core figure drawn according to Systems Biology Graphical Notation (SBGN) Activity Flow Specifications (Level 1 Version 1) using
Beacon Software76. The relationships, or arcs, express the nature of influence (positive, negative or unknown) between the glyphs and summarize this
paper's findings along with support from literature described in the text.

combined cold and depth stress triggered the simultaneous activation of a network of multiple
physiological stresses, which eventually overcame the cold tolerance mechanism of these
genotypes.

The tolerant *japonica* cultivar Spring showed higher accumulation of *ADH* and *alpha amy-
lase* at 6h and 24h of stress treatment; the tolerant *indica* ssp., showed a different expression
pattern (Figs 3C and 4C). *EXPA7* was generally induced equally in both ssp. while *EXPA12* was
detectable in all *indica* genotypes and in the sensitive *japonica* genotype. *ERF70* and *ERF68*
were highly induced in the *japonica* ssp. but only *ERF70* was detectable in the *indica* ssp. (Figs
3D and 4D). *SUB1B* was induced early at 6h in both ssp., peaked at 24h, and then declined at
96h. This suggests that the tolerant *japonica* genotypes are more vulnerable to sowing depth
+ cold stress than *indica*, indicating the presence of a stronger, innate tolerance mechanism in
the *indica* genotypes. However, upon exposure to cold + sowing depth stress both the *indica*
and *japonica* accessions showed an elevated level of all these genes suggesting that the com-
bined stress elicited a complex network of multiple stress-response pathways. These defense/
adaptive processes, however, were not sufficient to overcome the impact of the combined cold
and depth stress. The overall stress responsive gene expression and pathway in weedy rice is
summarized in Figs 5A–5D and 6.

Conclusion

This research showed that there are cold-tolerant and depth-tolerant *indica* weedy rice that
could be a more suitable genetic resource for the development of weed-competitive *indica* culti-
vars adapted to sub-tropical and temperate environments. No rice genotype tested (weedy or
cultivated) can withstand cold stress if placed deep in the soil profile. The cold stress responsive
gene expression of different rice and weedy rice genotypes has been summarized and

represented as heat map ([Fig 5A–5D](#)). Our research also revealed that cold stress signaling in *indica* spp. is more complex than that of *japonica* as it operates via both the CBF-dependent and CBF-independent pathways implicated through increased transcript accumulation of *OsNAC2*, *OsMYB46* and *OsF-BOX28*. The *indica weedy* rice appears to have a different cold tolerance mechanism than *japonica* rice. Furthermore, cold tolerance in the USA *indica* weedy rice is by a different mechanism from the Brazilian weedy rice. These warrant further investigation. Thus, our research provided a background to identify genes responsible for cold stress tolerance in weeds. Cold-tolerant weedy rice can be tapped for rice improvement, for example by marker-assisted breeding methods. More cold-tolerant weedy rice can be discovered with a wide-scale screening of weedy rice germplasm, using molecular markers derived from this study, for developing weed-competitive rice cultivars.

Supporting Information

S1 Table. Genes used in the gene expression analysis by qRT-PCR from *indica* and *japonica* rice (*Oryza sativa* L.) genotypes exposed to cold and depth stress.
(DOCX)

S1 Fig. Phenol test to classify weedy rice accessions and cultivated rice as *indica* or *japonica* subspecies. The *indica* genotype has positive reaction to phenol, indicated by color change of the grain from light to dark [31]. The grain on the left-hand corner is the nontreated control. BH = black hull; SH = straw hull. Rice cultivars are 3011, Oro, Hayayuki, and Spring.
(TIFF)

S2 Fig. Phenotypic change in weedy and cultivated rice seedlings in response to sowing depth stress. Seeds were germinated in the dark at 25°C for 14d. Response to depth stress was indicated by reduction in shoot growth relative to those planted at 1.5-cm depth.
(TIF)

S3 Fig. Phenotypic change in weedy and cultivated rice seedlings in response to cold + sowing depth stress. Seeds were germinated in the dark at 18°C/13°C, 16/8h temperature cycle for 14d. Response to cold + depth stress was indicated by reduction in shoot growth relative to those planted at 1.5-cm depth.
(TIFF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: NRB AP PDZ. Performed the experiments: CBB SB TMT. Analyzed the data: SB CBB. Contributed reagents/materials/analysis tools: NRB AP PDZ. Wrote the paper: SB NRB AP CBB. Collected weedy rice accessions from the southern USA: NRB TMT. Collected weedy rice accession from Brazil: PDZ. Obtained rice cultivars from the US GRIN: NRB. Maintained the AR weedy rice germplasm: TMT. Helped plan, implement, and collect data for the cold and depth stress phenotyping experiment: TMT.

References

1. Ramankutty N, Foley JA, Norman J, McSweeney K (2002) The global distribution of cultivable lands: current patterns and sensitivity to possible climate change. Glob Ecol Biogeogr 11: 377–392.

2. Sanghera GS, Wani SH, Wasim B, Singh N (2011) Engineering cold stress tolerance in crop plants. Current Genomics 12: 30–43. doi: 10.2174/138920211794520178 PMID: 21886453

3. Levitt J (1980) Responses of Plants to environmental stresses: Vol. 1, Acad. Press 496.

4. Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the *Arabidopsis* CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol 124: 1854–1865. PMID: 11115899

5. Shinozaki K, Yamaguchi-Shinozaki K (1996) Molecular responses to drought and cold stress. Curr Opin Biotechnol 7: 161–67. PMID: 8791336

6. Yamaguchi-Shinozaki Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57: 781–803. PMID: 16669782

7. Hannah MA, Heyer AG, Hincha DK (2005) A global survey of gene expression during cold acclimation in *Arabidopsis thaliana*. PloS Genet 1: e26. PMID: 16121258

8. Zhang J, Li J, Wang X, Chen J (2011) *OVP1*, a vacuolar H+-translocating inorganic pyrophosphatase (V-PPase), overexpression improved rice cold tolerance. Plant Physiol Biochem: 49: 33–38. doi: 10.1016/j.plaphy.2010.09.014 PMID: 20974539

9. Karki S, Rizal G, Quick WP (2013) Improvement of photosynthesis in rice (*Oryza sativa* L.) by inserting the C4 pathway. Rice 6:28. doi: 10.1186/1939-8433-6-28 PMID: 24280149

10. Ehleringer JR, Monson RK (1993) Evolutionary and ecological aspects of photosynthetic pathway variation. Annu Rev Ecol Syst 24: 411–439.

11. Cheng C, Yun KY, Ressom HW, Mohanty B, Bajic VB, Jia Y, et al.(2007) An early response regulatory cluster induced by low temperature and hydrogen peroxide in seedlings of chilling-tolerant japonica rice. BMC Genomics 8:175. PMID: 17577400

12. Gealy DR, Moldenhauer KAK, Jia MH (2013) Field performance of STG06L-35-061, a new genetic resource developed from crosses between weed-suppressive indica rice and commercial southern U.S. long-grains. Plant and Soil 370: 277–293.

13. Shivrain VK, Burgos NR, Gealy DR, Smith KL, Scott RC, Mauromoustakos, et al.,(2009) Red rice (*Oryza sativa* L.) emergence characteristics and influence on rice (O. sativa L.) yield at different planting dates. Weed Sci 57: 94–102.

14. Satake T (1976) Sterility-type cool injury in paddy rice plants. In International Rice Research Institute, Eds, Climate and Rice. Int'l Rice Research Institute, Los Banos, Philippines pp 281–298.

15. Board JE, Peterson ML, Ng E (1980) Floret sterility in rice in a cool environment. Ag.ron J 72: 483–48.

16. Jacobs BC, Pearson CJ (1999) Growth, development and yield of rice in response to cold temperature. J Agron and Crop Sci 18: 79–88.

17. Shimono H, Hasegawa T, Moriyama M, Fujimura S, Nagata T (2005) Modeling spikelet sterility induced by low temperature in rice. Agron J 97: 1524–1536.

18. Mackill DJ, Lei X (1997) Genetic variation for traits related to temperate adaptation of rice cultivars. Crop Sci 37: 1340–1346.

19. Mori M, Onishi K, Tokizono Y, Shinada H, Yushimura T, Numao Y, et al., (2011) Detection of a novel quantitative trait loci for cold tolerance at the booting stage derived from a tropical japonica rice variety Silewah. Breeding Sci 61: 61–68.

20. Kaw RN, Khush GS (1986) Combining ability for low temperature tolerance in rice. In Rice Genetics I. International Rice Research Institute, Manila, Philippines, pp 593–612.

21. Andaya VC, Mackill DJ (2003) Mapping of QTLs associated with cold tolerance during the vegetative stage in rice. J Exp Bot 54: 2579–2585. PMID: 12966040

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

23. Lee BH, Lee H, Xiong L, Zhu JK (2002) A mitochondrial complex I defect impairs cold-regulated nuclear gene expression. Plant Cell 14: 1235–1251. PMID: 12084824

24. Baruah AR, Ishigo ON, Adachi M, Oguma Y, Tokizono Y, Onishi K, et al.,(2009) Cold tolerance at the early growth stage in wild and cultivated rice. Euphytica 165: 459–470.

25. Su CF, Wang YC, Hsich TH, Lu CA, Tseng TH, Yu SM (2010) A novel MYBS3-dependent pathway confers cold tolerance in rice. Plant Physiol 153: 145–158. doi: 10.1104/pp.110.153015 PMID: 20130099

26. Shi J, Cao Y, Fan X, Li M, Wang Y, Ming F (2012) A rice microsomal delta-12 fatty acid desaturase can enhance resistance to cold stress in yeast and *Oryza sativa*. Mol Breed 29: 743–757.

27. Souza PR (1990) Alguns aspectos da influência do clima temperado sobre a cultura do arroz irrigado, no sul do Brasil. Lavoura Arrozeira 43: 9–11.

28. Xu LM, Zhou L, Zeng YM, Wang FM, Zhang HL, Shen SQ, et al., (2008) Identification and mapping of quantitative trait loci for cold tolerance at the booting stage in a japonica rice near-isogenic line. Plant Sci 174: 340–347.

29. Nahar K, Biswas JK, Shamsuzzaman AMM, Hasanuzzaman M, Barman HN (2009) Screening of indica rice (*Oryza sativa* L.) genotypes against low temperature stress. Bot Res Int'l 2: 295–303.

30. Bosetti F, Montebelli C, Novembre ADLC, Chamma P, Pinheiro JB (2012) Genetic variation of germination cold tolerance in Japanese rice germplasm. Breeding Sci 62: 209–215.

31. Sthapit BR, Witcombre JR (1998) Inheritance of tolerance to chilling stress in rice during germination and plumule greening. Crop Sci 38: 660–665.

32. Gross BL, Skare KA, Olsen KM (2009) Novel Ph1 mutations and the evolution of phenol reaction variation in US weedy rice (*Oryza sativa*). New Phytol 184: 842–850. doi: 10.1111/j.1469-8137.2009.02957.x PMID: 19674331

33. Vaughan LK, Ottis BV, Prazak-havey AM, Sneller C, Chandler JM, Park WD (2001) Is all red rice found in commercial rice really *Oryza sativa*? Weed Sci 49: 468–476.

34. Gealy DR, Talbert RE, Saldain NE (2000) Emergence of red rice (*Oryza sativa*) ecotypes under dry-seeded rice (*Oryza sativa*) culture. Weed Technol 14: 406–412.

35. Malone G, Zimmer PD, Meneghello GE, Castro MAS, Peske ST (2007) Differential expression of isoenzymes during germination of rice seeds in deep sowing. Revista Brasileira de Sementes 29: 61–67.

36. Gregorio GB, Senadhira D, Mendoza RD (1997) Screening rice for salinity tolerance. IRRI Discussion Paper Series 22: 1–30.

37. Cruz RP, Milach SCK (2004) Cold tolerance at the germination stage of rice: methods of evaluation and characterization of genotypes. Scientia Agricola 61: 1–8.

38. Cruz RP, Milach SCK, Luiz CF (2006) Inheritance of rice cold tolerance at the germination stage. Genet Mol Biol 29: 314–320.

39. Lourenço T, Sapeta H, Figuerido DD, Rodriguez M, Cordeiro A, Abreu IA (2013) Isolation and characterization of rice (*Oryza sativa* L.) E3-ubiquitin ligase *OsHOS1* gene in the modulation of cold stress response. Plant Mol Biol: 83: 351–63. doi: 10.1007/s11103-013-0092-6 PMID: 23780733

40. Ahamed A, Murai HM, Ishikawa SJ, Hayashi H, Kawamura Y, Ucmura M, (2012) Cold stress-induced acclimation in rice is mediated by root-specific aquaporins. Plant Cell Physiol 53: 1445–56. doi: 10.1093/pcp/pcs089 PMID: 22711693

41. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402–408. PMID: 11846609

42. Basu S, Roychoudhury A (2014) Expression profiling of abiotic stress-inducible genes in response to multiple stresses in rice (*Oryza sativa* L.) varieties with contrasting level of stress tolerance. Biomed Res Int 706890:12 pages.

43. Ranawake AL, Nakamura C (2012) Cold tolerance of an inbred line population of rice (*Oryza sativa* L.) at different growth stages. Tropical Agric'l Res and Ext 14: 25–33.

44. Srivastava AK, Goering CE, Rohbach RP Buckmaster DR (2006) Engineering Principles of Agricultural Machines, (2nd ed), Amer Soc Agric and Biol Eng, Michigan, USA.

45. Soltani A, Galeshi S, Zeinali E, Latifi N (2002) Germination, seed reserve utilization and seedling growth of chickpea as affected by salinity and seed size. Seed Sci Technol 30: 51–60.

46. Herbek JH, Bitzer MJ (1988) Soybean Production in Kentucky. Part III: Planting Practices and Double Cropping. http://www.ca.uky.edu/agc/pubs/agr/agr130/agr130.htm

47. Steponkus PL, Uemura M, Webb MS (1993) A contrast of the cryostability of the plasma membrane of winter rye and spring oat- two species that widely differ in their freezing tolerance and plasma membrane lipid composition. In: Steponkus PL, (Ed.), Advances in Low-Temperature Biology, Vol. 2, JAI Press, London pp 211–312.

48. Baek J-S, Chung N-J (2012) Seed wintering and deterioration characteristics between weedy and cultivated rice. Rice 5: 21–31.

49. Fowler S, Thomashow MF (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. PMID: 12172015

50. Kreps JA, Wu Y, Chang HS, Zhu T, Wang X, Harper J (2002) Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. Plant Physiol 130: 2129–2141. PMID: 12481097

51. Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, et al. (2002) Monitoring the expression profiles of ca 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J 31: 279–292. PMID: 12164808

52. Dumas B, Sailland A, Cheviet JP, Freyssinet G, Pallett K (1993) Identification of barley oxalate oxidase as a germin-like protein. CR Acad Sci Paris 316: 793–798.

53. Druka A, Kudrna D, Kannangara CG, Wettstein DV, Kleinhofs A (2002) Physical and genetic mapping of barley (Hordeum vulgare) germin-like cDNAs. Proc Natl Acad Sci USA 99: 850–855. PMID: 11792854

54. Zimmermann G, Bäumlein H, Mock HP, Himmelbach A, Schweizer P (2006) The multigene family encoding germin-like proteins of barley. Regulation and function in basal host resistance. Plant Physiol 142: 181–192. PMID: 16844832

55. Himmelbach A, Liu L, Zierold U, Altschmied L, Maucher H, Beier F, et al. (2010) Promoters of the barley germin-like GER4 gene cluster enable strong transgene expression in response to pathogen attack. Plant Cell 22: 937–952. doi: 10.1105/tpc.109.067934 PMID: 20305123

56. Frank G, Pressman E, Ophir R, Althan L, Shaked R, Freedman M, et al. (2009) Transcriptional profiling of maturing tomato (Solanum lycopersicum L.) microspores reveals the involvement of heat shock proteins, ROS scavengers, hormones, and sugars in the heat stress response. J Exp Bot 60: 3891–3908. doi: 10.1093/jxb/erp234 PMID: 19628571

57. Pnueli L, Hongjian L, Mittler R (2003) Growth suppression, abnormal guard cell response, and augmented induction of heat shock proteins in cytosolic ascorbate peroxidase (Apx1)-deficient *Arabidopsis* plants. Plant J 34: 187–203. PMID: 12694594

58. Davletova S, Rizhsky L, Liang H, Shenqiang Z, Oliver DJ, Coutu J, et al. (2005) Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of *Arabidopsis*. Plant Cell 17: 268–281. PMID: 15608336

59. Moons A, Prinsen E, Bauw G, Van Montagu M (1997) Antagonistic effects of abscisic acid and jasmonates on salt stress-inducible transcripts in rice roots. The Plant Cell 9: 2243–2259. PMID: 9437865

60. Roychoudhury A, Basu S, Sarkar SN, Sengupta DN (2008) Comparative physiological and molecular responses of a common aromatic indica rice cultivar to high salinity with non-aromatic indica rice cultivars. Plant Cell Rep 27: 1395–1410 doi: 10.1007/s00299-008-0556-3 PMID: 18509653

61. Roychoudhury A, Basu S, Sengupta DN (2009) Effects of exogenous abscisic acid on some physiological responses in a popular aromatic indica rice compared with those from two traditional non-aromatic indica rice cultivars. Acta Physiol Plant 31: 915–926.

62. Allagulova C, Gimalov FR, Shakirova FM, Vakhitov VA (2003) The plant dehydrins: structure and putative functions. Biochemistry 68: 945–951. PMID: 14606934

63. Masclaux-Daubresse C, DaniVedele F, Dechorgnat J, Chardon F, Gaufichon Suzuki A (2010) Nitrogen uptake, assimilation and remobilisation in plants: challenges for sustainable and productive agriculture. Annals Bot 105: 1141–1158.

64. Skopelitis DS, Paranychianakis NV, Paschalidis KA, Pliakonis ED, Yakoumakis D, Delis ID, et al. (2006) Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis in tobacco and grapevine. The Plant Cell Online 18: 2767–2781.

65. Miflin BJ, Habash D (2002) The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. J Exp Bot 53: 979–987. PMID: 11912240

66. Skopelitis DS, Paranychianakis NV, Paschalidis KA, Pliakonis ED, Yakoumakis D, Delis ID, et al. (2006) Abiotic stress generated ROS signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis. Plant Cell 18: 2767–2781. PMID: 17041150

67. Zhang H, Shen G, Kuppu S, Gaxiola R, Payton P (2011) Creating drought- and salt-tolerant cotton by overexpressing a vacuolar pyrophosphatase gene. Plant Signal Behav 6: 861–863. doi: 10.4161/psb.6.6.15223 PMID: 21849817

68. Stockinger EJ, Gilmour SJ, Thomashow MF (1997) *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Natl Acad Sci, USA 94: 1035–1040. PMID: 9023378

69. Zhang J, Li J, Wang X, Chen J (2011) *OVP1*, a vacuolar H+-translocating inorganic pyrophosphatase (V-PPase), overexpression improved rice cold tolerance. Plant Physiol Biochem: 49: 33–38. doi: 10.1016/j.plaphy.2010.09.014 PMID: 20974539

70. Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi SK, et al. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain, separate two cellular signal

transduction pathways in drought- and low temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10: 1391–1406. PMID: 9707537

71. Lee B-H, Henderson DA, and Zhu J-K (2005) The *Arabidopsis* cold-responsive transcriptome and its regulation by ICE1. Plant Cell 17: 3155–3175. PMID: 16214899

72. Maruyama K, Sakuma Y, Kasuga M, Ito Y, Seki M, Goda H, et al. (2004) Identification of cold-inducible downstream genes of the *Arabidopsis* DREB1A/CBF3 transcriptional factor using two microarray systems. Plant J 38: 982–993. PMID: 15165189

73. Lee SJ, Kang JY, Park HJ, Kim MD, Bae MS, Choi HI, et al. (2010) DREB2C interacts with ABF2, a bZIP protein regulating abscisic acid-responsive gene expression, and its overexpression affects abscisic acid sensitivity. Plant Physiol 153: 716–727. doi: 10.1104/pp.110.154617 PMID: 20395451

74. Lata C, Yadav A, Prasad M (2011) Role of plant transcription factors in abiotic stress tolerance. Abiotic Stress Response in Plants—Physiological, Biochemical and Genetic Perspectives 261–296.

75. Matsukura S, Mizoi J, Yoshida T, Todaka D, Ito Y et al. (2010) Comprehensive analysis of rice DREB2-type genes that encode transcription factors involved in the expression of abiotic stress-responsive genes. Molec Genet and Genomics 283: 185–196.

76. Vannini C, Locatelli F, Bracale M, Magnani E, Marsoni M, Osmato M, et al. (2004) Overexpression of the rice Osmyb4 gene increases chilling and freezing tolerance of *Arabidopsis thaliana* plants. Plant J 37: 115–127. PMID: 14675437

77. Sasidharan R, Voesenek LACJ, Pierik R (2011) Cell wall modifying proteins mediate plant acclimatization to biotic and abiotic stresses. Critic Rev Plant Sci 30: 548–562.

78. Kim WC, Kim JY, Ko JH, Kang H, Han KH (2014) Identification of direct targets of transcription factor MYB46 provides insights into the transcriptional regulation of secondary wall biosynthesis. Plant Mol Biol 85: 589–99. doi: 10.1007/s11103-014-0205-x PMID: 24879533

79. Nuruzzaman M, Sharoni AM, Kikuchi S (2013) Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. Front Microbiol 4: 248. doi: 10.3389/fmicb.2013.00248 PMID: 24058359

80. Hu H, Dai M, Yao J, Xiao B, Li X, Zhang Q, et al. (2006) Overexpressing a NAM, ATAF and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proc Natl Acad Sci USA 103: 12987–12992. PMID: 16924117

81. Zheng X, Chen B, Lu G, Han B (2009) Overexpression of a NAC transcription factor enhances rice drought and salt tolerance. Biochem Biophys Res Commun 379: 985–989. doi: 10.1016/j.bbrc.2008.12.163 PMID: 19135985

82. Hu R, Qi G, Kong Y, Kong D, Gao Q, Zhou G (2010) Comprehensive analysis of NAC domain transcription factor gene family in *Populus trichocarpa*. BMC Plant Biol 10:145. doi: 10.1186/1471-2229-10-145 PMID: 20630103

83. Cosgrove DJ (1999) Enzymes and other agents that enhance cell wall extensibility. Annu Rev Plant Physiol Plant Mol Biol 50: 391–417. PMID: 11541953

84. Huang J, Takano T, Akita S (2000) Expression of alpha-expansin genes in young seedlings of rice (*Oryza sativa* L.). Planta 211: 467–473. PMID: 11030545

85. Loreti E, Poggi A, Novi G, Alpi A, Perata P (2005) A genome-wide analysis of the effects of sucrose on gene expression in *Arabidopsis* seedlings under anoxia. Plant Physiol 137: 1130–1138. PMID: 15734908

86. Xu K, Xu X, Fukau T, Canlas P, Maghirang RR, Heuer S, et al. (2006) *Sub1A* is an ethylene-response-factor-like gene that confers submergence tolerance to rice. Nature 442: 705–708. PMID: 16900200

87. Beck E, Ziegler P (1989) Biosynthesis and degradation of starch in higher plants. Annu Rev Plant Physiol Plant Mol Biol 40: 95–117.

88. Yu SM, Lee YC, Fang SC, Chan MT, Hwa SF, Liu LF (1996) Sugars act as signal molecules and osmotica to regulate the expression of alpha-amylase genes and metabolic activities in germinating cereal grains. Plant Mol. Biol 30: 1277–1289. PMID: 8704135

89. Lasanthi-kudahettige R, Magneschi L., Loreti E, Gonzali S, Licausi F, Novi G, et al. (2007) Transcript profiling of the anoxic rice shoot. Plant Physiol 144: 218–231. PMID: 17369434

90. Sato A, Okubo H (2006) Increase in the expression of an alpha-amylase gene and sugar accumulation induced during cold period reflects shoot elongation in hyacinth bulbs. J Amer Soc Hort Sci 131: 185–191.

91. Dolferus R, Jacobs M, Peacock WJ, Dennis ES (1994) Differential interactions of promoter elements in stress responses of the *Arabidopsis Adh* gene. Plant Physiol 105: 1075–1087. PMID: 7972489

92. Chistie PJ, Hahn M, Walbot V (1991) Low-temperature accumulation of alcohol dehydrogenase-1 mRNA and protein activity in maize and rice seedlings. Plant Physiol 95: 699–706. PMID: 16668042

93. Lata C, Prasad M (2011) Role of DREBs in regulation of abiotic stress responses in plants. J Exp Bot 62: 4731–48. doi: 10.1093/jxb/err210 PMID: 21737415

94. Lim CJ, Hwang JE, Chen H, Hong JK, Yang KA, Choi MS, et al. (2007) Over-expression of the *Arabidopsis* DRE/CRT-binding transcription factor DREB2C enhances thermotolerance. Biochem Biophys Res Commun 362: 431–6. PMID: 17716623

95. Zhong R, Richardson EA, Ye ZH (2007) The MYB46 transcription factor is a direct target of SND1 and regulates secondary wall biosynthesis in *Arabidopsis*. Plant Cell 19: 2776–92. PMID: 17890373

96. Franco-Zorrilla JM, Lopez VI, Carrasco JL, Godoy M, Vera P, Solano R (2014) DNA-binding specificities of plant transcription factors and their potential to define target genes. Proc Natl Acad Sci USA 111:2367–72. doi: 10.1073/pnas.1316278111 PMID: 24477691

97. Jain M, Nijhawan A, Arora R, Agarwal P, Ray S, Sharma P, et al. (2007) F-box proteins in rice. Genome-wide analysis, classification, temporal and spatial gene expression during panicle and seed development, and regulation by light and abiotic stress. Plant Physiol 143: 1467–83. PMID: 17293439

98. Ganguly M, Datta K, Roychoudhury A, Gayen D, Sengupta DN, Datta SK. (2012) Overexpression of Rab16A gene in indica rice variety for generating enhanced salt tolerance. Plant Signal Behav 7: 502–9. doi: 10.4161/psb.19646 PMID: 22499169

99. Cheng C, Yun KY, Ressom HW, Mohanty B, Bajic VB, Jia Y, et al. (2007) An early response regulatory cluster induced by low temperature and hydrogen peroxide in seedlings of chilling-tolerant japonica rice. BMC Genomics 8: 175. PMID: 17577400

100. Kumar RG, Shah K, Dubey RS (2000) Salinity induced behavioural changes in malate dehydrogenase and glutamate dehydrogenase activities in rice seedlings of differing salt tolerance. Plant Sci 156: 23–34. PMID: 10908802

101. Caverzan A, Passaia G, Rosa SB, Ribeiro CW, Lazzarotto F, Margis PM (2012) Plant responses to stresses: Role of ascorbate peroxidase in the antioxidant protection. Genet Mol Biol 35: 1011–9. PMID: 23412747

102. Sato Y, Masuta Y, Saito K, Murayama S, Ozawa K (2011) Enhanced chilling tolerance at the booting stage in rice by transgenic overexpression of the ascorbate peroxidase gene, *OsAPXa*. Plant Cell Rep 30: 399–406. doi: 10.1007/s00299-010-0985-7 PMID: 21203887

103. Ismond KP, Dolferus R, de Pauw M, Dennis ES, Good AG (2003) Enhanced low oxygen survival in *Arabidopsis* through increased metabolic flux in the fermentative pathway. Plant Physiol 132: 1292–302. PMID: 12857811

104. ZhiMing Y, Bo K, Xiao WH, Shao LL, You HB, Wo ND, et al. (2011) Root hair-specific expansins modulate root hair elongation in rice. Plant J 66: 725–34. doi: 10.1111/j.1365-313X.2011.04533.x PMID: 21309868

105. Pegoraro C, Rosa FD, Mertz L, Santos R, Maia L, Rombaldi C, et al. (2013) Ethylene response factors gene regulation and expression profiles under different stresses in rice. Theoret Exp'l Plant Physiol 25: 261–74.

106. Dubois M, Skirycz A, Claeys H, Maleux K, Dhondt S, DeBodt S, et al. (2013) Ethylene Response Factor6 acts as a central regulator of leaf growth under water-limiting conditions in *Arabidopsis*. Plant Physiol 162: 319–32. doi: 10.1104/pp.113.216341 PMID: 23553636

107. Steffens B (2014) The role of ethylene and ROS in salinity, heavy metal, and flooding responses in rice. Front Plant Sci 5: 685. doi: 10.3389/fpls.2014.00685 PMID: 25538719

108. Fukao T, Yeung E, Bailey-Serres J (2011) The submergence tolerance regulator SUB1A mediates crosstalk between submergence and drought tolerance in rice. Plant Cell 23: 412–27. doi: 10.1105/tpc.110.080325 PMID: 21239643