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TESE

Investigação de mecanismos de tolerância ao frio em genótipos de arroz (*Oryza sativa* L.) da subespécie *indica* durante as fases iniciais do desenvolvimento.

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LISTA DE ABREVIATURAS E SIGLAS

ABS/RC	*Energy flux absorption per reaction center*
APX	Peroxidase do ascorbato
ATP	Adenosina trifosfato
CAT	Catalase
CO_2	Dióxido de carbono
DI_0/RC	*Energy dissipation per reaction center*
EROs	Espécies reativas de oxigênio
ET_0/ABS	*Quantum yield of electron transport from Q_A^- to the intersystem electron acceptors*
ET_0/CS	*Flow of electron transport per CS in t = 0*
ET_0/RC	*Electron transport flux per reaction center*
ET_0/TR_0	*Probability, at t = 0, that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-;*
F_0	Fluorescência inicial, quando todos os centros de reação do PSII estão abertos
F_I	Intensidade de fluorescência no passo I
F_j	Intensidade de fluorescência no passo J
F_M	Fluorescência máxima, quando todos os centros de reação do PSII estão fechados
FSI	Fotossistema I
FSII	Fotossistema II
GO	*Gene Ontology*
H_2O_2	Peróxido de hidrogênio
NADPH	Nicotinamida adenina dinucleotídeo fosfato
$^1O_2$	Oxigênio singleto
OsAXN	*Annexin*
OsAUX	*OsIAA13 - Auxin-responsive Aux/IAA gene family member*
OsAQU	*Aquaporin protein*
OsAUX	*OsIAA13 - Auxin-responsive Aux/IAA gene family member*
OsCAT	*Catalase domain-containing protein*
OsCBP	*Calmodulin-binding protein*

OsCES, CESA1	*Cellulose synthase*
OsCDKB2;1	*Cyclin-dependent kinase B2-1*
OsCYC	*Cyclin*
OsCSLE1	*Cellulose synthase-like family E*
OsDHN	*Dehydrin*
OsEXD	*Expressed protein*
OsEXP	*Expansin precursor*
OsFAD	*Omega-3 fatty acid desaturase*
OsFBX221	*F-box domain-containing protein*
OsGAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*
OsLEA	*Late embryogenesis abundant protein*
OsPRX	*Peroxidase precursor*
OsRBC	*Ribulose bisphosphate carboxylase small chain*
OsUBQ5	*Ubiquitin 5*
OsWIP	*Wound-induced protein Wi12*
OsKET	*3-ketoacyl-CoA synthase*
PI_{ABS}	*Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors*
PI_{Total}	*Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors*
Q_A	Quinona A
Q_A^-	Quinona A em estado reduzido
RC	Centro de reação
ROS	Espécies reativas de oxigênio
SOD	Superóxido dismutase
TBARS	*Thiobarbituric acid reactant substances*
TR_0/CS	*Flow of energy captured by CS in t = 0*
TR_0/RC	*Energy captured flow per reaction center*
φD_0	*Quantum yield, at t = 0, of energy dissipation*

RESUMO

Plantas de arroz possuem grande importância para a alimentação humana, para a economia mundial e para a ciência. O Brasil é o maior produtor de arroz fora da Ásia, e mais de 60% do arroz brasileiro é produzido no Rio Grande do Sul, onde as plantas estão sujeitas a estresse por baixas temperaturas nas fases iniciais do desenvolvimento. Genótipos de arroz que apresentam tolerância ao frio são principalmente da subespécie *japonica*, enquanto que quase todo arroz produzido no Brasil é da subespécie *indica*, sensível ao frio. Neste trabalho, avaliou-se um grande número de genótipos de arroz *indica* quanto à tolerância ao frio durante a fase de germinação e em plantas jovens com três a quatro folhas. Foi constatada grande variação entre os genótipos, e duas linhagens oriundas do mesmo cruzamento, porém contrastantes quanto à tolerância ao frio, IRGA 959-1-2-2F-4-1-4-A (tolerante) e IRGA 959-1-2-2F-4-1-4-D-1-CA-1 (sensível), foram identificadas e escolhidas para caracterização. Análises fisiológicas mostraram que a performance fotossintética foi fortemente influenciada pelo frio nos dois genótipos. No entanto, o genótipo tolerante foi capaz de recuperá-la mais rapidamente após a exposição ao frio. A melhor performance parece estar relacionada com uma menor fração de redução de Q_A^- e com o maior tamanho do pool de aceptores finais de elétrons do PSI. Adicionalmente, maiores níveis de ácido linoleico e maior deposição de celulose nas paredes celulares, assim como maior eficiência do sistema antioxidante foram observados no genótipo tolerante. O sequenciamento de bibliotecas de cDNA permitiu identificar genes diferencialmente expressos entre os dois genótipos, nas duas fases de desenvolvimento. Na fase de germinação, foram detectados 1.361 transcritos diferencialmente expressos. Destes, 758 mostraram-se mais expressos no genótipo tolerante ao frio, e 603 mostraram-se mais expressos no genótipo sensível. Este estudo revelou que vários processos são mais ativos no genótipo tolerante, incluindo taxas de divisão celular e de crescimento do coleóptilo. Na fase vegetativa, 1.305 genes exibiram padrões de expressão diferencial. Os produtos destes genes são importantes em várias rotas metabólicas, incluindo fotossíntese, metabolismo de carboidratos, síntese de parede celular, degradação proteica, detoxificação de espécies reativas de oxigênio e sinalização mediada por hormônios e Ca^{2+}. A identificação de genes diferencialmente expressos nos dois genótipos nas fases de germinação e vegetativa, sob estresse por frio, será útil no planejamento de futuras abordagens biotecnológicas visando a tolerância ao frio em genótipos de arroz da subespécie *indica*.

Palavras-chave: divisão celular, enzimas antioxidantes, estresse abiótico, expressão gênica, fotossíntese, parede celular, teste JIP.

ABSTRACT

Rice plants are of great importance for human nutrition, for the world economy and for science. Brazil is the largest rice producer outside Asia, and more than 60% of Brazil's rice is produced in Rio Grande do Sul State, where the plants are subject to stress by low temperatures in the early stages of development. Rice genotypes with cold tolerance belong mainly to the *japonica* subspecies, while almost all rice produced in Brazil is from the *indica* subspecies. In this work, we screened a large number of *indica* rice genotypes for cold tolerance during germination and initial vegetative growth. Large variation between genotypes was observed. Two sister lines with contrasting cold tolerance levels, IRGA 959-1-2-2F-4-1-4-A (tolerant) e IRGA 959-1-2-2F-4-1-4-D-1-CA-1 (sensitive), were identified and chosen for further characterization. Physiological analyses showed that the photosynthetic performance was heavily affected by cold in both genotypes. Recovery of performance indexes after cold exposure was faster in the tolerant genotype. The better performance seems to be related to a lower fraction of the reduced Q_A^- and with a larger pool of the end electron acceptors at the PSI. Additionally, higher linoleic acid levels, higher deposition of cellulose in cell walls, and more efficient antioxidant system were observed in the tolerant genotype. Sequencing of cDNA libraries from plants of both genotypes allowed the identification of genes differentially expressed in the two developmental phases. At the germination stage, 1,361 differentially expressed transcripts were detected: 758 showed higher expression and 603 showed lower expression in seedlings from the cold-tolerant genotype than in the cold-sensitive one. This study revealed that several processes are more active in the cold-tolerant than in the cold-sensitive seedlings, including cell division and expansion of the coleoptiles. At the vegetative stage, 1,305 genes exhibited differential expression patterns. Their gene products are important in several metabolic pathways, including photosynthesis, carbohydrate metabolism, cell wall synthesis, fatty acid desaturation, protein degradation, detoxification of reactive oxygen species and Ca^{2+} and hormone-signaling. The identification of differentially expressed genes in both genotypes during germination and initial vegetative growth, under cold stress, will be useful for planing future biotechnological approaches aiming to cold tolerance in *indica* rice genotypes.

Keywords: abiotic stress, antioxidant enzymes, cell division, cell wall, gene expression, JIP-Test.

INTRODUÇÃO

INTRODUÇÃO

O arroz (*Oryza sativa* L.) é um dos cereais mais produzidos e consumidos no mundo, sendo a principal fonte de carboidratos da dieta para a metade da população mundial (IRRI, 2007). O Brasil é o maior produtor de arroz fora da Ásia, e o Rio Grande do Sul é responsável por 68,3% da produção nacional (IBGE, 2014). Além de sua importância comercial e nutricional, o arroz é a principal espécie-modelo para estudos em monocotiledôneas, tendo sido a primeira planta deste grupo a ter o seu genoma totalmente seqüenciado (IRGSP, 2005) e possuindo alta relação de sintenia com os genomas de outros cereais cultivados (Moore et al 1995), havendo também uma grande quantidade de ferramentas disponíveis, como bancos de mutantes e de cDNAs de arroz (Rice Full-Length cDNA Consortium et al. 2003; An et al. 2005).

Estresses abióticos afetam direta e indiretamente as plantas de arroz e alteram negativamente o seu metabolismo, gerando perdas na produção de grãos. Dentre estes, a baixa temperatura é particulamente prejudicial devido a origem tropical das espécies de arroz. Geralmente, o grau de dano depende do tempo de ocorrência (fase de crescimento), da intensidade e da duração do estresse (Li et al. 1981). O frio tem potencial para afetar o crescimento e o desenvolvimento das plantas durante qualquer estádio de desenvolvimento, ou seja, desde a germinação até a fase reprodutiva (Ye et al. 2009).

As variedades de arroz cultivadas no Brasil, apesar de apresentarem alto potencial de rendimento e qualidade de grãos, são extremamente sensíveis ao frio (Lopes 2008). A semeadura antecipada (de final de setembro ao final de outubro) da cultura do arroz irrigado no Rio Grande do Sul é uma das práticas de manejo mais importantes no sentido de garantir altas produtividades, pela coincidência dos períodos de perfilhamento e início do período reprodutivo com a maior radiação solar (Mariot et al 2005). Entretanto, como a ocorrência de temperaturas mínimas abaixo de 17 °C nos meses de setembro a novembro (período de crescimento vegetativo) é freqüente no Rio Grande do Sul, as plantas apresentam germinação e desenvolvimento inicial lentos. Além disso, o desenvolvimento das ervas daninhas é mais acelerado do que o do arroz sob baixas temperaturas, e torna-se necessária a utilização de herbicidas em maiores quantidades e em repetidas aplicações. O crescimento inicial lento também resulta em ciclo vegetativo mais longo e, consequentemene, há maior consumo da água de irrigação (Toro, 2006; Cruz et al., 2013). Considerando-se as atuais previsões de aumento da frequência de eventos de curta duração

de temperaturas extremas (muito baixas ou elevadas) (IPCC, 2007; Marengo et al. 2008), os danos por frio na cultura do arroz podem ser ainda mais significativos.

Neste contexto, a tolerância ao frio nas fases de germinação e vegetativa em arroz irrigado é uma característica de fundamental importância para garantir o estabelecimento rápido e uniforme da lavoura no sistema de plantio antecipado.

Os genótipos de arroz com maiores níveis de tolerância ao frio são pertencentes à subespécie *japonica* (Mackill e Lei, 1997). No entanto, grande parte da produção de grãos de arroz no Brasil é da subespécie *indica*, preferida no mercado pelo consumidor brasileiro. Por este motivo, é extremamente importante identificar mecanismos de tolerância ao frio em genótipos de arroz da subespécie *indica*, assim como identificar genes capazes de contribuir para o melhor desempenho destas plantas em condições de baixas temperaturas (Cruz et al. 2013).

Uma solução aparente para o problema seria a utilização de cruzamentos entre as subespécies *indica* e *japonica*. No entanto, além de diferirem em muitos aspectos, as duas subespécies possuem barreiras reprodutivas que dificultam o processo de melhoramento. Problemas como esterilidade das progênies, tipo de planta resultante e ligação gênica limitam a transferência de genes úteis das cultivares *japonica* para as *indica* por métodos convencionais (Toro, 2006). Além disso, os caracteres relacionados com rendimento e caracteres agronômicos são herdados quantitativamente, estão relacionados entre si, e influenciados pelo ambiente (Kobayashi et al., 2003).

Estudos moleculares e de expressão gênica têm contribuído muito para aumentar o conhecimento a respeito das bases fisiológicas relacionadas aos estresses ambientais em plantas, entre eles a tolerância a baixas temperaturas (Gao et al., 2008). Vários genes de arroz já foram isolados e caracterizados como responsivos ao estresse por frio. Muitos deles codificam enzimas necessárias para a biossíntese de osmoprotetores (MaNeil et al. 1999), componentes de sinalização (Saijo et al. 2000; Wen et al. 2002; Xiong e Yang 2003; Chen et al. 2011; Xie et al. 2012), chaperonas (Lee et al. 2005; Mittal et al. 2009) e fatores de transcrição (Huang et al. 2005; Ohnishi et al. 2005; Nakashima et al. 2007; Chaikam and Karlson 2008; Huang et al. 2008; Kim et al. 2009; Ye et al. 2009; Hossain et al. 2010; Tao et al. 2011), especialmente da família DREB1/CBF (C-repeat binding factor/dehydrationresponsive element binding; Chen et al. 2003; Dubouzet et al. 2003; Lee et al. 2004a,b; Ito et al. 2006; Wang et al. 2008; Zhang et al. 2009). Muitos trabalhos também têm mostrado que a superexpressão destes genes resulta em tolerância ao frio: *OsCOIN* (*Oryza sativa* cold-inducible) (Liu et al. 2007); *OsDREB1F* (Wang et al. 2008);

OsPRP3 (proline-rich protein) (Gothandam et al. 2010); zinc finger protein ZFP245 (Huang et al. 2009); *OsAsr1* (Kim et al. 2009) e *OsMYB3R-2* (Ma et al. 2009). Embora já tenham sido identificados genes responsivos ao frio em plantas de arroz, a grande maioria dos estudos utilizou cultivares da subespécie *japonica*.

As análises das respostas das plantas em função da super-expressão destes genes também têm mostrado que muitas alterações moleculares e fisiológicas ocorrem durante o processo de aclimatação e que várias vias metabólicas são afetadas durante o estresse por baixa temperatura. Isto indica que a tolerância a este tipo de estresse é bastante complexa, e um melhor entendimento dos mecanismos envolvidos ainda é necessário.

Atualmente, uma ferramenta bastante utilizada para traçar um determinado perfil transcricional é o sequenciamento em grande escala (deep sequencing ou RNAseq). A técnica de RNAseq permite a avaliação bastante exata dos níveis de transcritos e suas isoformas. A população de RNAs mensageiros de uma amostra é utilizada como molde para a síntese de uma biblioteca de fragmentos de cDNA. Em seguida, cada molécula é seqüenciada em uma ou ambas extremidades, com a obtenção de sequências curtas. Após o sequenciamento, os resultados das leituras são alinhados com o genoma de referência para a espécie em estudo, produzindo um mapa da transcrição genômica com informações sobre o nível de expressão de cada gene. O uso desta tecnologia necessita de análises de bioinformática para dar sentido à imensa quantidade de dados que são gerados (Wang et al. 2009). Vários estudos envolvendo análise das respostas aos diferentes estresses ambientais em plantas já utilizaram esta ferramenta (Cruz-Jaramillo et al., 2014; Vitulo et al., 2014; Xia et al., 2014).

Considerando a importância das variedades de arroz da subespécie *indica* para a agricultura brasileira e o impacto negativo das baixas temperaturas sobre estas plantas, é necessário entender os seus mecanismos de resposta e de tolerância ao frio, assim como identificar genes relacionados à tolerância. O conhecimento adquirido poderá ser utilizado em futuras abordagens biotecnológicas visando a tolerância ao frio em genótipos de arroz da subespécie *indica*.

Nas cultivares de arroz sensíveis, o frio desencadeia diversas alterações fisiológicas que afetam a produtividade. A exposição a baixa temperatura causa inibição do transporte de elétrons através do FSII, comprometendo a produção de NADPH e ATP. Tal situação favorece a produção excessiva de espécies reativas de oxigênio provocando danos ao aparato fotossintético e aumento da fotoinibição (Bonnecarrere et al., 2011).

Avaliações da eficiência fotossintética permitem avaliar as respostas das plantas a estresses ambientais (Guo e Tan, 2013; Millan-Almaraz et al., 2009). Dentre os métodos disponíveis, os sinais da fluorescência da clorofila *a* são amplamente utilizados para a avaliação de estresse por frio sobre o metabolismo fotossintético em arroz (Guo-li e Zhen-fei, 2005; Bonnecarrere et al., 2011) e, assim, determinar a resposta (grau de tolerância) das plantas. A ampla utilização da fluorescência da clorofila *a* é decorrente do fato de ser um método não destrutivo, relativamente simples e por permitir fazer inferências sobre processos fundamentais da fase luminosa da fotossíntese (Yusuf et al., 2010). Esta técnica tem permitido aumento do conhecimento dos processos fotoquímicos e não fotoquímicos que ocorrem na membrana dos tilacóides dos cloroplastos (Rohácek, 2002), além de possibilitar o estudo de características relacionadas à capacidade de absorção e transferência de energia luminosa na cadeia de transporte de elétrons (Krause e Weis, 1991).

A energia da luz absorvida por moléculas de clorofila associadas ao FSII pode ser utilizada na cadeia de transporte de elétrons da fotossíntese, onde um elétron é transferido do centro de reação para o aceptor primário de elétrons do FSII (Q_A). Alternativamente, a luz absorvida pode ser perdida como fluorescência da clorofila ou calor (Baker, 2008). Quando uma amostra fotossintética previamente mantida no escuro (F_0) é iluminada com um pulso de luz saturante, observa-se um aumento rápido da fluorescência da clorofila até um ponto máximo (F_M). Neste ponto, todos os centros de reação estão "fechados", ou seja, o conteúdo de Q_A está completamente reduzido ($Q_A^+ \xrightarrow{e^-} Q_A^-$). Quando o aumento da intensidade de fluorescência é plotado em uma escala logaritmica de tempo, é observada uma curva polifásica, onde é possível identificar os pontos O, J, I e P (Lazár, 2006). Esses pontos são obtidos aproximadamente a 50 μs, 2 ms, 30 ms e 300 ms, respectivamente, após a incidência de luz na amostra. Sua amplitude pode estar diretamente ligada à intensidade e duração do estresse (Strasser, 1997; Jiang et al., 2006). Adicionalmente, é possível analisar os dados através do Teste JIP, que traduz os valores da curva polifásica em parâmetros biofísicos, fluxos específicos, rendimentos e eficiências das etapas da cadeia transportadora de elétrons, além dos índices de performance (PI_{ABS} e PI_{TOTAL}) (Strasser et al., 2004; Tsimilli-Michael e Strasser, 2008).

Análises de parâmetros da fluorescência têm demonstrado alterações funcionais e estruturais do aparato fotossintético de diferentes genótipos ou plantas transgênicas de arroz submetidos a tratamentos com baixa temperatura (Saijo et al. 2000; Ji et al. 2003;

Hirotsu et al. 2004; Lee et al. 2004a,b; Wang and Guo 2005; Kim et al. 2009; Lee et al. 2009a,b; Bonnecarrere et al. 2011; Saad et al. 2012).

Outro método de avaliação da atividade fotossintética em plantas é baseado nas trocas gasosas, sendo também utilizado para analisar a fotossíntese em plantas de arroz de diferentes genótipos ou linhagens transgênicas submetidas ao estresse por frio (Wang and Guo 2005; Saad et al. 2012). Para tanto, utiliza-se de um analisador de gás por absorção de luz na região do infravermelho (IRGA).

A exposição das plantas ao estresse por frio pode perturbar a homeostase celular e aumentar a produção de diversas espécies reativas de oxigênio (EROs), como o superóxido ($O_2^{\bullet-}$), os radicais hidroxila ($^{\bullet}OH$), o oxigênio singleto ($^1O_2$) e o peróxido de hidrogênio (H_2O_2) (Song et al. 2011; Theocharis et al. 2012; Yang et al. 2012). As EROs podem causar severos danos aos componentes celulares, incluindo lipídios de membrana, proteínas estruturais e enzimas (Cruz et al. 2013). No entanto, as plantas possuem um sistema de defesa antioxidante enzimático que permite a detoxificação das ERO_S e a proteção das células vegetais de danos oxidativos (Gratão et al., 2005). A destruição eficiente das ERO_S requer a ação de diversas enzimas antioxidantes atuando em sincronia, como a superóxido dismutase (SOD), catalase (CAT) e ascorbato peroxidase (APX) (Deuner, et al., 2008). A SOD vem sendo considerada como a primeira enzima de defesa antioxidante em plantas, dismutando dois radicais superóxido ($O_2^{\bullet-}$) até oxigênio molecular (O_2) e peróxido de hidrogênio (H_2O_2) (Sinha, 2006). CAT e APX são enzimas que catalisam a conversão do H_2O_2 e O_2 em água. O sistema antioxidante vem sendo usado como ferramenta de análise da tolerância ao frio em várias cultivares de arroz (Takesawa et al. 2002; Kuk et al. 2003; Morsy et al. 2007; Yang et al. 2012) e em plantas transformadas (Song et al. 2011; Lee et al., 2009a,b). Por outro lado, as EROs, quando em concentrações baixas, podem atuar como moléculas sinalizadoras, modulando a expressão de vários genes, incluindo aqueles que codificam enzimas antioxidantes, aclimatando a planta ao estresse (Neill et al. 2002; Quan et al. 2008; Theocharis et al. 2012).

As membranas celulares são os locais primeiramente afetados pelo frio, o que desencadeia uma cascata de processos celulares com efeitos adversos para a planta. O aumento da permeabilidade das membranas e o conseqüente vazamento de eletrólitos são considerados injúrias graves para as células e tecidos. Quando a exposição a baixa temperatura é breve, o efeito pode ser transitório e a planta sobrevive. No entanto, haverá necrose e morte celular se a exposição for mantida (Theocharis et al. 2012). Os ácidos graxos insaturados, presentes na membrana plasmática das células, estão relacionados com

a estabilidade da membrana durante estresse por baixa temperatura, impedindo o vazamento de eletrólitos e, assim, o dano celular (Cruz et al., 2013). Analisando diferentes genótipos de arroz seníveis e tolerantes ao frio, Cruz et al. (2010) observaram diferenças no conteúdo de ácidos graxos saturados e insaturados somente após exposição das plantas ao frio. No trabalho, também foi observado que os níveis de ácido linoleico aumentaram, enquanto que os níveis de ácido palmítico diminuíram nos genótipos tolerantes, confirmando a importância dos ácidos graxos insaturados na manutenção da integridade da membrana plasmática durante estresse por frio.

Os objetivos deste trabalho foram selecionar dois genótipos de arroz da subespécie *indica* com níveis contrastantes de tolerância ao frio, caracterizá-los fisiológica e bioquimicamente, e identificar novos genes responsivos ao frio para possível uso futuro em abordagens biotecnológicas ou em programas de melhoramento genético.

REFERÊNCIAS BIBLIOGRÁFICAS

An G, Jeong DH, Jung KH, Lee S (2005): Reverse genetic approaches for functional genomics of rice. Plant Molecular Biology 59: 111-123.

Baker NR (2008): Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annual Review of Plant Biology 59: 89-113.

Bonnecarrèrea V, Borsanib O, Díazb P, Capdeviellea F, Blanco P, Monza J (2011): Response to photoxidative stress induced by cold in japonica rice is genotype dependent. Plant Science: 180: 726–732.

Chaikam V, Karlson D (2008): Functional characterization of two cold shock domain proteins from *Oryza sativa*. Plant Cell Environ. 31: 995-1006.

Chen JQ, Dong Y, Wang YJ, Liu Q, Zhang JS, Chen SY (2003) An AP2/EREBP-type transcription-factor gene from rice is cold inducible and encodes a nuclear-localized protein. Theoretical and Applied Genetics 107: 972-979.

Chen NA, Xu Y, Wang X, Du C, Du J, Yuan M, et al. (2011): *OsRAN2*, essential for mitosis, enhances cold tolerance in rice by promoting export of intranuclear tubulin and maintaining cell division under cold stress. Plant Cell Environ. 34: 52-64.

Cruz-Jaramillo JL, Ruiz-Medrano R, Rojas-Morales L, Lópes-Buenfil JA, Morale-Galván O, Chavarín-Palacio C et al. (2014): Characterization of a proposed dichorhavirus associated with the citrus leprosis disease and analysis of the host response. Viruses 6: 2602-2622.

Cruz RP, Golombieski JI, Bazana MT, Cabreira C, Silveira TF, da Silva LP (2010): Alterations in fatty acid composition due to cold exposure at the vegetative stage in rice. Braz. J. Plant Physiol. 22: 199–207.

Cruz, RP, Sperotto, RA, Cargnelutti D, Adamski, JM, Terra, TF, Fett, JP (2013): Avoiding damage and achieving cold tolerance in rice plants. Food and Energy Security 2: 96-119.

Deuner S, Alves JD, Fries DD, Zanandrea I, Lima AA, Henrique PC, Goulart PF (2008): Peróxido de hidrogênio e ácido ascórbico influenciando a atividade de enzimas antioxidantes de mudas de cafeeiro. Revista Ceres 55: 135-140.

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought, high-salt- and cold-responsive gene expression. *The Plant Journal* 33: 751-763.

Gao JP, Chao DY, Lin HX (2008): Toward understanding molecular mechanisms of abiotic stress responses in rice. Rice 1: 36-51.

Gothandam KM, Nalini E, Karthikeyan S, Shin JS (2010): OsPRP3, a flower specific proline-rich protein of rice, determines extracellular matrix structure of floral organs and its overexpression confers cold-tolerance. Plant Molecular Biology 72: 125-135.

Gratão PL, Polle A, Lea PJ, Azevedo RA (2005): Making the life of heavy metal-stressed plants a little easier. Functional Plant Biology 32: 481-494.

Guo-li W, Zhen-fei G (2005): Effects of Chilling Stress on Photosynthetic Rate and Chlorophyll Fluorescence Parameter in Seedlings of Two Rice Cultivars Differing in Cold Tolerance. Rice Science, 12(3): 187−191.

Guo Y, Tan J (2013): Applications of Delayed Fluorescence from Photosystem II. Sensors, v. 13, p.17332-17345.

Hirotsu N, Makino A, Ushio A, Mae T (2004): Changes in the thermal dissipation and the electron flow in the water–water cycle in rice grown under conditions of physiologically low temperature. Plant Cell Physiol. 45: 635-644.

Hossain MA, Cho JI, Han M, Ahn CH, Jeon JS, An G, et al. (2010): The ABRE-binding bZIP transcription factor OsABF2 is a positive regulator of abiotic stress and ABA signaling in rice. J. Plant Physiol. 167: 1512-1520.

Huang J, Sun SJ, Xu DQ, Yang X, Bao YM, Wang ZF, Tang HJ, Zhang H (2009): Increased tolerance of rice to cold, drought and oxidative stresses mediated by the overexpression of a gene that encodes the zinc finger protein ZFP245. Biochemical and Biophysical Research Communications 389: 556-561.

Huang M, Guo Z (2005): Responses of antioxidative system to chilling stress in two rice cultivars differing in sensitivity. Biologia Plantarum 49 (1): 81-84.

Huang J, Wang MM, Jiang Y, Bao MY, Huang X, Sun H, et al. (2008): Expression analysis of rice A20/AN1-type zinc finger genes and characterization of *ZFP177* that contributes to temperature stress tolerance. Gene 420: 135-144.

IBGE - Intituto Brasileiro de Geografia e Estatística (2014): Available: http://www.ibge.gov.br/home/estatistica/indicadores/agropecuaria/lspa/default.shtm. [Accessed 20 Oct 2014].

IPCC- Intergovernmental Panel on Climate Change (2007): Summary for policymakers. In: Solomon S. et al., eds. Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change), Cambrige University Press.

IRGSP - International Rice Genome Sequencing Project (2005): The map-based sequence of the rice genome. Nature03895, v. 436/11.

IRRI (2007): Rice Around the World. http://www.irri.org/science/cnyinfo/index2.asp.

Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, Shinozaki K, Yamagushi-Shinozaki K (2006): Functional analysis of rice DREB1/CBF-type transcription factors

involved in cold-responsive gene expression in transgenic rice. Plant and Cell Physiology 47: 141-153.

Ji BH, Cao YY, Xie HS, Zhu SQ, Ma Q, Jiao DM (2003): Changes in violaxanthin deepoxidase activity and unsaturation of thylakoid membrane lipids in *indica* and *japonica* rice under chilling condition and strong light. Acta Bot. Sin. 45: 1063-1070.

Jiang C, Jiang G, Wang X, Lia DK, Biswasa L, Lia Y (2006): Increased photosynthetic activities and thermostability of photosystem II with leaf development of elm seedlings (Ulmus pumila) probed by the fast fluorescence rise OJIP. Environmental and Experimental Botany 58: 261-268.

Kim SJ, Lee SC, Hong SK, An K, An G, Kim SR (2009): Ectopic expression of a cold-responsive *OsAsr1* cDNA gives enhanced cold tolerance in transgenic rice plants. Mol. Cells 27: 449-458.

Kobayashi S, Fukuta Y, Sato T, Osaki M, Khush GS (2003): Molecular markerdissection of rice (*Oryza sativa* L.) plant architecture under temperate and tropical climates. Theoretical and Applied Genetics**,** Berlin, v. 107, n. 8, p. 1350-1356.

Krause GH, Weis E (1991): Chlorophyll fluorescence and photosynthesis: the basics. Annual Review Plant Physiology Plant Molecular Biology 42: 313-349.

Kuk YI, Shin JS, Burgos NR, Hwang TE, Han O, Cho BH, Jung S, Guh JO (2003): Antioxidative Enzymes Offer Protection from Chilling Damage in Rice Plants. Crop Sci 43: 2109-2117.

Lazár D (2006): The polyphasic chlorophyll a fluorescence rise measured under high intensity of exciting light. Functional Plant Biology 33: 9-30.

Lee SC, Kim JY, Kim SH, Kim SJ, Lee K, Han SK, et al. (2004a). Trapping and characterization of cold-responsive genes from T-DNA tagging lines in rice. Plant Sci. 166: 69-79.

Lee SC, Huh KW, An K, An G, Kim SR (2004b). Ectopic expression of a cold-inducible transcription factor, CBF1/DREB1b, in transgenic rice (*Oryza sativa* L.). Mol. Cells 18: 107-114.

Lee SC, Lee MY, Kim SJ, Jun SH, An G, Kim SR (2005): Characterization of an abiotic stress-inducible dehydrin gene, *OsDhn1*, in rice (*Oryza sativa* L.). Mol. Cells 19: 212-218.

Lee DG, Ahsan N, Lee SH, Lee JJ, Bahk JD, Kang KY, et al. (2009a): Chilling stress-induced proteomic changes in rice roots. J. Plant Physiol. 166: 1-11.

Lee SC, Kwon SY, Kim SR (2009b): Ectopic expression of a cold-responsive Cu/Zn superoxide dismutase gene, *SodCc1*, in transgenic rice (*Oryza sativa* L.). J. Plant Biol. 52: 154-160.

Li TG, Visperas RM, Vergara BS (1981). Correlation of cold tolerance at different growth stages in rice. Acta Bot. Sin. 23: 203-207.

Liu K, Wang L, Xu Y, Chen N, Ma Q, Li F, Chong K (2007): Overexpression of OsCOIN, a putative cold inducible zinc finger protein, increased tolerance to chilling, salt and drought, and enhanced proline level in rice. Planta 226: 1007-1016.

Lopes SIG, Carmona PS, Lopes MCB, Cruz RP, Funck GRD, Rosso AF, Avozani AO, Kempf D, Fagundes CAA (2008): Contribuição das cultivares lançadas pelo IRGA para a lavoura orizícola do Rio Grande do Sul. Boletim Técnico, n.06.

Ma Q, Dai X, Xu Y, Guo J, Liu Y, Chen N, Xiao J, Zhang D, Xu Z, Zhang X, Chong K (2009): Enhanced tolerance to chilling stress in *OsMYB3R-2* transgenic rice is mediated by alteration in cell cycle and ectopic expression of stress genes. Plant Physiology 150: 244-256.

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

MaNeil SD, Nuccio ML, Hanson, AD (1999): Betaines and related osmoprotectants. Targets for metabolic engineering of stress resistance. Plant Physiol. 120: 945–949.

Marengo JA, Camargo C (2008): Surface air temperature trends in Southern Brazil for 1960-2002. International Journal of Climatology 28: 893-904.

Mariot CHP, Menezes VG, Lima AL et al. (2005): Influência da época de semeadura no rendimento de grãos de cultivares de arroz irrigado. In: CONGRESSO BRASILEIRO DE ARROZ IRRIGADO, 2005, Santa Maria, Editora Orium, p. 251-253.

Millan-Almaraz JR, Guevara-Gonzalez RG, Romero-Troncoso R, Osornio-Rios RA, Torres-Pacheco I (2009): Advantages and disadvantages on photosynthesis measurement techniques: A review. African Journal of Biotechnology 8: 7340-7349.

Mittal D, Chakrabarti S, Sarkar A, Singh A, Grover A (2009): Heat shock factor gene family in rice: genomic organization and transcript expression profiling in response to high temperature, low temperature and oxidative stresses. Plant Physiol. Biochem. 47: 785-795.

Moore G, Devos KM, Wang Z, Gale MD (2005): Cereal genome evolution. Grasses, line up and form a circle. Current Biology 5: 737-739.

Morsy MR, Jouve L, Hausman JF, Hoffmann L, Stewart JD (2007): Alteration of oxidative and carbohydrate metabolism under abiotic stress in two rice (*Oryza sativa* L.) genotypes contrasting chilling tolerance. J. Plant Physiol. 164: 157-167.

Nakashima K, Tran LSP, Nguyen DV, Fujita M, Maruyama K, Todaka D, et al. (2007): Functional analysis of a NAC-type transcription factor *OsNAC6* involved in abiotic and biotic stress-responsive gene expression in rice. Plant J. 51: 617-630.

Neill S, Desikan R, Hancock J (2002): Hydrogen peroxide signalling. Current Opinion in Plant Biology 5: 388-395.

Ohnishi T, Sugahara S, Yamada T, Kikuchi K, Yoshiba Y, Hirano HY, et al. (2005): *OsNAC6*, a member of the NAC gene family, is induced by various stresses in rice. Genes Genet. Syst. 80: 135-139.

Quan L-J, Zhang B, Shi W-W, Li H-Y (2008): Hydrogen Peroxide in Plants: a Versatile Molecule of the Reactive Oxygen Species Network. Journal of Integrative Plant Biology 50 (1): 2-18.

Rice Full-Length cDNA Consortium; National Institute of Agrobiological Sciences Rice Full-Length cDNA Project Team, Kikuchi S, Satoh K, Nagata T, Kawagashira N, Doi K, Kishimoto N, Yazaki J, Ishikawa M, Yamada H, Ooka H, Hotta I, Kojima K, Namiki T, Ohneda E, Yahagi W, Suzuki K, Li CJ, Ohtsuki K, Shishiki T; Foundation of Advancement of International Science Genome Sequencing & Analysis Group, Otomo Y, Murakami K, Iida Y, Sugano S, Fujimura T, Suzuki Y, Tsunoda Y, Kurosaki T, Kodama T, Masuda H, Kobayashi M, Xie Q, Lu M, Narikawa R, Sugiyama A, Mizuno K,

Yokomizo S, Niikura J, Ikeda R, Ishibiki J, Kawamata M, Yoshimura A, Miura J, Kusumegi T, Oka M, Ryu R, Ueda M, Matsubara K; RIKEN, Kawai J, Carninci P, Adachi J, Aizawa K, Arakawa T, Fukuda S, Hara A, Hashizume W, Hayatsu N, Imotani K, Ishii Y, Itoh M, Kagawa I, Kondo S, Konno H, Miyazaki A, Osato N, Ota Y, Saito R, Sasaki D, Sato K, Shibata K, Shinagawa A, Shiraki T, Yoshino M, Hayashizaki Y, Yasunishi A (2003): Collection, mapping, and annotation of over 28,000 cDNA clones from japonica rice. Science 301: 376-379.

Rohácek K (2002): Chlorophyll fluorescence parameters: the definitions, photosynthetic meaning, and mutual relationships. Photosynthetica 40: 13-29.

Saad BR, Fabre D, Mieulet D, Meynard D, Dingkuhn M, Al-Doss A, et al. (2012): Expression of the *Aeluropus littoralis AlSAP* gene in rice confers broad tolerance to abiotic stresses through maintenance of photosynthesis. Plant Cell Environ. 35: 626-643.

Saijo Y, Hata S, Kyozuka J, Shimamoto K, Izui K (2000). Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J. 23: 319-327.

Song SY, Chen Y, Chen J, Dai XY, Zhang WH (2011): Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress. Planta 234: 331-345.

Sinha S, Saxena R (2006): Effect of iron on lipid peroxidation, and enzymatic and non-enzymatic antioxidants and bacoside-A content in medicinal plant *Bacopa monnieri* L. Chemosphere 62: 1340-1350.

Strasser BJ (1997): Donor side capacity of Photosystem II probed by chlorophyll a fluorescence transients. Photosynthesis Research 52: 147-155.

Strasser RJ, Srivastava A, Tsimilli-Michael M (2004): Analysis of the Chlorophyll a fluorescence transient. In: Papageorgiou, C., Govindjee, (Eds.), Chlorophyll Fluorescence: A Signature of Photosynthesis. Springer, Netherlands, p. 321–362.

Su CF, Wang YC, Hsieh TH, Lu CA, Tseng TH, Yu SM (2010): A novel MYBS3-dependent pathway confers cold tolerance in rice. Plant Physiology 153: 145-158.

Takesawa T, Ito M, Kanzaki H, Kameya N, Nakamura I (2002): Over-expression of glutathione S-transferase in transgenic rice enhances germination and growth at low temperature. Mol. Breeding 9: 93-101.

Tao Z, Kou Y, Liu H, Li X, Xiao J, Wang S (2011): *OsWRKY45* alleles play different roles in abscisic acid signaling and salt stress tolerance but similar roles in drought and cold tolerance in rice. J. Exp. Bot. 62: 4863-4874.

Theocharis A, Clément C, Barka EA (2012): Physiological and molecular changes in plants at low temperatures. Planta 235: 1091-1105.

Toro EAT (2006): Avaliação de linhagens de arroz (*Oryza sativa* L.) suscetíveis e tolerantes a baixas temperaturas em cruzamentos dialélicos parciais. [Tese] . Piracicaba: Universidade de São Paulo. 144 p.

Tsimilli-Michael M, Strasser RJ (2008): In vivo assessment of plants vitality: applications in detecting and evaluating the impact of Mycorrhization on host plants. In: Varma, A. Mycorrhiza. Uttar Pradesh: Springer, 3ª. Ed., p.679-703.

Vitulo N, Forcato C, Carpinelli EC, Telatin A, Campagna D, D'Angelo M et al. (2014): A deep survey of alternative splicing in grape reveals changes in the splicing machinery related to tissue, stress condition and genotype. BMC Plant Biol**.** 14, (99).

Wang G, Guo Z (2005): Effects of chilling stress on photosynthetic rate and chlorophyll fluorescence parameter in seedlings of two rice cultivars differing cold tolerance. Rice Sci. 12: 187-191.

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

Wang Z, Gerstein M, Snyder M (2009): RNA-Seq: a revolutionary tool for transcriptomics. Nature Rev Genet. 10: 57-63.

Wen JQ, Oono K, Imai R (2002): Two novel mitogen-activated protein signaling components, OsMEK1 and OsMAP1, are involved in a moderate low-temperature signaling pathway in rice. Plant Physiol. 129: 1880-1891.

Yang A, Dai X, Zhang WH (2012): A R2R3-type MYB gene, *OsMYB2*, is involved in salt, cold, and dehydration tolerance in rice. J. Exp. Bot. 63: 2541-2556.

Ye H, Du H, Tang N, Li X, Xiong L (2009): Identification and expression profiling analysis of TIFY family genes involved in stress and phytohormone responses in rice. Plant Mol. Biol. 71: 291-305.

Yusuf MA, Kumar D, Rajwanshi R, Strasser R J, Tsimilli-Michael M, Govindjee, Sarin NB (2010): Overexpression of γ-tocopherol methyl transferase gene in transgenic Brassica juncea plants alleviates abiotic stress: Physiological and chlorophyll a fluorescence measurements. Biochimica et Biophysica Acta 1797: 1428-1438.

Xia J, Zeng C, Chen Z, Zhang K, Chen X, Zhou Y et al. (2014): Endogenous small-noncoding RNAs and their roles in chilling response and stress acclimation in Cassava. BMC Genomics 15 (634).

Xie G, Kato H, Imai R (2012): Biochemical identification of the OsMKK6-OsMPK3 signaling pathway for chilling stress tolerance in rice. Biochem. J. 443: 95-102

Xiong L, Yang Y (2003): Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. Plant Cell 15: 745-759.

Zhang Y, Chen C, Jin XF, Xiong AS, Peng RH, Hong YH, et al. (2009): Expression of a rice *DREB1* gene, *OsDREB1D*, enhances cold and high-salt tolerance in transgenic Arabidopsis. BMB Reports 42: 486-492.

CONTEÚDOS ABORDADOS

Capítulo I: manuscrito submetido ao período *Canadian Journal of Plant Science*. Este capítulo descreve o *screening* inicial dos genótipos de arroz *indica* em relação à tolerância ao frio durante a germinação e em plantas jovens com três a quatro folhas. Duas linhagens irmãs, provenientes do mesmo cruzamento mas com níveis contrastantes de tolerância ao frio, foram escolhidas para caracterização fisiológica e bioquímica.

Capítulo II: manuscrito publicado no periódico *Plant Science*. Este capítulo descreve a identificação (através da técnica de RNAseq) e caracterização de novos genes envolvidos na resposta ao frio durante o estádio de germinação nos dois genótipos de arroz *indica*, previamente identificados como tolerante e sensível ao frio.

Capítulo III: manuscrito a ser submetido ao periódico *Plant Science*. Este capítulo descreve a identificação (através da técnica de RNAseq) e caracterização de novos genes envolvidos na resposta ao frio durante o estádio vegetativo nos dois genótipos de arroz *indica*, previamente identificados como tolerante e sensível ao frio.

Capítulo IV: manuscrito a ser submetido ao período *Photosynthetica*. Este capítulo descreve análises do funcionamento do aparelho fotossíntético em plantas dos dois genótipos de arroz *indica* durante exposição ao frio e em período de recuperação sob temperatura controle.

CAPÍTULO I

Artigo submetido para publicação:

Canadian Journal of Plant Science

Identification and Physiological Characterization of Two Sister Lines of *indica* Rice (*Oryza sativa* L.) with Contrasting Levels of Cold Tolerance

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Adamski, J.M., Cargnelutti, D., Sperotto, R.A., Terra, T.F., Rosa, L.M.G., Cruz, R.P., Fett, J.P. 2015. **Identification and physiological characterization of two sister lines of *indica* rice (*Oryza sativa* L.) with contrasting levels of cold tolerance.** Exposure to low temperature during germination and vegetative growth is a limiting factor to the establishment and development of rice seedlings. Higher cold tolerance of *japonica* than *indica* subspecies is well documented. However, reports of cold tolerance in *indica* genotypes are rare. We screened a large number of *indica* rice genotypes for cold tolerance during germination and initial vegetative growth. The *indica* genotypes IRGA 959-1-2-2F-4-1-4-A and IRGA 959-1-2-2F-4-1-4-D-1-CA-1, derived from the same cross, were characterized, respectively, as tolerant and sensitive to low temperature. Indexes of photosynthetic performance during light absorption were heavily affected by cold in both genotypes, but recovered after cold exposure only in the tolerant genotype. Activities of the antioxidant enzymes SOD and CAT (at the vegetative stage) and CAT and APX (at the germination stage) were higher in the tolerant than in the sensitive genotype. Expression

of twenty genes previously related to cold response in rice was evaluated. Expression of *OsLIP9* and *OsWCOR413* were higher in the tolerant genotype upon or prior to cold exposure, respectively. The two sister lines show different molecular and physiological responses to low temperature stress. Further in-depth studies with these lines may help to identify new cold tolerance mechanisms in rice.

Key words: Abiotic stress, antioxidant enzymes, cold, gene expression, *indica* rice, JIP-test.

Adamski, J.M., Cargnelutti, D., Sperotto, R.A., Terra, T.F., Rosa, L.M.G., Cruz, R.P., Fett, J.P. 2015. **Identification et caractérisation de deux lignes sœurs de riz *indica* (*Oryza sativa* L.) avec des niveaux contrastés de tolérance au froid.** L'exposition à basse température pendant la germination et la croissance végétative est un facteur limitant à l'établissement et le développement des plantules de riz. La tolérance supérieure au froid observée en *japonica* par rapport à sous-espèces *indica* c'est bien documentée. Cependant, les rapports de la tolérance adéquate au froid dans les génotypes de *indica* sont encore rares. Cette étude a examiné plusieurs génotypes de *indica* en relation à leurs tolérances au froid pendant la germination et la croissance végétative initiale. Les génotypes *indica* IRGA 959-1-2-2F-4-1-4-A et IRGA 959-1-2-2F-4-1-4-D-1-CA-1, qui sont dérivées d'un meme croisement, ont été caractérises comme tolérant et sensible à basse température. Les indices de performance photosynthétiques pendant l'absorption de lumière ont été fortement affectés par le froid dans les deux génotypes. Cependant, seulement le génotype tolérant a été capable de se récupérer après l'exposition au froid. L'activité des enzymes antioxydantes SOD et CAT (au stade végétatif) et CAT et APX (au stade germinatif) ont été plus élevés pour le génotype tolérant que pour le génotype sensible. L'expression de vingt gènes précédemment liés à réponse au froid dans le riz a été évaluée. Les profils d'expression de *Os*LIP9 et *Os*WCOR413 ont été plus élevés dans le génotype tolérant avant ou après l'exposition au froid. Ces deux lignées sœurs présentent des différents mécanismes moléculaires et physiologiques pour répondre au stress engendré par la basse température. De nouvelles études approfondies avec ces lignées peuvent aider l'identification des nouveaux mécanismes de tolérance au froid dans le riz.

Mots clés: Stress abiotique, enzymes antioxydantes, froid, l'expression du gène, le riz *indica*, test JIP

Abbreviations: **ABS/RC**, energy flux absorption per reaction center; **APX**, ascorbate peroxidase; **CAT**, catalase; **DI_0/RC**, energy dissipation per reaction center; **ET_0/ABS**, quantum yield of electron transport from Q_A^- to the intersystem electron acceptors; **ET_0/CS**, flow of electron transport per CS in t = 0; **ET_0/RC**, electron transport flux per reaction center; **ET_0/TR_0**, probability, at t = 0, that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-; **H_2O_2**, hydrogen peroxide; **PI_{ABS}**, performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors; **$PI_{ABS,total}$**, performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase; **TBARS**, thiobarbituric acid reactant substances; **TR_0/CS**, flow of energy captured by CS in t = 0; **TR_0/RC**, energy captured flow per reaction center; **φD_0**, quantum yield, at t = 0, of energy dissipation.

Rice is a staple food for half of the world population. Abiotic stresses have negative impacts on rice plants, often leading to decreased grain yield. Among these, low temperatures can be particularly harmful due to the tropical origin of the rice species (Cruz et al. 2013).

Plants of tropical or subtropical origin can be injured or killed by non-freezing low temperatures. Chlorosis, necrosis, and growth retardation are symptoms of chilling injury (Iba 2002). In contrast, plants from chilling tolerant species are able to grow at temperatures ranging from 0 to 15 ^oC (Sanghera et al. 2011). Cold air and cold irrigation water can damage rice plants during any developmental stage such as germination, seedling, vegetative, reproductive and grain maturity (Xu et al. 2008), although cold exposure at different developmental stages may result in different symptoms and degrees of injury (Li et al. 1981). The rice subspecies *indica* and *japonica* differ widely in cold tolerance. Several studies have demonstrated that cold tolerance is higher among *japonica* than in *indica* genotypes (Li et al. 1981; Lee 2001; Cruz and Milach 2004). Therefore, *Japonica* cultivars are the main source of cold tolerance for the rice species.

Cold stress is a serious problem for rice growing in twenty-five countries, including Korea and Japan, and even in tropical countries such as the Philippines and Thailand

(Kaneda and Beachell 1974). In Australia, losses due to low temperature during the reproductive stage range from 0.5 to 2.5 t/ha in 75% of the years (Singh 2005). Considering the total area cultivated with rice in Australia, cold damage results on average losses of $ 23.2 million per year to the rice industry, with the available genotypes. In Korea, low temperatures in the mountain areas can damage rice plants at any stage between germination and maturity. In years when low temperatures are extreme, rice growing in all regions of Korea is affected at the reproductive stage, with up to 26% losses in grain yield (Lee 2001).

In Brazil, the largest rice producer outside Asia, cold occurrence is common in the southernmost state of Rio Grande do Sul (RS), where over 68% of the Brazilian rice grain is produced annually in about one million hectares of irrigated land (IBGE 2015). The negative impact of low temperature on final yield can be as high as 25% (Cruz et al. 2013). The incidence of low temperatures during early stages of rice development (germination and vegetative stages) delays the establishment of the crop and lowers plant stand (Cruz et al. 2013). To ensure the best light incidence during the reproductive phase, rice sowing in RS is performed when low temperatures still occur in the cooler periods of the day (Cruz and Milach 2004). Losses due to cold stress during germination and vegetative growth are increased because the varieties cultivated in this region belong to the *indica* subspecies, sensitive to cold (Cruz et al. 2000; Saito et al. 2004). *Indica* rice accounts for almost the totality of cultivated rice in RS, as well as in Uruguay and Argentina (Bierlen et al. 1997).

The effects of low temperatures on metabolism, physiology and gene expression of rice cultivars from the *japonica* subspecies have been shown (Xie et al. 2009; Saad et al. 2012). Although cold stress has an important impact on *indica* rice production in sub-tropical areas, reports about cold responses in different *indica* genotypes are rare (Zhang et al. 2012a). Therefore, comparative studies between *indica* genotypes sensitive and tolerant to low temperature may help to understand possible tolerance mechanisms and to provide useful information to breeding programs aiming at *indica* genotypes adapted to temperature oscillations.

During the early stages of development (germination and vegetative), rice plants are damaged by chilling (temperatures lower than 10 to 13 °C) (Yoshida 1981). In the germination stage, the most common symptoms of cold temperature damage are delayed and lower percentage of germination (Cruz and Milach 2000). In the vegetative stage, one of the major effects is the inhibition of photosynthetic activity through reduced electron transport in the photosystem II (PSII) (Jeong et al. 2002). Due to reduced activity of

electron transport proteins, the PSII (P680) is overloaded with energy excess, damaging PSII proteins and compromising ATP and NADPH production (Hüner et al. 2012). Moreover, as a consequence of excess excitation energy, there is formation of triplet chlorophyll, which reacts with molecular oxygen generating singlet oxygen. Oxygen reduction also leads to the formation of superoxide anion, hydroxyl radical and hydrogen peroxide (Foyer and Noctor 2005). Additionally, the photo-oxidative stress is considerably enhanced when lower temperatures are combined with higher illumination (Lukatkin 2005), inducing photoinhibition of PSII in chilling-sensitive plants (Jeong et al. 2002). To decrease free radical toxicity, plant cells may increase the activities of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase and catalase, which participate in scavenging of reactive oxygen species (ROS) and hence protect cells from injury (Mittler 2002).

Several rice genes have been identified as responsive to low temperature stress. Most of these genes are transcription factors involved in cold tolerance through the control of other cold-responsive genes. One class of cold related transcription factors present in several species is the DREB1/CBF (Dubouzet et al. 2003). Manipulation of *DREB1/CBF* rice genes (*OsDREBL, OsDREB1A*, *OsDREB1B*, *OsDREB1F*) contributed to increased cold tolerance (Dubouzet et al. 2003; Ito et al. 2006; Wang et al. 2008). Other transcription factors isolated and characterized as cold-responsive in *japonica* rice cultivars include *OsLIP19*, *OsCOIN* and *OsABF2* (bZIP-like transcription factors) (Wen et al. 2002; Liu et al. 2007; Hossain et al. 2010); *OsZFP245* (C2H2-type zinc finger protein) (Huang et al. 2009) and *OsMYB3R-2* (Ma et al. 2009).

A MYBS3-dependent and mediated signaling pathway has been identified in rice (Su et al. 2010). Rice plants overexpressing this gene are able to tolerate 4 °C for at least one week without compromising yield. Interestingly, MYBS3 represses the expression of the DREB1/CBF pathway. According to the authors, MYBS3 signaling is sufficient for cold tolerance in rice (Su et al. 2010). It was suggested that DREB1 proteins take part in a rapid response to cold, while MYBS3 acts in a slow response. Therefore, different pathways probably act sequentially in the adaptation of rice plants to low temperature stress.

Several other gene families are involved in responses to cold stress in rice, and overexpression of some of the genes resulted in cold tolerance: *OsLIP5* and *OsLIP9* (low-temperature-induced dehydrin proteins) (Aguan et al. 1991); *OsCDPK7* (Ca^{2+}-dependent protein kinase) (Saijo et al. 2000); *OsMEK1* and *OsMAP1* (mitogen-activated protein

signaling components) (Wen et al. 2002); *OsWCOR413* (unknown function membrane protein) (de Los Reyes et al. 2003); and *OsAsr1* (hydrophilin) (Kim et al. 2009).

Despite the large number of cold-responsive genes identified in rice, the vast majority of studies was performed with *japonica* genotypes. Here, we screened *indica* rice genotypes for cold tolerance during germination and initial vegetative growth. We identified two sister lines with contrasting cold tolerance levels, and we provided initial physiological characterization of these two genotypes in response to cold exposure, in an attempt to identify possible features related to cold tolerance mechanisms in *indica* rice.

MATERIAL AND METHODS

Plant Materials and Growth Conditions

The experiments were conducted under growth chamber and greenhouse conditions at Instituto Rio-Grandense do Arroz (IRGA), Cachoeirinha, Rio Grande do Sul, Brazil. All *indica* rice genotypes were obtained from the IRGA germplasm bank.

Screening of Rice Genotypes According to Low Temperature Tolerance

To identify low temperature sensitive and tolerant *indica* rice genotypes at the germination stage, 45 genotypes were evaluated according to the percentage of germinating seeds with coleoptile length ≥ to 5 mm after 26 days at 13 °C in a growth chamber in the dark, following the protocol established by Cruz and Milach (2004). Seeds were germinated in Petri dishes containing filter paper moistened with distilled water. Each genotype was evaluated in three replicates consisting of Petri dishes containing 30 seeds each.

At the vegetative stage, cold tolerance was accessed as percentage of plant survival (recovery capacity) after cold treatment. For this purpose, 90 genotypes were evaluated according to the percentage of three leaf-stage plant survival after ten days at 10 °C under white light with a photoperiod of 16/8 h light/dark (irradiance of approximately 40 µMol m^{-2} s^{-1}) and seven days of recovery in greenhouse conditions (temperature range from 20 to 30 °C, irradiance of approximately 1,000 µMol m^{-2} s^{-1}, average day length of 11:30 h). Three replicates of 10 plants were evaluated per genotype. One cold tolerant *japonica* rice genotype (Diamante) was used as positive control. *Indica* genotypes with no statistical difference in relation to Diamante using both criteria described above were considered cold tolerant.

Cold Treatment

To characterize the response of selected genotypes to cold, seeds were germinated for five days, planted into soil and grown in a greenhouse under ambient temperature (20 to 30 °C), under full day light, as described above. Plants were grown in plastic trays of 16.5 X 16.5 cm, divided in 25 conic cells (5 cm deep, open bottom) filled with soil. Each cell contained one individual plant. Trays with soil were kept inside regular plastic trays filled with enough water to maintain the soil permanently covered by a water layer of about 1 cm. After 20 days of growth in the greenhouse, plants at the three leaf stage were transferred to the climatic chamber with low light intensity (40 µMol m^{-2} s^{-1}), and maintained at 10 °C (cold treatment) for ten days, followed by seven days of recovery in the greenhouse. These plants were evaluated to assess oxidative stress, chlorophyll *a* fluorescence and gene expression. To assess oxidative stress (concentrations of MDA and H_2O_2; activities of CAT, SOD and APX) during seed germination, two genotypes selected as sensitive and tolerant to low temperature stress were subjected to germination in the dark at 28 ºC for two days (control) and at 13 ºC for 14 days (cold treatment). At the end of these periods, seedlings from both treatments were at the S3 stage (Counce et al. 2000).

Chlorophyll *a* Fluorescence Transients

The chlorophyll fluorescence transients were evaluated on the youngest fully expanded leaves (using one leaf per plant) of ten plants from each genotype (sensitive and tolerant), using a portable pulse modulated fluorometer (OS30p, Optisciences, UK). Before the measurements, leaves were dark adapted for 20 minutes. Fluorescence intensity was quantified by applying a saturating pulse of 3,000 µmol m^{-2} s^{-1} and the resulting fluorescence of the chlorophyll *a* measured from 0 to 1 s. The data was used to calculate the parameters of the JIP test (Strasser et al. 2000), in which J corresponds to fluorescence intensity at 2 ms, I corresponds to fluorescence intensity at 30 ms and P corresponds to maximal fluorescence intensity, F_M. The JIP test translates the changes observed in the fluorescence transients to quantitative changes in formulated parameters derived from the energy flux theory. This quantitative analysis of the fluorescence transients can be used to explain the stepwise flow of energy through PSII at the reaction center (RC) level (ABS/RC, TR0/RC, ET0/RC and DI0/RC) as well as the level of a PSII cross-section (CS) (ABS/CS0, TR0/CS0, ET0/CS0 and DI0/CS0) (Strasser et al. 2000) and to evaluate the performance indexes (PI) of photosystem in plants. Most of the sensitive plants showed leaf rolling and wilting after ten days under low temperature. Therefore, fluorescence

analysis of the sensitive genotype was performed only in three periods: before plant exposure to low temperature (control), after three days and after eight days of cold treatment (10 °C, light intensity of 40 µMol m^{-2} s^{-1} and photoperiod of 16/8 h light/dark). In plants from the tolerant genotype, fluorescence was also evaluated after ten days of low temperature stress, and after three and seven days of recovery under room temperature (temperature range from 20 to 30 °C, irradiance of approximately 1,000 µMol m^{-2} s^{-1}, average day length of 11:30 h).

Indicators of Oxidative Stress

Seedlings were grown and subjected to cold stress as described above. Leaf samples were collected after 0, 24, 48 and 72 h of cold treatment for quantification of MDA and H_2O_2.

Malondialdehyde (MDA) concentration was quantified as a final product of lipid peroxidation through reaction with thiobarbituric acid (TBA) (El-Moshaty et al. 1993). Leaves of the rice genotypes (100 mg fresh weight, previously ground in liquid nitrogen) were homogenized with 0.2 M citrate-phosphate buffer (pH 6.5) containing 0.5% Triton X-100. The homogenate was centrifuged at 20,000 g for 15 min. One hundred µL of supernatant was added to equal volume of 20% TCA (w/v) and 0.5% TBA (w/v). The mixture was heated at 95 °C for 40 min and cooled in ice bath for 15 min. After centrifugation at 10,000 g for 15 min, the supernatant absorbance was registered at 532 nm. Correction of non-specific turbidity was made by subtracting the absorbance value taken at 600 nm. The lipid peroxides were expressed as nmol MDA (mg protein)$^{-1}$, using the extinction molar coefficient of 155 L^{-1} mol^{-1} cm^{-1}.

Hydrogen peroxide concentration was determined following the method described by Loreto and Velikova (2001) with modifications. Approximately 100 mg of germinating seedlings (or leaves - volumes within brackets) previously ground in liquid nitrogen were homogenized with 2 mL of 0.1% trichloroacetic acid (TCA) (w/v). The homogenate was centrifuged at 12,000 g for 15 min at 4 °C. Then, 0.5 mL (or 250 µL) of supernatant was added to 0.5 mL (or 250 µL) of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL (or 500 µL) of 1M KI. Hydrogen peroxide concentration was assessed by comparing this absorbance with a calibration curve at 390 nm. The H_2O_2 concentration was expressed as µmol g^{-1} fresh weight.

Activity of Antioxidant Enzymes

Approximately 1 g of germinating seeds previously ground in liquid nitrogen were homogenized with 50 mM sodium phosphate buffer (pH 7.0), containing 10 g L^{-1} polyvinylpyrrolidone, 0.2 mM ethylenediaminetetraacetic acid, and 10 mL L^{-1} Triton X-100. The homogenate was centrifuged at 12,000 g for 20 min at 4 °C. The resultant supernatant (extract) was used in assays to determine the activity of antioxidant enzymes. Catalase (CAT, EC 1.11.1.6) activity was determined in germinating seeds (or leaves - volumes within brackets) following the method of Aebi (1984) with modifications. The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.0), 15 mM (or 25 mM) H_2O_2 and 30 µL (or 10 µL) of extract, in a final volume of 2 mL (or 200 µL). CAT activity was determined by monitoring the consumption of H_2O_2 at 240 nm. Superoxide dismutase (SOD, E.C 1.15.1.1) activity was assayed as described by Beyer and Fridovich (1987), using 15 min of illumination and recording the absorbance at 560 nm. Ascorbate peroxidase (APX, EC 1.11.1.11) activity in germinating seeds (or leaves - volumes within brackets) was assayed in a reaction mixture with final volume of 2 mL (or 200 µL), which contained 25 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbate, 1.0 mM H_2O_2 and 100 µL (or 10 µL) of extract (Zhu et al. 2004). H_2O_2-dependent ascorbate oxidation was recorded by decreasing in absorbance at 290 nm using the molar extinction coefficient of 2.8 mM cm^{-1}. Protein concentration was determined by the Comassie Brilliant Blue method (Bradford 1976) using bovine serum albumin (BSA) as standard in all enzymatic preparations.

Gene Expression Analysis by Real-Time Quantitative PCR

Fully expanded leaves of control plants (before cold exposure) and from plants subjected to 6 h of chilling treatment (10 °C) were harvested and total RNA was extracted using Concert Plant RNA Reagent (Invitrogen) and treated with DNase I (Invitrogen). First-strand cDNA synthesis was performed with reverse transcriptase (M-MLV, Invitrogen) using 1 µg of RNA. RT-qPCRs were carried out in a StepOne Real-Time Cycler (Applied Biosystems). Expression of seventeen previously described cold-related genes and three additional genes which have their expression modified in response to low temperature stress in rice leaves according to the expression databank Genevestigator (http://www.genevestigator.com/) were evaluated in the tolerant and sensitive rice genotypes. All primers (listed in Table 2) were designed to amplify 100-150 bp of the 3'-

UTR of the transcripts and to have similar Tm values (60 °C ± 2). Reaction settings were composed of an initial denaturation step of 5 min at 94 °C, followed by 40 cycles of 10 s at 94 °C, 15 s at 60 °C, 15 s at 72 °C and 35 s at 60 °C (fluorescence data collection); samples were held for 2 min at 40 °C for annealing of the amplified products and then heated from 55 to 99 °C with a ramp of 0.1 °C/s to produce the denaturing curve of the amplified products. RT-qPCRs were carried out in 20 µl final volume composed of 10 µl of each reverse transcription sample diluted 100 times, 2 µl of 10X PCR buffer, 1.2 µl of 50 mM $MgCl_2$, 0.1 µl of 5 mM dNTPs, 0.4 µl of 10 µM primer pairs, 4.25 µl of water, 2.0 µl of SYBR green (1:10,000, Molecular Probe), and 0.05 µl of Platinum Taq DNA polymerase (5 U/µl, Invitrogen, Carlsbad, CA, USA). Gene expression was evaluated using a modified $2^{-\Delta CT}$ method (Schmittgen and Livak 2008), which takes into account the PCR efficiencies of each primer pair (Relative Expression $_{\text{Tested Gene / Control Gene}}$ = $(PCR_{eff}\ CG)^{Ct\ CG}$ / $(PCR_{eff}\ TG)^{Ct\ TG}$) (Schmittgen and Livak 2008). Each data point corresponds to three true biological replicate samples.

Statistical Analysis

The data were subjected to analyses of variance (ANOVA) and mean values were compared by the Tukey test ($P \leq 0.05$) or Student's *t* test ($P \leq 0.05$, 0.01 and 0.001) using the SPSS Base 17.0 for Windows (SPSS Inc., USA). The Levene's test (for homogeneity of variance) was used prior to ANOVA.

RESULTS

Rice Genotypes Sensitive and Tolerant to Low Temperature

Cold tolerance at the germination stage was evaluated in 45 genotypes and tolerance at the vegetative stage was evaluated in 90 genotypes (Figure 1 and Table 1). There was large variation among genotypes (Figure 1). Only three *indica* genotypes were considered cold tolerant on both developmental stages evaluated (Table 1), with no significant differences in relation to the *japonica* positive control (Diamante). Genotypes IRGA 959-1-2-2F-4-1-4-A and IRGA 959-1-2-2F-4-1-4-D-1-CA-1 were characterized, respectively, as tolerant and sensitive to low temperature stress. These two genotypes were chosen for further characterization because they were originated from the same cross (LEMONT / IRGA 117-72-1P-3-2A // P1790-5-1M-4-5M-1B-3M-1B). Differences in gene expression not related

to cold responses should be less abundant when comparing these two sister lines than in comparisons within a pair of genetically distant lines.

The final percentage of germination 26 days after imbibition at 13 °C was not significantly different between the two genotypes (ranging from 80 to 87%). However, progression of coleoptile growth was slower in the cold-sensitive genotype (Dametto et al. 2015), resulting in 80% and 15.8% of the seeds with coleoptile length ≥ to 5 mm, respectively in the tolerant and sensitive genotypes (26 days at 13 °C - Table 1). Both genotypes reached above 93% germination after one week of imbibition at 28 °C, with 100% of seeds with coleoptile length ≥ to 5 mm.

At the vegetative stage, percentage of plant survival after ten days of cold treatment and seven days of recovery was about 90% and 15%, respectively, for the tolerant and sensitive genotypes (Table 1).

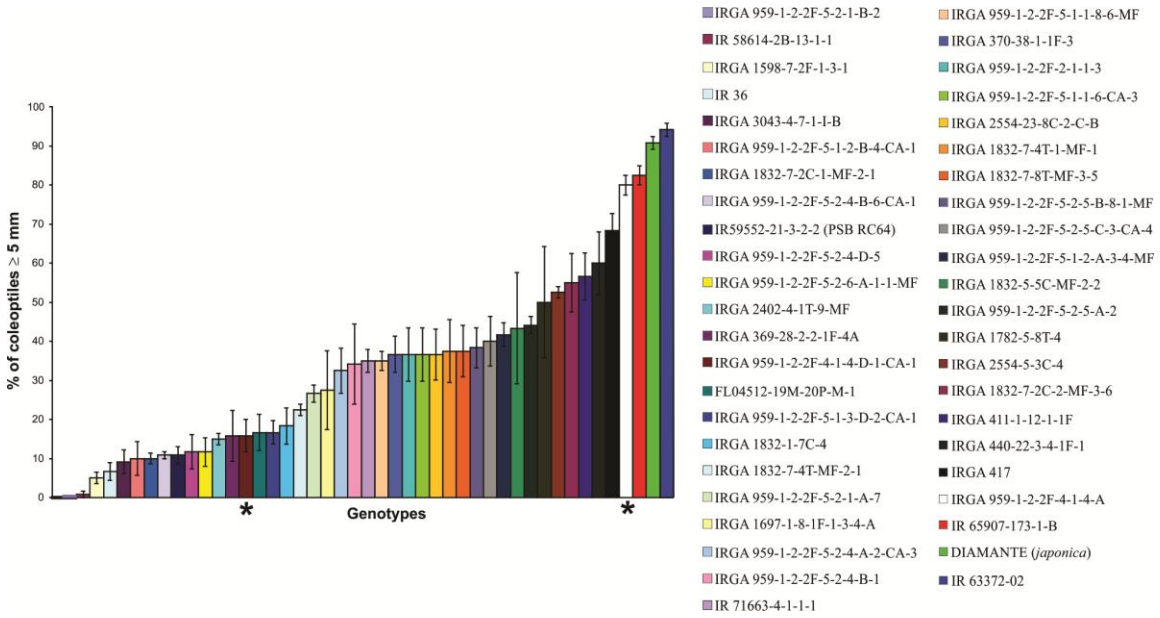


Figure 1. Screening of *indica* rice genotypes for cold tolerance at the germination stage. Percentage of germinating seeds with coleoptile length ≥ to 5 mm after 26 days at 13 °C was evaluated for 45 genotypes (values are averages ± SE of tree replicates with at least 30 seeds each). One *japonica* genotype (Diamante) was used as positive control for cold tolerance. Two genotypes resulting from the same cross and with contrasting levels of cold tolerance are indicated by asterisks. These genotypes were used in further experiments. The value for the first genotype (IR63372-02) is zero in all replicates.

Table 1. Cold tolerance evaluation of *indica* rice genotypes from the germplasm collection at IRGA. The ***japonica*** **genotype "Diamante" was used as positive control for cold tolerance. Plant survival after ten days at 10 °C and seven days of recovery was evaluated for 90 genotypes (values are averages from three replicates with at least 10 plants each). Percentage of germinating seeds with coleoptile length ≥ to 5 mm after 26 days at 13 °C was evaluated for 45 genotypes (values are averages from three replicates with at least 30 seeds each)**

Genotype	Survival at vegetative stage (%)	Germinating seeds with coleoptile length ≥ 5 mm (%)
AGULHÃO ARGENTINO	50.00	ND
BR-IRGA 412	66.60	ND
BRS TAIM	76.60	ND
CICA 4	63.30	ND
COLOMBIANO	60.00	ND
CT 10491-12-4-2T-3P-2P-1	66.66	ND
CT 10693-C10-1-1F-10-1	76.66	ND
CT 10992-3-4-1T-3P-2P-3	96.66	ND
CT 11626-14-4-1-2-M-M	76.66	ND
CT 12245-26-2-4P-1P	90.00	ND
CT 12376-22-1P-M-3	0***	ND
CT 8008-16-31-8P-1	0***	ND
CT 8240-1-1-3P-2	6.66**	ND
CT 9145-4-21-5P-1-MI-F8-3P	0***	ND
CT 9736-9-7-2-2-1P-F	66.66**	ND
CT 9892-6-2-1E-2-F7	86.66	ND
CT13733-1-1C-1-B-B	0***	ND
CT9882-16-4-2-3-2P-M	0***	ND
Diamante (*japonica*)	**93.33**	**90.83**
EL PASO L 144	46.66	ND
FL00162-1P-5-3P	36.66*	ND
FL00440-47P-6-1P-M	30.00*	ND
FL00443-33P-4-3P-M-M-M	0***	ND
FL00593-4P-1-1P-M-M-M	0***	ND
FL00658-22P-2-1P-M	0***	ND
FL00797-9P-14-1P-M-M-M	76.66	ND
FL00867-10P-15-3P-M-M-M	76.66	ND
FL00898-6P-12-3P-M-M-M	0***	ND
FL01870-5P-3-1P-M-M-M	0***	ND
FL01928-5P-4-2P-M-M-M	10.00**	ND
FL02007-1P-1-3	63.33	ND
FL02095-4P-1-4P-2-M-1P-F12-1	73.33	ND
FL02095-4P-1-4P-2-M-1P-F12-3	50.00	ND
FL02759-16P-4-1P-3-M-1P-F12	0***	ND
FL02759-16P-4-1P-3-M-1P-F12-4	20.00*	ND
FL02955-5P-16-3P-1P-F11	10.00**	ND
FL04512-19M-20P-M-1	83.33	16.66***
FL04518-7M-6P-4M-3	20.00*	ND
FL04540-2M-10P-5M-1-1-M	10**	ND
FL04616-1P-11-M-2	3.33***	ND
FL04870-1P-17-1P-3P-M-F7	0***	ND
FL05310-1P-11-M-1-1	43.33*	ND
FL05372-7P-1-2P-1P-M	16.66*	ND
FL05372-7P-1-2P-1P-M-F7	6.66**	ND
G368094	0***	ND
IR 1552 (purple leaves)	76.66	ND
IR 36	0***	6.66***
IR 58614-2B-13-1-1	93.33	0.83***
IR 63372-02	**90.00**	**94.17**
IR 65907-173-1-B	**86.66**	**82.5**
IR 71663-4-1-1-1	86.66	35.0***
IR50	0***	ND

IR59552-21-3-2-2 (PSB RC64)	83.33	10.83***
IR72158-11-5-2-3	0***	ND
IR72903-121-2-1-2	0***	ND
IR77700-84-2-2-2	23.33*	ND
IRGA 1085-3-3-2F-1-A-1	30.00*	ND
IRGA 1555-23-1F-1-5-1	16.66*	ND
IRGA 1580-33-1-3-2	3.33***	ND
IRGA 1588-1-5-2-2-6	6.66**	ND
IRGA 1598-7-2F-1-3-1	0***	5.00***
IRGA 1697-1-8-1F-1-3-4-A	10.00**	27.50*
IRGA 1782-5-8T-4	3.33***	50.00
IRGA 1832-7-8T-MF-3-5	16.66**	37.50**
IRGA 1832-7-2C-2-MF-3-6	ND	55.00*
IRGA 1832-5-5C-MF-2-2	ND	43.33
IRGA 1832-7-4T-1-MF-1	ND	37.50**
IRGA 1832-7-4T-MF-2-1	ND	22.50***
IRGA 1832-1-7C-4	ND	18.33***
IRGA 1832-7-2C-1-MF-2-1	ND	10.00***
IRGA 2402-4-1T-9-MF	3.33***	15.00***
IRGA 2423-2-10V-2V-2	50.00	ND
IRGA 2554-23-8C-2-C-B	5.00**	36.66**
IRGA 2554-5-3C-4	20.00*	52.50***
IRGA 2694-25-3	6.66**	ND
IRGA 2807-1-16-4-2V	0***	ND
IRGA 2852-20-4-3-3V	76.66	ND
IRGA 2855-20-3-3-6-I-TM-PM-2	0***	ND
IRGA 2912-19-7-I-3	0***	ND
IRGA 3043-4-7-1-I-B	83.33	9.16***
IRGA 3125-9-9-2-I	50.00	ND
IRGA 3165-6-1-1 (short Amy)	65.00	ND
IRGA 3217-10-1Pg-4Pg-3 (hairy)	20.00*	ND
IRGA 369-28-2-2-1F-4A	0***	15.83**
IRGA 370-38-1-1F-3	16.66*	36.66***
IRGA 411-1-12-1-1F	3.33***	56.66**
IRGA 416	0***	ND
IRGA 417	83.33	68.33*
IRGA 419-16-1-17-1	0***	ND
IRGA 440-22-3-4-1F-1	93.33	60.00*
IRGA 575-12-3-3-6-3A	6.66***	ND
IRGA 653-1-13-1-2B	23.33*	ND
IRGA 959-1-2-2F-2-1-1-3	90.00	36.66**
IRGA 959-1-2-2F-4-1-4-A	**90.00**	**80.00**
IRGA 959-1-2-2F-4-1-4-D-1-CA-1	15.00*	15.83***
IRGA 959-1-2-2F-5-2-1-B-2	ND	0.00***
IRGA 959-1-2-2F-5-2-5-A-2	ND	44.17***
IRGA 959-1-2-2F-5-1-2-A-3-4-MF	ND	41.67***
IRGA 959-1-2-2F-5-2-5-C-3-CA-4	ND	40.00**
IRGA 959-1-2-2F-5-2-5-B-8-1-MF	ND	38.33**
IRGA 959-1-2-2F-5-1-1-6-CA-3	ND	36.67**
IRGA 959-1-2-2F-5-1-1-8-6-MF	ND	35.00***
IRGA 959-1-2-2F-5-2-4-B-1	ND	34.17*
IRGA 959-1-2-2F-5-2-4-A-2-CA-3	ND	32.50**
IRGA 959-1-2-2F-5-2-1-A-7	ND	26.67***
IRGA 959-1-2-2F-5-1-3-D-2-CA-1	ND	16.67***
IRGA 959-1-2-2F-5-2-4-D-5	ND	11.67***
IRGA 959-1-2-2F-5-2-6-A-1-1-MF	ND	11.67***
IRGA 959-1-2-2F-5-2-4-B-6-CA-1	ND	10.83***
IRGA 959-1-2-2F-5-1-2-B-4-CA-1	ND	10.00***
IRGA 975-2-2-3F-1-2-2-A	23.33*	ND
ORIZYCA 1	3.33***	ND

ORYZICA LLANOS 5	23.33**	ND

ND: not determined.
Mean values with one, two or three asterisks are different by the Student's T test ($p \leq 0.05$, 0.01 and 0.001, respectively) compared to Diamante (*japonica* cultivar used as a positive control for cold tolerance). Percentage values of individual replicates (n = 3) were transformed as square root of (x+1) prior to statistical comparisons. Genotypes considered tolerant (not different from Diamante) by both screening criteria are marked in bold. Tolerant and sensitive genotypes used in further experiments are shaded in gray.

Chlorophyll *a* Fluorescence Transients

Some parameters obtained from the JIP Test are shown in radar graphs (Figure 2). After eight days of cold stress, the energy flux absorption (ABS/RC) and the energy dissipation per reaction center (DI_0/RC) increased in comparison to the control ($p \leq 0.01$), in both genotypes (Figure 2A and B). These two specific fluxes per reaction center (ABS/RC and DI_0/RC) increased further in the tolerant genotype after ten days of cold exposure (data not shown), while the sensitive plants showed leaf rolling and wilting, impeding the measurements. After eight days of cold stress, the electron transport flux per reaction center (ET_0/RC) was smaller than in the control treatment in both genotypes ($p \leq 0.05$) and the energy captured flow per reaction center (TR_0/RC) had similar values to the control, also in both genotypes (Figure 2A and B).

The cold tolerant plants were selected based on their ability to recover after cold treatment. To investigate the mechanisms involved in stress recovery, we evaluated JIP test parameters in tolerant plants after three and seven days of recovery (Figure 2C and D). The data was normalized in relation to the values obtained before the cold treatment (Figure 2C) and to the values obtained after ten days of stress (Figure 2D). After seven days of recovery, parameters of specific flow per reaction center (DI_0/RC and ET_0/RC) were equivalent to the values from before cold treatment (Figure 2C). Also, ABS/RC ($p \leq 0.01$) and TR_0/RC ($p \leq 0.0006$) were higher than before cold exposure in the tolerant genotype (Figure 2C). The parameter which describes the energy flux efficiency (ET_0/TR_0, at t = 0, probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-) decreased in both genotypes ($p \leq 0.01$) after eight days of cold stress (Figure 2A and B). In the recovery period, this parameter was still below the control (before cold treatment, $p \leq 0.0001$) in the tolerant genotype (Figure 2C).

The yield or flux ratio, $\varphi D_0 = F_0/F_M$ (quantum yield of energy dissipation at t = 0), increased in both genotypes ($p \leq 0.0001$) upon cold treatment (Figure 2A and B). After

seven days of recovery, this parameter was smaller than before cold treatment ($p \leq 0.0001$) in the tolerant genotype (Figure 2C).

ET_0/ABS, the parameter which describes the quantum yield of electron transport from Q_A^- to the intersystem electron acceptors, decreased in both genotypes ($p \leq 0.0001$) after eight days of cold stress (Figure 2A and B). In the tolerant genotype, ET_0/ABS increased about 65% after seven days of recovery in greenhouse conditions, when compared to the values after ten days of cold exposure (Figure 2D) ($p \leq 0.0002$). However, the recovery was not complete in relation to the conditions before cold treatment, reaching 83% of the original value (Figure 2C).

The parameters of phenomenological energy flow based on the excited sample area (CS) decreased in both genotypes ($p \leq 0.0001$) after eight days of cold. These parameters are TR_0/CS (flow of energy captured by CS in t = 0) and ET_0/CS (flow of electron transport per CS in t = 0) (Figure 2A and B). At the recovery period, these parameters increased and reached over 86% of control levels (ET_0/CS) or even levels 14% higher than control ones (TR_0/CS) in the tolerant genotype (Figure 2C).

The parameter PI_{ABS}, which includes partial potential of energy conservation, and the $PI_{ABS,total}$, which adds to their components the parameter that indicates the reduction of the final acceptor PSI, were extremely affected by low temperature in both genotypes, decreasing under cold treatment ($p \leq 0.0002$) (Figure 2A and B). However, at the recovery period, PI_{ABS} and $PI_{ABS,total}$ for the tolerant genotype were, respectively, about 4-fold and 3.4-fold higher than under 10 °C (Figure 2D), although these values were still below the ones determined before the onset of the cold treatment (Figures 2C and 3). The tolerant plants were able to recover 63% (PI_{ABS}) and 58% ($PI_{ABS,total}$) of the original values (before cold treatment), which was not possible for the sensitive plants (Figure 3). Plants from the tolerant genotype remained green after cold exposure, whereas plants from the sensitive genotype showed severe leaf rolling and wilting, and died.

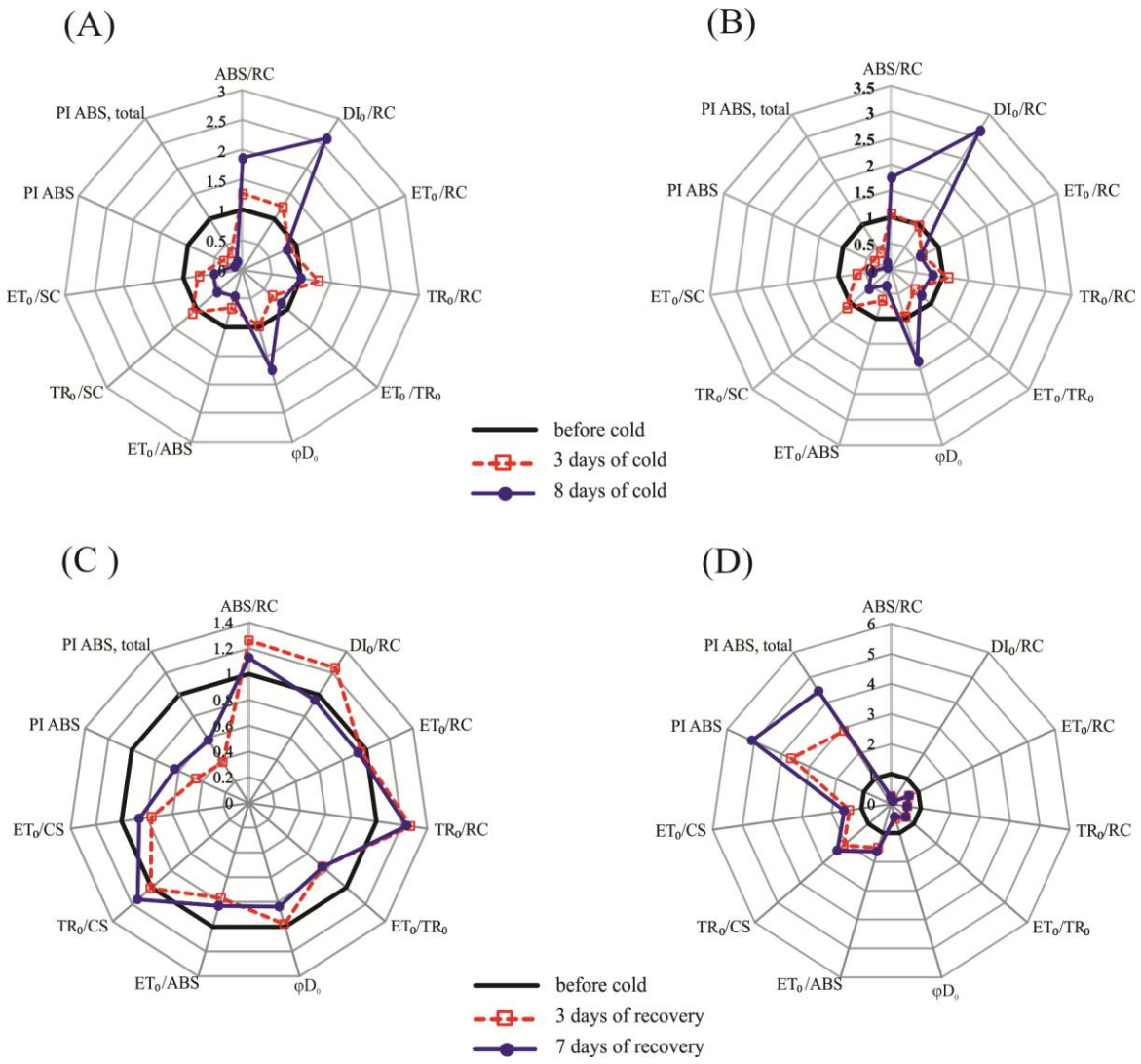


Figure 2. JIP-test parameters calculated from the chlorophyll *a* fluorescence transient in rice seedlings of *indica* genotypes. (A) Tolerant genotype IRGA 959-1-2-2F-4-1-4-A and (B) sensitive genotype IRGA 959-1-2-2F-4-1-4-D-1-CA-1 subjected to cold stress at 10 °C for different periods (three or eight days). For each parameter in (A) and (B), values were normalized relative to values measured before the onset of the chilling treatment. (C) and (D): Tolerant Genotype IRGA 959-1-2-2F-4-1-4-A subjected to cold stress for ten days followed by recovery at 28 °C for seven days. For each parameter, data were normalized using values measured before the onset of the chilling treatment (C) or using the values measured after ten days of cold stress (D). **ABS/RC**, energy flux absorption per reaction center; **DI_0/RC**, energy dissipation per reaction center; **ET_0/RC**, electron transport flux per reaction center; **TR_0/RC**, energy captured flow per reaction center; **ET_0/TR_0**, probability, at t = 0, that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-; **φD_0**, quantum yield, at t = 0, of energy dissipation; **ET_0/ABS**, quantum yield of

electron transport from Q_A^- to the intersystem electron acceptors; **TR_0/CS**, flow of energy captured by CS in t = 0; **ET_0/CS**, flow of electron transport per CS in t = 0; **PI_{ABS}**, performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors; **$PI_{ABS,total}$**, performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors.

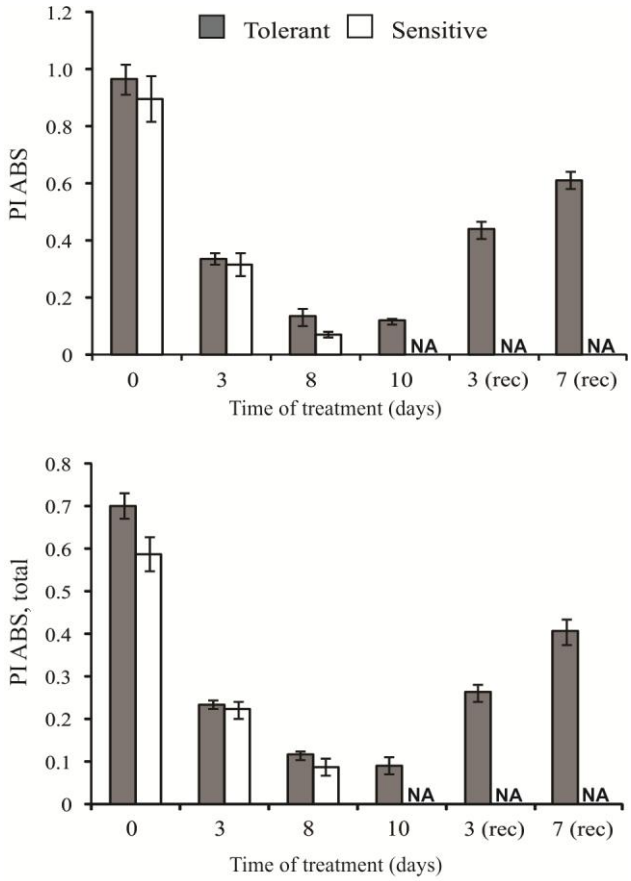


Figure 3. Effects of cold treatment (exposed to 10 °C) followed by recovery period (Rec) on PI_{ABS} and $PI_{ABS,\ total}$ of rice leaves of *indica* genotypes. Actual values were normalized relative to values measured before the onset of the chilling treatment. Bars indicate SE (*n* = 8). **NA** = not analyzed, due to the lack of live plants. **PI_{ABS}** = performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors. **$PI_{ABS,total}$** = performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors.

Lipid Peroxidation and Hydrogen Peroxide Concentration

At the germination stage, H_2O_2 and TBARS (thiobarbituric acid reactant substances, expressed as nmol MDA per mg of protein) levels were not altered in plants from both genotypes. There were also no significant differences in MDA levels between the two *indica* genotypes in the periods of cold stress evaluated during the vegetative stage (data not shown).

Both tolerant and sensitive genotypes presented similar H_2O_2 levels and maintained these levels until 48 h after the beginning of chilling treatment at the vegetative stage. However, after 72 h at 10 °C, the H_2O_2 levels were higher than at 48 h of treatment in the tolerant genotype, while no significant changes were seen in the sensitive genotype (Figure 5).

Activities of Antioxidant Enzymes

The genotypes selected as tolerant and sensitive to cold were germinated under control conditions (28 ºC) or under cold treatment (13 ºC) for 14 days prior to evaluation of antioxidant enzyme activities. SOD activity did not change between the two *indica* genotypes (data not shown). However, CAT and APX activities were higher in the tolerant than in the sensitive genotype regardless of cold treatment (Figure 4).

SOD, CAT and APX activities of the two *indica* genotypes under low temperature stress at the vegetative stage were also evaluated (Figure 5). Leaves of the tolerant genotype showed a slight decrease in SOD activity over the first 24 h of cold exposure. However, a significant increase in SOD activity (53%) was detected from 24 to 48 h at 10 °C. After 72 h of chilling exposure, SOD activity decreased, reaching values equivalent to the ones seen at the onset of the cold treatment. In the sensitive genotype, on the other hand, SOD activity was similar throughout the period evaluated. CAT activity of the cold-tolerant genotype increased gradually up to 46% after 72h at 10 ºC. However, no significant change in CAT activity was observed in the sensitive genotype during cold exposure. No differences were found in APX activity between the two *indica* genotypes at the evaluated cold stress periods at the vegetative stage.

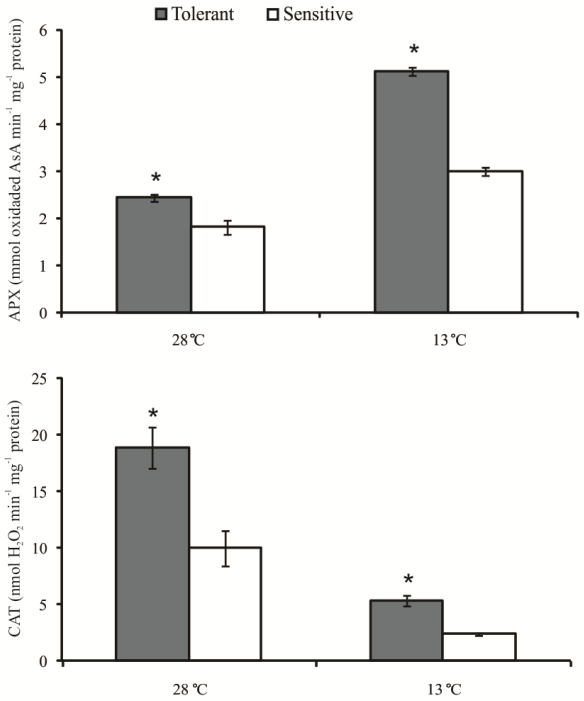


Figure 4. Enzyme activities (CAT and APX) in seeds of the two *indica* rice genotypes subjected to germination at 28 °C for three days and 13 °C for 14 days. All seedlings were at the S3 stage of development after treatments. The results are expressed as means ± SE ($P \leq 0.05$, $n = 3$). Asterisks indicate a statistical difference between genotypes at the same period. AsA: Ascorbic Acid.

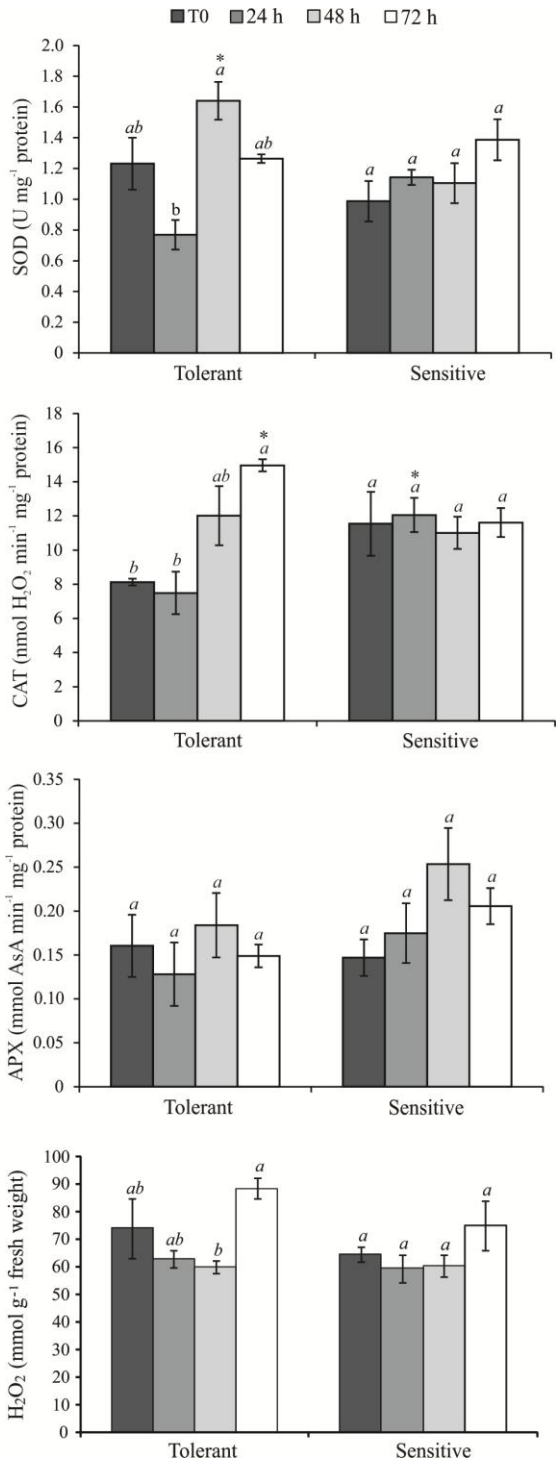


Figure 5. Enzyme activities (SOD, CAT and APX) and H_2O_2 concentration in rice seedlings of *indica* genotypes subjected to cold stress (10 °C under white light with a photoperiod of 16/8 h light/dark and irradiance of approximately 40 µMol m^{-2} s^{-1}). Results are expressed as means ± SE ($P \leq 0.05$, $n = 3$). Different letters indicate statistical difference among hours of treatment in the same genotype. Asterisks indicate a statistical difference between genotypes at the same period. AsA: Ascorbic Acid.

Low-Temperature Gene Expression

In order to investigate the molecular regulation underlying cold tolerance in the described genotypes, we evaluated the expression of twenty genes already described as cold-responsive in rice (Table 2).

At the vegetative stage, expression of *OsLIP9*, *OsDREB1B*, *OsGNV1*, *OsGNV2*, *OsGNV3* and *OsMEK1* increased under cold treatment in both genotypes, while expression of *OsWCOR413*, *OsCDPK7*, *OsMYBS3*, *OsABF2*, *OsMAP1*, *OsASR1* and *OsCOIN* decreased in both genotypes after six hours of exposure to cold. Interestingly, only one gene (*OsLIP9*) reached significantly higher expression in the tolerant genotype than in the sensitive one under cold exposure at the vegetative stage (Figure 6). Considering that *OsLIP9* expression seems to be regulated by the CBF1/DREB1B transcription factor (Lee et al. 2004), it would be expected to see higher *OsDREB1B* expression in the tolerant genotype than in the sensitive one after cold exposure. However, the difference between genotypes was not significant by the statistical test used in this work (Figure 6). Higher expression of *OsWCOR413* was seen before the onset of low temperature stress in the tolerant genotype (Figure 6), which indicates that this gene may maintain a higher basal expression level in this genotype than in the sensitive one. Surprisingly, neither of the tested genotypes presented increased levels of *OsWCOR413* expression after 6 h of cold treatment, as would be expected based on the literature (de Los Reyes et al. 2003). A plausible explanation for such difference in *OsWCOR413* expression could be the different subspecies used (*japonica* versus *indica* in our work) and also the different developmental stages analyzed (5, 8 and 12 days post-imbibition versus three leaf stage in our work, with plants being under cold treatment from 20 to 30 days post-imbibition). Therefore, *OsLIP9* and *OsWCOR413* were the only two genes with significantly higher expression in the tolerant genotype, respectively after and before cold exposure.

At the germination stage, expression of *OsDREB1A*, *OsMYB3R2*, *OsMYBS3* and *OsGNV1* was induced by cold treatment (seven days at 13 °C) in both genotypes, although not detected in seedlings germinating for two days at 28 °C. On the other hand, *qLTG3* expression was repressed by over 200-fold on both genotypes after cold treatment. However, no significant differences in gene expression were observed between genotypes (Supplementary Figure 1).

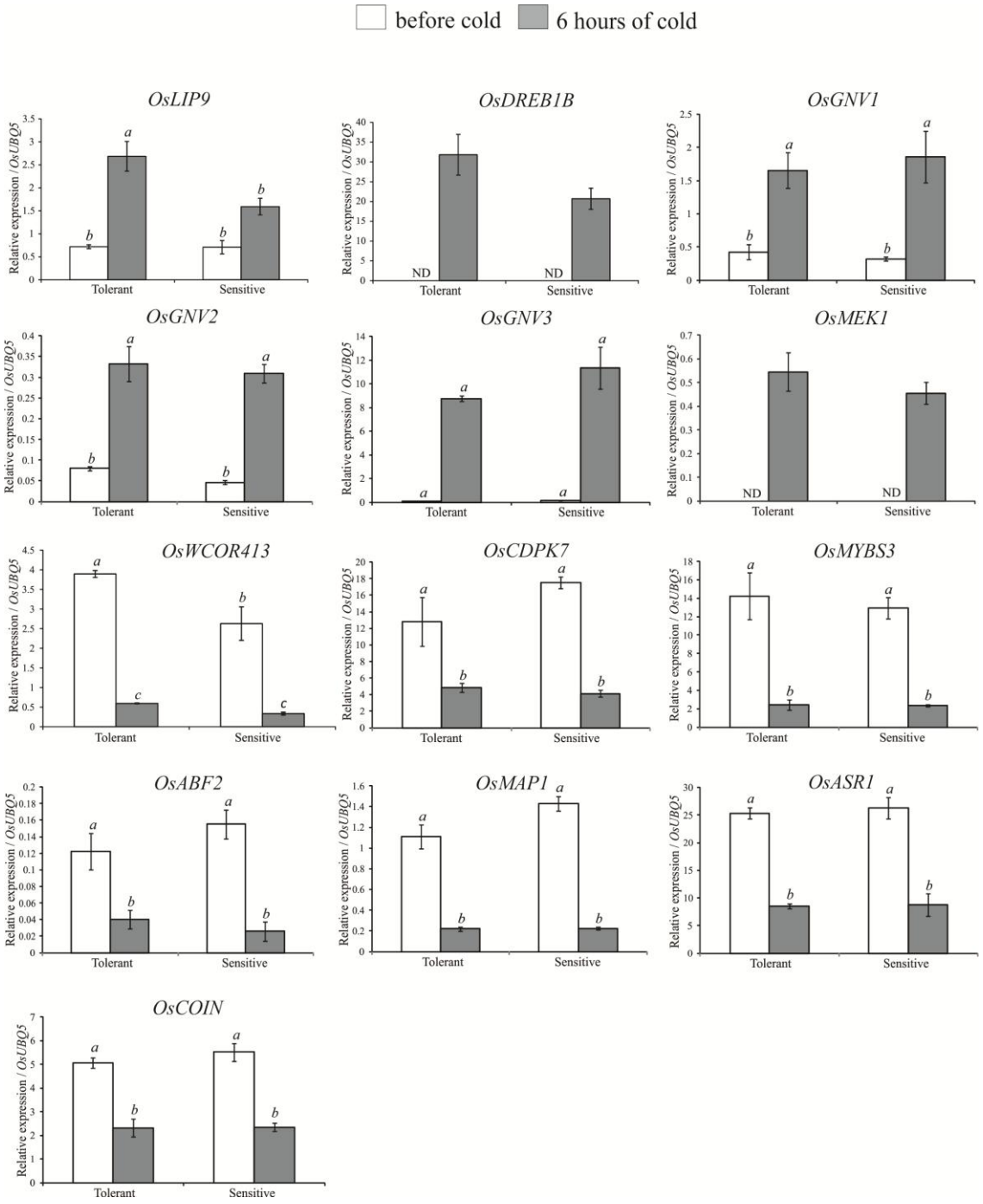
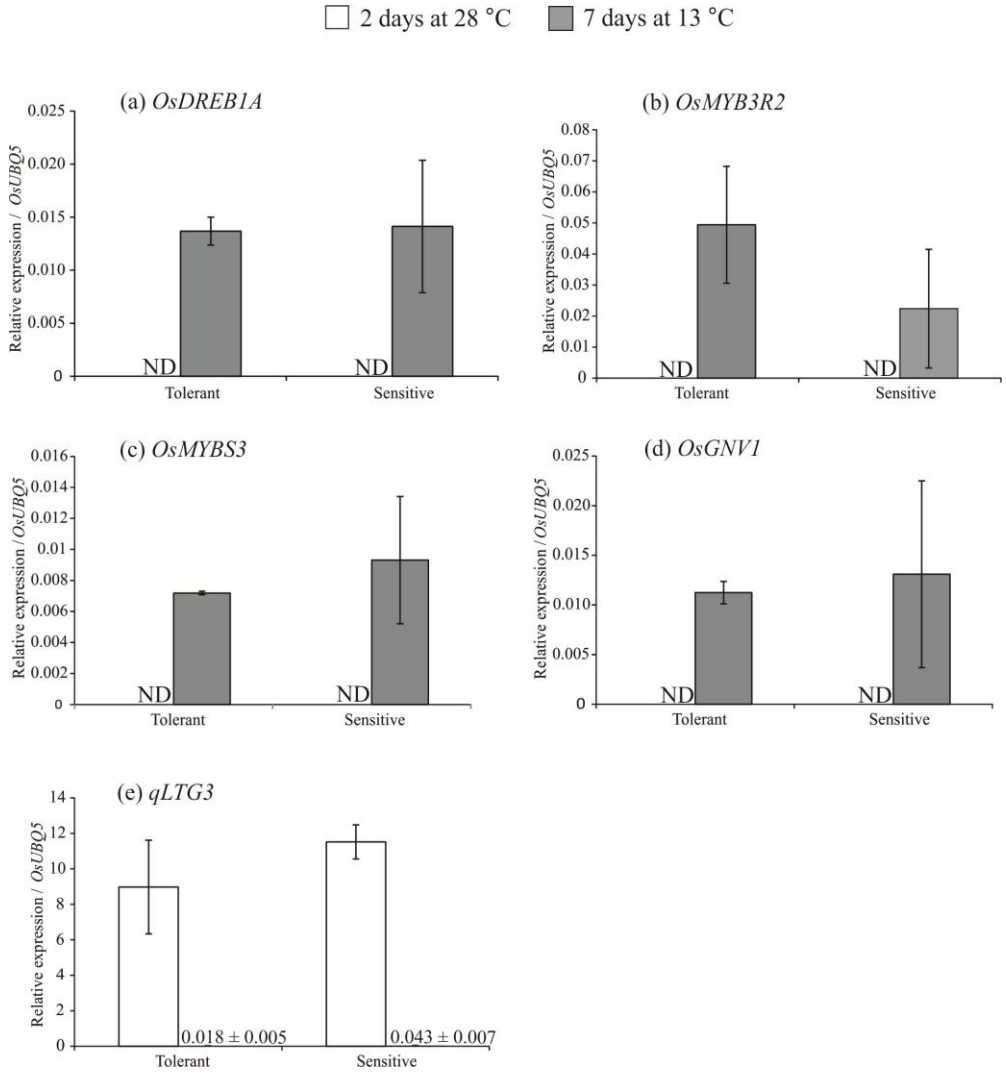


Figure 6. Relative expression levels (RT-qPCR, relative to *OsUBQ5* expression) of previously described cold-related genes in leaves of IRGA 959-1-2-2F-4-1-4-A (cold-tolerant) and IRGA 959-1-2-2F-4-1-4-D-1-CA-1 (cold-sensitive) genotypes before (white bars) and after (gray bars) six hours of cold treatment (10 °C under white light with a photoperiod of 16/8 h light/dark and irradiance of approximately 40 µMol m^{-2} s^{-1}). Values are the averages of three biological replicates ± SE. Different letters indicate that the mean values are different by the Tukey test ($P \leq 0.05$).



Supplementary Figure 1. Relative expression levels (RT-qPCR, relative to *OsUBQ5* expression) of previously described cold-related genes in germinating seeds of IRGA 959-1-2-2F-4-1-4-A (cold-tolerant) and IRGA 959-1-2-2F-4-1-4-D-1-CA-1 (cold-sensitive) genotypes after seven days of imbibition at 13 °C (white bars) or two days of imbibition at 28 °C (gray bars). All seedlings were at the S1 stage of development after treatments. Values are the averages of three biological replicates ± SE. No significant differences were detected using Student's *t* test ($P \leq 0.05$).

Table 2. Gene-specific PCR primers used for qRT-PCR evaluation of gene expression in rice genotypes tolerant and sensitive to cold stress. Gene expression analyses were performed during vegetative and germination stages as indicated

Gene (locus)	Sequence	Vegetative stage	Germination stage	Reference
1) *OsMEK1* (LOC_Os01g32660)	*OsMEK1-F:* TTGGGAGGAATGAGGTTTTG *OsMEK1-R:* TACTGCAACTCCTCCCGAAT	X		Wen et al. (2002)
2) *OsMAP1* (LOC_Os03g17700)	*OsMAP1-F:* AGAGCCGGGTAGACTGAACA *OsMAP1-R:* TCCAAAGTTCCAAACCCTTT	X		Wen et al. (2002)
3) *OsLIP19* (LOC_Os05g03860)	*OsLIP19-F:* GATGTGTTCATCTCCAGTGCTC *OsLIP19-R*: TCACATCACAAACCAGCACA	X (ND)		Wen et al. (2002)
4) *OsDREB1A* (LOC_Os09g35030)	*OsDREB1A-F:* GCATGGGTTGTAGGTTCGAT *OsDREB1A-R*: TGAGCTCCTGGTGACCCTTA	X (ND)	X	Ito et al. (2006)
5) *OsDREB1B* (LOC_Os09g35010)	*OsDREB1B-F:* TCTCGCACTGAAAAGTGTGG *OsDREB1B-R*: GAGAAATCTGGCACATTCCAA	X	X (ND)	Ito et al. (2006)
6) *OsCOIN* (LOC_Os01g01420)	*OsCOIN-F*: TGCTCCAGATCGAATGAATG *OsCOIN-R*: GTGCCTCAGGGACTCAAAGA	X		Liu et al. (2007)
7) *OsDREB1F* (LOC_Os01g73770)	*OsDREB1F-F*: CGACGATGACCATTACCACA *OsDREB1F-R*: CATTCTTCGGGATGTAGAAGC	X (ND)		Wang et al. (2008)
8) *OsZFP245* (LOC_Os07g0588700)	*OsZFP245-F*: GGAGGCTCTGTCAAGGAGAA *OsZFP245-R*: GTGAGCTCTCAGCCTCGTCT	X (ND)		Huang et al. (2009)
9) *OsAsr1* (LOC_Os11g0672)	*OsASR1-F*: CGTGTCGTCGATTTGTGTGT *OsASR1-R*: GGCTGCAGAGAAAATCAAGC	X		Kim et al. (2009)
10) *OsMYB3R-2* (LOC_Os01g62410)	*OsMYB3R2-F*: CGGTGGAGAAGAATTCCAGA *OsMYB3R2-R*: AAAGTGCAGCGAGCAATGTA	X (ND)	X	Ma et al. (2009)
11) *qLTG3-1* (LOC_Os03g01320)	*qLTG3_1-F*: CACCAGGCCACGTACAACTA *qLTG3_1-R*: TTACGGGATGAAGGAGGAAA		X	Fujino et al. (2008)
12) *MYBS3* (LOC_Os10g41200)	*OsMYBS3-F*: CCCAGGCAAAATAAGCTTTG *OsMYBS3-R:* TGTGTGCCAGACAAACAACA	X	X	Su et al. (2010)
13) *OsCDPK7* (LOC_Os04g49510)	*OsCDPK7-F*: CAACGAACTCCTGCGATTTT *OsCDPK7-R*: ACAACCCAGCTTCGTTTGAG	X		Saijo et al. (2000)
14) *OsWCOR413* (LOC_Os03g55850)	*OsWCOR413-F:* TTCAGCGCTTCTCTACACCA *OsWCOR413-R*: AGGCAGGAGGTCCAAACATA	X		de Los Reyes et al. (2003); Lee et al. (2004)
15) *OsLIP5* (LOC_Os03g45280)	*OsLIP5-F*: TGCAATACGTGATGCAGTGA *OsLIP5-R*: TATTACAAGGCACCGTGCAG	X (ND)		Aguan et al. (1991)
16) *OsLIP9* (LOC_Os02g44870)	*OsLIP9-F*: TTGGTGCTTTTTCTGCACTG *OsLIP9-R*: TACCCCACACGAAACACAAA	X		Aguan et al. (1991)
17) *OsABF2* (LOC_Os06g10880)	*OsABF2-F*: TCCTTGTTGGTGAATGGTGA *OsABF2-R*: CCATGCCAGATTTCCCTAAA	X		Hossain et al. (2010)
18) *Os.11719.1.S2_at* (LOC_Os07g46670)	*GNV1-F*: CGCCCTTGTTAATGTTGGAT *GNV1-R*: CACAGACAACAAAAGAGCAGGT	X	X	Tyagi et al (2007)
19) *Os.27759.1.S2_at* (LOC_Os01g46720)	*GNV2-F*: CATCCTTCCGAATCAGGAGA *GNV2-R*: TTTATGCTTCCACCCTGGAC	X	X (ND)	Tyagi et al (2007)
20) *Os.52150.1.S1_at* (LOC_Os04g45970)	*GNV3-F*: GTCCGAAAATGTTGGACGAC *GNV3-R*: TATTATTTTTCGCCGGAACG	X	X (ND)	Tyagi et al (2007)

ND = gene expression not detected.

DISCUSSION

The *indica* genotypes IRGA 959-1-2-2F-4-1-4-A and IRGA 959-1-2-2F-4-1-4-D-1-CA-1 were identified, respectively, as tolerant and sensitive to low temperature stress at two stages (germination and vegetative). These two genotypes originated from the same cross, and therefore are sister lines. The identification of genetically similar genotypes with contrasting levels of cold tolerance may be very useful for the characterization of cold stress responses related to tolerance in *indica* rice, which may rely on distinct mechanisms compared to *japonica* rice. Here we report the characterization of two genotypes that can be useful in understanding such mechanisms.

Exposure to low temperature initiates numerous physiological alterations and photosynthesis is largely affected by cold, although some responses to low temperature may be different in herbaceous and woody plants (Ensminger et al. 2006). Results from chlorophyll fluorescence studies suggest that the reaction center of photosystem II (PSII) is one of the main targets of low temperature stress (Strauss et al. 2007). When rice seedlings are chilled, the superstructure of chloroplasts is altered and the electron transfer activity is decreased (Guo-Li and Zhen-Fei 2005). Additionally, the combination of low temperature and high light results in an imbalance between the light energy absorbed through photochemistry versus the energy utilized through metabolism (Hüner et al. 1998), inducing photodamage (Chytyk et al. 2011; Jeong et al. 2002). The experiments described here were conducted under conditions of low temperature (10 °C) and low light (40 µMol m^{-2} s^{-1}, avoiding excessive photo-oxidative stress). The cold stress in the two evaluated rice genotypes, characterized as tolerant and sensitive to cold, resulted in increases in the parameters ABS/RC and DI_0/RC (Figure 2A and B). ABS/RC values express the total absorption of PSII antenna chlorophylls divided by the number of active (in the sense of Q_A reducing) reaction centers (RCs) (Strasser et al. 2000). The increase in ABS/RC could mean that a fraction of the RCs is inactive or that there was a size increase of the antenna that provides excitation energy for active RCs (Yusuf et al. 2010). In both genotypes, the increase in ABS/RC may be a consequence of inactivation of some RCs promoted by cold stress. DI_0/RC values indicate loss of the energy absorbed by PSII through heat, fluorescence emission, or even energy transfer to other systems (Strasser et al. 2000). In this work, the increase in DI_0/RC indicates that part of the excessive excitation energy under cold treatment was lost (Figure 2A and B). Additionally, plants from both genotypes showed increased quantum yield of energy dissipation (φ_{D0}) (Figure 2A and B). Guo-li and Zhen-fei (2005) analyzed two rice cultivars differing in cold tolerance, and concluded

that increased cold tolerance of Xiangnuo 1 relative to IR50 was related to higher *q*P (ratio of opened reaction centers to total reaction centers in PSII) and *q*NP (dissipation and the heat emission). On the other hand, Jeong et al. (2002) showed that differential susceptibility of rice cultivars to chilling is not dependent on the non-radiative dissipation of light. In the present study, increased DI_0/RC and φ_{D0} was seen in both genotypes, suggesting that dissipation of excess light is not a determining factor for cold tolerance. Nevertheless, other mechanisms of energy dissipation may be important in the cold tolerant genotype. A previous study has suggested that cold tolerance in one *japonica* rice genotype may be a consequence of active non-harmful energy dissipation processes through the xanthophyll cycle (Bonnecarrère et al. 2011).

Comparison between chilling-sensitive (*indica*) and chilling-resistant (*japonica*) cultivar about the extents of PSII photoinhibition led to the conclusion that the differences depended on the relative capacity of photochemical utilization of absorbed light (Jeong et al. 2002). On the other hand, our data indicate that cold tolerance and sensitivity in the *indica* genotypes evaluated are not related to differential capacities of photochemical utilization of absorbed light at PSII, because plants from both genotypes showed similar values of capture, dissipation, absorption and utilization of light energy, per reaction centers, under the light conditions used in this experiment.

The photosynthetic performance indexes on the basis of light absorption (PI) were shown to be very sensitive to different stresses and therefore very useful for physiological, environmental and biotechnological screenings (Stirbet and Govindjee 2011). Among other parameters, photosynthetic performance index was used to evaluate the cold tolerance of transgenic rice expressing the *AlSAP* gene (Saad et al. 2012). In the present study, the photosynthetic performance index in relation to absorption (PI_{ABS}) (Strasser et al. 2000) and total photosynthetic performance index ($PI_{ABS,total}$), that measures the electrons flux till the final electron acceptors of PSI (Stirbet and Govindjee 2011), decreased greatly, indicating that the photosynthetic activity was affected by cold stress in both genotypes (Figure 2A and B). However, during the recovery period, the tolerant genotype showed considerable increase in these parameters, demonstrating the ability to recover the photosystems performance (Figures 2D and 3). PIs were not evaluated in the sensitive genotype after ten days of cold stress and during the recovery period due to complete leaf senescence. Cold stress reduces hydraulic conductivity of roots and causes dehydration in rice plants even when the soil is abundant in water. It has been suggested that low root temperature has a negative influence in the activity of aquaporins, leading to

decreased hydraulic conductivity in rice plants (Murai-Hatano et al. 2008). Dehydrated plants close their stomata, rapidly reducing CO_2 levels within the leaf, which in turn inhibit light-dependent reactions, decreasing photosynthesis (Saibo et al. 2009). High hydraulic conductance was shown to positively influence the rate of leaf photosynthesis in rice plants (Taylaran et al. 2011). In the present work, ten days of cold treatment resulted in leaf rolling and wilting only in plants from the sensitive genotype. It is possible that recovery of photosynthetic performance in the tolerant genotype is related to a lower impact of cold exposure on root water uptake in this genotype than in the sensitive one. This hypothesis is reinforced by a reduction in root length and dry weight (decreasing root surface area) only in the cold-sensitive genotype after six days of cold treatment (data not shown).

At the germination stage, APX and CAT activities were higher in the cold tolerant genotype than in the sensitive one, both at 28 °C and 13 °C (Figure 4). High APX activities are related to high scavenging of the hydrogen peroxide produced in response to low temperature (Okuda et al. 1991). Our results suggest that the tolerant seedlings exhibited an effective protection mechanism and mitigated oxidative stress by reaching higher APX activity than the sensitive ones during germination.

Oxidative damage during cold exposure is caused by increased ROS production (Suzuki and Mittler 2006). H_2O_2, a non-radical form of ROS, although being rather inert to biomolecules such as proteins and lipids (Pospíšil 2014), is still toxic to the cell. H_2O_2 can be involved in the specific cleavage of the PSII reaction center protein D1 (Jeong et al. 2002). In addition to oxidative stress, biochemical and genetic studies confirm that hydrogen peroxide is a signaling molecule in plants and could also play a key role in mediating important signal transduction events (Suzuki and Mittler 2006). We detected a significant increase in H_2O_2 concentration from 48 to 72 hours of cold treatment only in the tolerant genotype (Figure 5). The relevance of this variation as a signal needs further investigation.

High SOD activity has been associated with stress tolerance in plants, catalyzing the conversion of O_2^- to H_2O_2 (Bowler et al. 1992). Chilling-tolerant or drought-tolerant rice cultivars have higher SOD activity than sensitive cultivars under chilling or drought stress, suggesting that higher SOD activity in tolerant cultivars is an indicator of higher O_2^- scavenging capacity under stresses (Guo et al 2006). In the present work, a significant increase in leaf SOD activity was observed in the tolerant genotype, from 24 to 48 hours of cold exposure, suggesting higher potential for O_2^- scavenging activity of the tolerant genotype during the vegetative stage (Figure 5). Increased activity of this enzyme after 48

hours of cold may be related to the increased levels of H_2O_2 in plants from the tolerant genotype after 72 hours of cold.

The antioxidant enzymes APX and CAT are involved in H_2O_2 scavenging (Møller et al. 2007). CAT is mainly present in peroxisomes, and is indispensable for detoxification of high levels of ROS produced during stress. Due to its low affinity for peroxide, CAT activity is increased when substrate concentrations are high, in the mM range (Mittler 2002). The results observed at the vegetative stage, when 72 hours of low temperature led to increased CAT activity in the tolerant genotype, may be related to the increased H_2O_2 levels (Figure 5). The balance between SOD and the different H_2O_2-scavenging enzymes in cells is considered to be crucial in determining the steady state level of O_2^- and H_2O_2 (Mittler 2002). During temperature stresses, almost all H_2O_2 is derived from the O^{2-} and it has been suggested that SOD and CAT play important roles in disposing of these ROS species (Suzuki and Mittler 2006). Therefore, the synchronised actions of SOD and CAT may have contributed to the better performance of the tolerant genotype after 48 and 72 hours of stress. Other mechanisms of detoxification of H_2O_2 could be acting in the cold-tolerant plants, such as the ascorbate/glutathione cycle, the peroxiredoxin system and higher activities of glutathione peroxidases (Møller et al. 2007). Bonnecarrère et al. (2011) analyzed two cold-tolerant *japonica* rice cultivars and concluded that increased cold tolerance of L2825CA relative to INIA Tacuarí was related to higher activity of the enzymes superoxide dismutase, ascorbate peroxidase and catalase. As expected, no changes were observed in CAT and SOD activities in the sensitive genotype upon exposure to cold (Figure 5).

No differences were observed in APX activity in both genotypes at the vegetative stage (Figure 5). APX requires an ascorbate and/or a glutathione (GSH) regenerating cycle. This cycle uses electrons directly from the photosynthetic apparatus or NAD(P)H as reducing power (Mittler 2002). Thus, because this enzyme requires a supply of reducing equivalents for its function, increases in APX activity may have been hindered under cold treatment, when photosynthetic activity was impaired.

Based on the different physiological traits evaluated in this study, IRGA 959-1-2-2F-4-1-4-A (cold-tolerant) and IRGA 959-1-2-2F-4-1-4-D-1-CA-1 (cold-sensitive), derived from the same cross, clearly differ in their response to chilling stress. It has been established that low temperature responses in plants are mainly controlled at the transcriptional level through extensive reprogramming of gene expression (Chinnusamy et al. 2010). By evaluating the expression of genes previously described as cold-responsive,

we identified two genes with leaf expression levels that could be directly related to the cold tolerance/sensitivity characteristics of the tested genotypes: *OsLIP9* and *OsWCOR413* (Figure 6).

Dehydrins are thought to act as chaperons, and thus to stabilize vesicles, proteins, and membrane structures in stressed plants (Koag et al. 2003). Cold-induced dehydrins have been reported in several plants, including cereals as barley and wheat (Bravo et al, 2003). Among rice dehydrins, the LIP9 protein was identified as responsive to cold stress and the *OsLIP9* gene was shown to be induced after 6 h of temperature down-shift, being triggered at 13 ^{o}C (Aguan et al. 1991). In the tolerant genotype, cold treatment resulted in higher *OsLIP9* expression in relation to the control treatment and to the sensitive genotype (Figure 6). It is possible that the tolerance mechanism expressed by this genotype includes higher LIP9 protein levels.

It has been suggested that *OsLIP9* is a target of DREB1 signaling (Lee et al. 2004). Over-expression of *OsDREB1B* in *japonica* rice plants significantly improves tolerance to cold stress and enhances *OsLIP9* expression (Ito et al. 2006). We found higher *OsDREB1B* expression in relation to the control treatment in leaves from both genotypes (Figure 6). However, no difference in expression level was found between the two genotypes (Figure 6). It may be possible that enhancement of *OsLIP9* expression by *OsDREB1* is more efficient in plants from the cold-tolerant genotype (or *OsLIP9* expression is more responsive to *OsDREB1* in the tolerant plants). However, the mechanisms of cold stress responses and tolerance are complex, and other factors besides *OsDREB1B* do likely impact on *OsLIP9* expression. Before cold stress, *OsLIP9* expression was detected in both genotypes, despite no detectable levels of *OsDREB1* expression.

High gene expression prior to abiotic stress treatment might represent a constitutive tolerance mechanism (Zhang et al. 2012b). In this work, *OsWCOR413* gene expression was higher in the cold-tolerant than in the cold-sensitive genotype before cold treatment. After six hours of cold, *OsWCOR413* expression was down-regulated in both genotypes (Figure 6). WCOR413 is a transmembrane protein that provides membrane stability and also acts as a receptor of extracellular signals, being possibly involved in cold acclimation (Mishra et al. 2009). The higher expression of *OsWCOR413* in the cold-tolerant than in the cold-sensitive genotype before cold treatment at 10 $^{\circ}$C may be important for intrinsic tolerance to chilling stress. Additional studies with Arabidopsis over-expressing the

OsWCOR413 gene are being conducted by our group, and will help to understand its function.

Our screening of rice *indica* genotypes for cold tolerance in two stages during early development (germination and young seedlings) revealed a wide range of cold tolerance in this subspecies, and allowed us to identify two genotypes with contrasting levels of cold tolerance that share a very close genetic relation. The performance indexes (PI) were affected in both genotypes. However, the tolerant genotype was able to recover photosynthetic performance on the basis of light absorption after a period of intensive cold stress. Plants from the tolerant genotype reached higher SOD and CAT activities during the vegetative stage and higher APX and CAT activities during germination, allowing better coping with cold stress. The results suggest that the antioxidant system was more efficient in the tolerant than in the sensitive genotype at both stages of development, under cold exposure. We evaluated the expression of twenty genes previously described as related to cold responses in rice, two of which showed differential regulation in leaves from the tolerant genotype: cold induction of *OsLIP9* expression and constitutive high *OsWCOR413* expression. Our group is also using next generation sequencing to compare the transcriptome of both genotypes under cold stress during germination (Dametto et al. 2015) and vegetative stages. In future studies, the *indica* genotypes identified as tolerant and sensitive to cold in this work may help to identify new cold tolerance mechanisms in rice plants, possibly including pathways not known from the previous studies in *japonica* rice.

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Aebi, H. 1984. Catalase *in vitro*. Methods Enzymol. **105**: 121-126.

Aguan, K., Sugawara, K., Susuki, N. and Kusano, T. 1991. Isolation of genes for low-temperature-induced proteins in rice by a simple subtractive method. Plant Cell Physiol. **32**: 1285-1289.

Beyer, W.F. and Fridovich, I. 1987. Assaying of superoxide dismutase activity: some large consequences of minor changes in conditions. Anal. Biochem. **161**: 559-566.

Bierlen, R., Wailes. E.J. and Cramer, G.L. 1997. The Mercosur rice economy. Ark. Exp. Stat. Bull. **954**: 1-58.

Bonnecarrère, V., Borsani, O., Díaz, P., Capdevielle, F., Blanco, P. and Monza, J. 2011. Response to photoxidative stress induced by cold in *japonica* rice is genotype dependent. Plant Sci. **180**: 726-732.

Bowler, C., Montagu, M.V. and Inzé, D. 1992. Superoxide dismutase and stress tolerance. Ann. Rev. Plant Physiol. Plant Mol. Bioi. **43**: 83-116.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

Bravo, L.A., Gallardo, J., Navarrete, A., Olave, N., Martínez, J., Alberdi, M., Close, T.J., and Corcuera, L.J. 2003. Cryoprotective activity of a cold-induced dehydrin purified from barley. Physiol. Plant. **118**: 262-269.

Chinnusamy, V., Zhu, J.K. and Sunkar, R. 2010. Gene regulation during cold stress acclimation in plants. Methods Mol. Biol. **639**:39-55.

Chytyk C.J., Hucl P.J. Gray G.R. 2011. Leaf photosynthetic properties and biomass accumulation of selected western Canadian spring wheat cultivars. Can. J. Plant Sci. 91: 305-314.

Counce, P.A., Keisling, T.C.and Mitchell, A.J. 2000. A uniform, objective, and adaptive system for expressing rice development. Crop Sci. **40**: 436-443.

Cruz, R.P. da and Milach, S.C.K. 2000. Breeding for cold tolerance in irrigated rice. Ciência Rural **30**: 909-917.

Cruz, R.P. da and Milach, S.C.K. 2004. Cold tolerance at the germination stage of rice: methods of evaluation and characterization of genotypes. Sci. Agric. **61**: 1-8.

Cruz, R.P. da, Sperotto, R.A., Cargnelutt,i D., Adamski, J.M., Terra, T.F. and Fett, J.P. 2013. Avoiding damage and achieving cold tolerance in rice plants. Food Energy Security **2**: 96-119.

Dametto, D., Sperotto, R.A., Adamski, J.M., Blasi E.A.R., Cargnelutti, D., Oliveira, L.F.V., Ricachenevsky, F.K., Fregonezi, J.N., Mariath, J.E.A., Cruz, R.P., Margis, R.

and Fett, J.P. 2015. Cold tolerance in rice germinating seeds revealed by deep RNAseq analysis of contrasting *indica* genotypes. Plant Sci. **238**: 1-12.

de Los Reyes, B.G., Morsy, M., Gibbons, J., Varma, T.S., Antoine, W., McGrath, J.M., Halgren, R. and Redus, M. 2003. A snapshot of the low temperature stress transcriptome of developing rice seedlings (*Oryza sativa* L.) via ESTs from subtracted cDNA library. Theor. Appl. Genet. **107**: 1071-1082.

Dubouzet, J.G., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E.G., Miura, S., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. 2003. *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought, high-salt- and cold-responsive gene expression. Plant J. **33**: 751-763.

El-Moshaty, F.I.B., Pike, S.M., Novacky, A.J. and Sehgal, O.P. 1993. Lipid peroxidation and superoxide production in cowpea (*Vigna unguiculata*) leaves infected with tobacco ringspot virus or southern bean mosaic virus. Physiol. Mol. Plant Pathol. **43**: 109-119.

Ensminger, I., Busch, F., Huner, N.P.A. 2006. Photostasis and cold acclimation: sensing low temperature through photosynthesis. Physiol. Plant. 126: 28–44.

Foyer, C. and Noctor, G. 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiology responses. Current Perspective Assay, Plant Cell **17**: 1866-1875.

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

Guo-Li, W. and Zhen-Fei, G. 2005. Effects of chilling stress on photosynthetic rate and chlorophyll fluorescence parameter in seedlings of two rice cultivars differing in cold tolerance. Rice Sci. **12**: 187-191.

Guo, Z., Ou, W., Lu, S. and Zhong, Q. 2006. Differential responses of antioxidative system to chilling and drought in four rice cultivars differing in sensitivity. Plant Physiol. Biochem. **44**: 828-836.

Hossain, M.A., Cho, J.I., Han, M., Ahn, C.H., Jeon, J.S., An, G. and Park, P.B. 2010. The ABRE-binding bZIP transcription factor OsABF2 is a positive regulator of abiotic stress and ABA signaling in rice. J. Plant Physiol. **167**: 1512-1520.

Huang, J., Sun, S.J., Xu, D.Q., Yang, X., Bao, Y.M., Wang, Z.F., Tang, H.J. and Zhang, H. 2009. Increased tolerance of rice to cold, drought and oxidative stresses

mediated by the overexpression of a gene that encodes the zinc finger protein ZFP245. Biochem. Biophys. Res. Commun. **389**: 556-561.

Hüner, N.P.A., Öquist G. and Sarhan F. 1998. Energy balance and acclimation to light and cold. Trends Plant Sci.3: 224-230.

Hüner, N.P.A., Bode, R., Dahal, K, Hollis, L., Rosso, D. Krol, M., Ivanov, A.G. 2012. Chloroplast redox imbalance governs phenotypic plasticity: the "grand design of photosynthesis" revisited. Front Plant Sci. 3: 1-12.

Iba, K. 2002. Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. Ann. Rev. Plant Biol. **53**: 225-245.

IBGE - Intituto Brasileiro de Geografia e Estatística. 2015. Available: http://www.ibge.gov.br/home/estatistica/indicadores/agropecuaria/lspa/default.shtm [Accessed 9 June 2015].

Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. 2006. Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant Cell Physiol. **47**: 141-153.

Jeong, S.W., Choi, S.M., Lee, D.S., Ahn, S.N., Hur, Y., Chow, W.S. and Park, Y.I. 2002. Differential susceptibility of photosynthesis to light-chilling stress in rice (*Oryza sativa* L.) depends on the capacity for photochemical dissipation of light. Mol. Cells **13**: 419-428.

Kaneda, C. and Beachell, H.M. 1974. Breeding rice for cold tolerance. Saturday Seminar Paper 9. IRRI, Los Baños, Philippines.

Kim, S.J., Lee, S.C., Hong, S.K., An, K., An, G. and Kim, S.R. 2009. Ectopic expression of a cold-responsive *OsAsr1* cDNA gives enhanced cold tolerance in transgenic rice plants. Mol. Cells **27**: 449-458.

Koag, M.C., Fenton, R.D., Wilkens, S. and Close, T.J. 2003. The binding of maize DHN1 to lipid vesicles. Gain of structure and lipid specificity. Plant Physiol. **131**: 309-316.

Lee, M.H. 2001. Low temperature tolerance in rice: the Korean experience. Pages 109-117 in S. Fukai and J. Basnayake eds. Increased lowland rice production in the Mekong Region, Canberra, Australia.

Lee, S.C., Huh, K.W., An, K., An, G. and Kim, S.R. 2004. Ectopic expression of a cold-inducible transcription factor, CBF1/DREB1b, in transgenic rice (*Oryza sativa* L.). Mol. Cells **18**: 107-114.

Li, T.G., Visperas, R.M. and Vergara, B.S. 1981. Correlation of cold tolerance at different growth stages in rice. Acta Bot. Sin. **23**: 203-207.

Liu, K., Wang, L., Xu, Y., Chen, N., Ma, Q., Li, F. and Chong, K. 2007. Overexpression of *OsCOIN*, a putative cold inducible zinc finger protein, increased tolerance to chilling, salt and drought, and enhanced proline level in rice. Planta **226**: 1007-1016.

Loreto, F. and Velikova, V. 2001. Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quences ozone products, and reduces lipid peroxidation of cellular membranes. Plant Physiol. **127**: 1781-1787.

Lukatkin, A.S. 2005. Initiation and development of chilling injury in leaves of chilling-sensitive plants. Russ. J. Plant Physiol. **52**: 542-546.

Ma, Q., Dai, X., Xu, Y., Guo, J., Liu, Y., Chen, N., Xiao, J., Zhang, D., Xu, Z., Zhang, X. and Chong, K. 2009. Enhanced tolerance to chilling stress in OsMYB3R-2 transgenic rice is mediated by alteration in cell cycle and ectopic expression of stress genes. Plant Physiol. **150**: 244-256.

Mishra, M., Das, R. and Pandey, G.K. 2009. Role of ethylene responsive factors (ERFs) in abiotic stress mediated signaling in plants. - Journal of Biological Sciences **1**: 133-146.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. **7**: 405-410.

Møller, I.M., Jensen, P.E. and Hansson, A. 2007. Oxidative modifications to cellular components in plants. Annu. Rev. Plant Biol. **58**: 459-81.

Murai-Hatano, M., Kuwagata, T., Sakurai, J., Nonami, H., Ahamed, A., Nagasuga, K., Matsunami, T., Fukushi, K., Maeshima, M. and Okada, M. 2008. Effect of low root temperature on hydraulic conductivity of rice plants and the possible role of aquaporins. Plant Cell Physiol. **49**: 1294-1305.

Okuda, T., Matsuda, Y., Yamanaka, A. and Sagisaka, S. 1991. Abrupt increase in the level of hydrogen peroxide in leaves of winter wheat is caused by cold treatment. Plant Physiol. **97**: 1265-1267.

Pospíšil, P. 2014. The role of metals in production and scavenging of reactive oxygen species in photosystem II. Plant Cell Physiol. **55**: 1224-1232.

Saad, R.B., Fabre, D., Mieulet, D., Meynard, D., Dingkuhn, M., Al-Doss, A., Guiderdoni, E. and Hassairi, A. 2012. Expression of the *Aeluropus littoralis AlSAP* gene in rice confers broad tolerance to abiotic stresses through maintenance of photosynthesis. Plant Cell Environ. **35**: 626-643.

Saibo, N.J.N., Lourenço, T. and Oliveira, M.M. 2009. Transcription factors and regulation of photosynthetic and related metabolism under environmental stresses. Ann. Bot. **103**: 609-623.

Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K. and Izui, K. 2000. Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J. **23**: 319-327.

Saito, K., Hayano-Saito, Y., Maruyama-Funatsuki, W., Sato, Y. and Kato, A. 2004. Physical mapping and putative candidate gene identification of a quantitative trait locus *Ctb1* for cold tolerance at the booting stage of rice. Theor. Appl. Genet. **109**: 515-522.

Sanghera, G.S., Wani, S.H., Hussain, W. and Singh, N.B. 2011. Engineering cold stress tolerance in crop plants. Curr. Genomics **12**: 30-43.

Schmittgen, T.D. and Livak, K.J. 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. **3**: 1101-1108.

Singh, R.P., Brennan, J.P., Farrell, T., Williams, R., Reinke, R., Lewin, L. and Mullen, J. 2005. Economic analysis of breeding for improved cold tolerance in rice in Australia. Aust. Agribus. Rev. **13**: 1-9.

Stirbet, A. and Govindjee. 2011. On the relation between the Kautsky effect (chlorophyll a fluorescence induction) and Photosystem II: Basics and applications of the OJIP fluorescence transient. J. Photochem. Photobiol. B, Biol. **104**: 236-257.

Strasser, R.J., Srivastava, A. and Tsimilli-Michael, M. 2000. The fluorescence transient as a tool to characterize and screen photosynthetic samples. Pages 443-480 in M. Yunus, U. Pathre and P. Mohanty eds. Probing Photosynthesis. Pp Taylor & Francis, London, UK.

Strauss, A.J., Krüger, G.H.J., Strasser, R.J. and van Heerden, P.D.R. 2007. The role of low soil temperature in the inhibition of growth and PSII function during dark chilling in soybean genotypes of contrasting tolerance. Physiol. Plant. **131**: 89-105.

Su, C.F., Wang, Y.C., Hsieh, T.H., Lu, C.A., Tseng, T.H. and Yu, S.M. 2010. A novel MYBS3-dependent pathway confers cold tolerance in rice. Plant Physiol. **153**: 145-158.

Suzuki, N. and Mittler, R. 2006. Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. Physiol. Plant. **126**: 45-51.

Taylaran, R.D., Adachi, S., Ookawa, T., Usuda, H. and Hirasawa, T. 2011. Hydraulic conductance as well as nitrogen accumulation plays a role in the higher rate of leaf photosynthesis of the most productive variety of rice in Japan. J. Exp. Bot. **62**: 4067-4077.

Tyagi A.K., Kapoor S., Khurana J.P., Ray S. 2007. Expression data for stress treatment in rice seedlings. Gene Expression Omnibus, NCBI. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6901).

Wang, Q., Guan, Y., Wu, Y., Chen, H., Chen, F. and Chu, C. 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.

Wen, J.Q., Oono, K. and Imai, R. 2002. Two novel mitogen-activated protein signaling components, OsMEK1 and OsMAP1, are involved in a moderate low-temperature signaling pathway in rice. Plant Physiol. **129**: 1880-1891.

Xie, G., Kato, H., Sasaki, K., Imai, R. 2009. A cold-induced thioredoxin h of rice, OsTrx23, negatively regulates kinase activities of OsMPK3 and OsMPK6 in vitro. FEBS Lett. 583: 2734-2738.

Xu, L.M., Zhou, L., Zeng, Y.W., Wang, F.M., Zhang, H.L., Shen, S.Q. and Li, Z.C. 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.

Yoshida, S. 1981. Fundamentals of rice crop science. International Rice Research Institute, Los Baños, Laguna, Philippines.

Yusuf, M.A., Kumar, D., Rajwanshi, R., Strasser, R.J., Tsimilli-Michael, M., Govindjee and Sarin, N.B. 2010. Overexpression of γ-tocopherol methyl transferase gene in transgenic *Brassica juncea* plants alleviates abiotic stress: physiological and chlorophyll a fluorescence measurements. Biochim. Biophys. Acta **1797**: 1428-38.

Zhang, F., Huang, L., Wang, W., Zhao, X., Zhu, L., Fu, B. and Li, Z. 2012a. Genome-wide gene expression profiling of introgressed *indica* rice alleles associated with seedling cold tolerance improvement in a *japonica* rice background. BMC Genomics **13**: 461.

Zhang, T., Zhao, X., Wang, W., Pan, Y., Huang, L., Liu, X., Zong, Y., Zhu, L., Yang, D. and Fu, B. 2012b. Comparative transcriptome profiling of chilling stress responsiveness in two contrasting rice genotypes. PLoS ONE **7**: e43274.

Zhu, Z., Wei, G., Li, J., Qian, Q. and Yu, J. 2004. Silicon alleviates salt stress and increases antioxidant enzymes activity in leaves of salt-stressed cucumber (*Cucumis sativus* L.). Plant Sci. **167**: 527-533.

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Cold tolerance in rice germinating seeds revealed by deep RNAseq analysis of contrasting *indica* genotypes

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ABSTRACT

Rice productivity is largely affected by low temperature, which can be harmful throughout plant development, from germination to grain filling. Germination of *indica* rice cultivars under cold is slow and not uniform, resulting in irregular emergence and small plant population. To identify and characterize novel genes involved in cold tolerance during the germination stage, two *indica* rice genotypes (sister lines previously identified as cold-tolerant and cold-sensitive) were used in parallel transcriptomic analysis (RNAseq) under cold treatment (seeds germinating at 13 °C for 7 days). We detected 1,361 differentially expressed transcripts. Differences in gene expression found by RNAseq were confirmed for 11 selected genes using RT-qPCR. Biological processes enhanced in the cold-tolerant seedlings include: cell division and expansion (confirmed by anatomical sections of germinating seeds), cell wall integrity and extensibility, water uptake and membrane transport capacity, sucrose synthesis, generation of simple sugars, unsaturation of membrane fatty acids, wax biosynthesis, antioxidant capacity (confirmed by histochemical staining of H_2O_2), and hormone and Ca^{2+}-signaling. The cold-sensitive seedlings respond to low temperature stress increasing synthesis of HSPs and dehydrins, along with enhanced ubiquitin/proteasome protein degradation pathway and polyamine biosynthesis. Our findings can be useful in future biotechnological approaches aiming to cold tolerance in *indica* rice.

1. Introduction

Rice is one of the world's most important crops, and its grains are the staple food for more than half of the world population. To satisfy the growing demand of the ever-increasing population, more sustained production is needed. However, rice yield is largely affected by different abiotic stresses, including low temperature [1], which can be harmful during the entire development of rice plants, from germination to grain filling [2].

Cold (non-freezing) temperatures can injure or kill plants of tropical or subtropical origin, causing chlorosis, necrosis and growth retardation. Low temperature affects rice plants in 25 countries, with large negative impacts on yield [3]. In rice leaves, cold stress affects chlorophyll content and fluorescence, and thus interferes with photosynthesis. Also, reactive oxygen species and

Abbreviations: *OsEXD*, Expressed protein; *OsCYC*, Cyclin; *OsCDKB2 ;1*, Cyclin-dependent kinase B2-1; *OsCSLE1*, Cellulose synthase-like family E; *OsAQU*, Aquaporin protein; *OsKET*, 3-ketoacyl-CoA synthase; *OsPRX*, Peroxidase precursor; *OsFBX221*, F-box domain-containing protein; *OsAUX*, OsIAA13 – Auxin-responsive Aux/IAA gene family member; *OsDHN*, Dehydrin; *OsLEA*, Late embryogenesis abundant protein; *OsUBQ5*, Ubiquitin 5.

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malondialdehyde levels increase and can impair metabolism via cellular oxidative damage [4]. During the reproductive stage, exposure to low temperatures leads to spikelet sterility, which may result from pollen abortion, anther indehiscence or growth arrest of the pollen tube during anthesis. During grain filling, chilling temperatures may also cause delayed and incomplete grain maturation [3]. During germination, the most common symptoms of cold temperature damage are low percentage and delayed germination [3].

Seeds are a vital component of the world's diet and also play a central role in the life cycle of plants [5]. Seed germination is a complex process, which begins with water uptake by the dry seed and ends with the emergence of the radicle. During imbibition, three distinct phases can be observed: a rapid uptake of water (phase I), followed by a plateau phase of water uptake (phase II), and the initiation of growth (phase III). Seed imbibition restores the metabolic activity of the dried seed, leading to extensive physiological and biochemical changes [5]. When non-dormant seeds are provided with appropriate environmental conditions (water, atmosphere, and temperature), germination can efficiently occur.

Low temperature can delay seed germination and, consequently, plant emergence [6], resulting in yield decreases up to 25% of the final yield [7] and in increased weed competition [8]. It has been suggested that transcription factors and plant hormones play central roles in the response to low temperature in *japonica* rice varieties, through regulation of multiple processes including chromatin assembly, cell division, cell fate specification, and organ morphogenesis [9]. *Indica* rice varieties are well adapted to tropical climates, but are also cultivated in cold temperate climatic conditions. Under cold temperature, *indica* rice varieties usually present slow and not uniform germination, resulting in irregular emergence and low plant population. Genotypes belonging to the *japonica* subspecies are consistently more cold-tolerant than *indica* genotypes [6]. However, variability for this trait has been observed within both subspecies [3]. With that in mind, our group has previously screened for cold-tolerant genotypes in *indica* background, using percentage of seeds with coleoptile length ≥5 mm after 26 days of germination under cold temperature as an indicator. Two sister lines were isolated and characterized. However, the mechanisms that confer cold tolerance and sensitivity are still not understood, especially at the molecular level.

Several transcriptome and proteome analyses have been applied to elucidate the fundamental mechanism of seed germination in rice [10–12]. However, to the best of our knowledge, this is the first high-throughput analysis of transcripts in rice germinating seeds of two *indica* rice genotypes with different levels of cold tolerance. In this work, we used the above mentioned sister lines of rice (previously identified as cold-tolerant and cold-sensitive) in parallel transcriptomic analysis of germinating seeds under cold treatment (13 °C for 7 days), aiming to identify and characterize novel genes involved in rice cold tolerance during this stage.

2. Material and methods

2.1. Plant materials and cold treatment

We have previously evaluated 45 *indica* genotypes according to the percentage of seeds with coleoptile length ≥ to 5 mm after 26 days at 13 °C during germination. In that screening, the sister lines (derived from the same cross) IRGA 959-1-2-2F-4-1-4-A and IRGA 959-1-2-2F-4-1-4-D-1-CA-1 were characterized, respectively, as tolerant and sensitive to low temperature stress. Germination at 13 °C resulted in 80% and 16% of the seeds with coleoptile length ≥ to 5 mm, respectively, in cold-tolerant and cold-sensitive genotypes, after 26 days of imbibition (Supplementary Fig. 1). At the vegetative stage, percentage of plant survival after 10 days of cold treatment (10 °C) and 7 days of recovery was about 90% and 15%, respectively, for the same cold-tolerant and cold-sensitive genotypes (data not shown).

These two genotypes were developed by the IRGA (Rio Grande do Sul Rice Institute) breeding program. They present the same genealogy, which is a triple cross: "Lemont/IRGA117-72-1P-3-2A//P1790". The first cross (Lemont/IRGA117-72-1P-3-2A) was performed in 1988. One year later, the F1 of this cross was crossed to P1790. In the following years, the segregating generations were field cultivated and individual plants were selected according to agronomic traits of interest (including cold-response) using the pedigree method. After six generations of selection, many F7 lines were obtained, all designated IRGA 959. The two lines evaluated in this study were chosen among them.

In the present work, both selected genotypes were germinated in the dark for 7 days at 13 °C. Three biological replicates (each one containing at least ten germinating seeds) were collected and immediately frozen in liquid nitrogen. Samples were then maintained at −80 °C until RNA extraction.

2.2. RNA extraction and comparative transcriptomic profiling by RNAseq

Total RNA was extracted from rice germinating seeds using Concert Plant RNA Reagent (Invitrogen) and treated with DNase I (Invitrogen). Approximately 20 μg of total RNA was used to high-throughput cDNA sequencing by Illumina HiSeq 2000 technology (Fasteris SA, Plan-les-Ouates, Switzerland – http://www.fasteris.com/). We constructed one individual single-end cDNA library for each rice genotype. It is important to highlight that RNAs derived from three biological replicates were combined to generate each cDNA library. The cDNA libraries were prepared according to Illumina's protocols. Briefly, RNAseq was performed using the following successive steps: poly-A purification; cDNA synthesis using a poly-T primer, shotgun method to generate inserts of approximately 500 nt; 3p and 5p adapter ligations; pre-amplification; colony generation; and Illumina single-end 100 bp sequencing.

All low quality reads (FASTq value < 13) were removed, and 3p and 5p adapter sequences were trimmed using Genome Analyzer Pipeline (Fasteris). The remaining low quality reads with 'n' were removed using a Python script. After trimming the data (low quality reads, adapter sequences), mRNAseq data from the two libraries were aligned to the rice genome using software Bowtie version 0.12.7. Only sequences with up to two mismatches to the rice reference genome (ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/peudomolecules/version_6.1/all.dir/all.seq) were used. The SAM files from Bowtie were then processed using Python scripts to assign frequencies of each read and map them onto references. For data normalization, we used the scaling normalization method proposed by [13]. To assess whether genes were differentially expressed, we used the R package EdgeR [14]. We considered that genes were differentially expressed if they had an adjusted p-value < 1.0 e^{-12}, according to EdgeR.

2.3. Gene Ontology (GO) terms enrichment analysis

Comparison of differentially expressed genes loci in cold-tolerant or cold-sensitive datasets (Supplementary Table 2) was performed to find enriched Gene Ontology (GO) terms. The rice proteome was downloaded from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) and fully annotated *de novo* using Blast2GO (http://www.blast2go.com [15]). The enrichment analysis was performed using Blast2GO built-in Fisher's Exact Test with the following parameters: two-tailed test, remove double IDs ($p \leq 0.05$). From a total of 758 (cold-tolerant) and 603

(cold-sensitive) differentially expressed genes, 484 and 383 genes could be assigned a GO term, and thus considered in the enrichment analysis.

2.4. Gene expression analysis by RT-qPCR

To confirm the high-quality of deep sequencing results, RT-qPCR was used to check the gene expression of putative cold tolerance-related genes. Total RNA was extracted from rice germinating seeds after 2 days at 28 °C or after 7 days at 13 °C. Different exposure times to the treatments were necessary to ensure that coleoptiles were at the same developmental stage. After extraction with Concert Plant RNA Reagent (Invitrogen), RNA was treated with DNase I (Invitrogen). First-strand cDNA synthesis was performed with reverse transcriptase (M-MLV, Invitrogen) using 1 μg of RNA. RT-qPCRs were carried out in a StepOne Real-Time Cycler (Applied Biosystems). All primers (listed in Supplementary Table 1) were designed to amplify 100–150 bp of the 3′-UTR of the genes and to have similar Tm values (60 ± 2 °C). Reaction settings were composed of an initial denaturation step of 5 min at 94 °C, followed by 40 cycles of 10 s at 94 °C, 15 s at 60 °C, 15 s at 72 °C and 35 s at 60 °C (fluorescence data collection); samples were held for 2 min at 40 °C for annealing of the amplified products and then heated from 55 to 99 °C with a ramp of 0.1 °C/s to produce the denaturing curve of the amplified products. RT-qPCRs were carried out in 20 μl final volume composed of 10 μl of each reverse transcription sample diluted 100 times, 2 μl of 10× PCR buffer, 1.2 μl of 50 mM $MgCl_2$, 0.1 μl of 5 mM dNTPs, 0.4 μl of 10 μM primer pairs, 4.25 μl of water, 2.0 μl of SYBR green (1:10,000, Molecular Probe), and 0.05 μl of Platinum Taq DNA Polymerase (5 U/μl, Invitrogen, Carlsbad, CA, USA). Gene expression was evaluated using a modified $2^{-\Delta CT}$ method [16], which takes into account the PCR efficiencies of each primer pair (Relative expression$_{\text{TESTED GENE/CONTROL GENE}}$ = $(PCR_{eff}$ $CG)^{Ct\ CG}/(PCR_{eff}$ $TG)^{Ct\ TG}$). *OsUBQ5* gene expression was used as internal control to normalize the relative expression of tested genes [17]. Each data point corresponds to three true biological replicate samples, collected in a second experiment (i.e. distinct from the one for RNAseq).

2.5. In situ *histochemical localization of H_2O_2*

Accumulation of H_2O_2 was detected by histochemical staining with diaminobenzidine (DAB) according to [18], with minor modifications. For H_2O_2 detection, seedlings (10 days at 13 °C or 2 days at 28 °C) of both genotypes were immersed in DAB solution (1 mg ml^{-1}, pH 3.8) in 10 mM phosphate buffer (pH 7.8), and incubated at room temperature for 8 h under light until brown spots (derived from the reaction of DAB with H_2O_2) were visible. Seedlings were kept in 70% ethanol for taking pictures with a digital camera coupled to a stereomicroscope.

2.6. Measurement of longitudinal cell length and cell division percentage

To assess longitudinal cell length and the number of cells in mitotic phase during seed germination, rice seeds from both genotypes (selected as tolerant and sensitive to low temperature stress) were subjected to germination in the dark at 28 °C for one day (control) and at 13 °C for 10 days (cold treatment). At the end of these periods, both seedlings were at the S1 stage [19]. Seedlings were fixed for 24 h in a solution of absolute ethanol and glacial acetic acid (3:1) at room temperature. After fixation, coleoptiles were separated from the remaining seed parts, washed in distilled water and dehydrated using a graded ethanol series. Subsequently, coleoptiles were infiltrated and embedded in 2-hydroxyethyl methacrylate-based resin [20].

Longitudinal sections of the methacrylate-embedded coleoptiles were cut (5 μm thickness) using a Microm HM 340 microtome. To measure the length of the coleoptile epidermal cells, sections were stained with 0.1% (w/v) aqueous solution of toluidine blue O. In order to count the cells in mitotic phase, sections were stained with 2 μg ml^{-1} DAPI (4′,6-diamidino-2-phenylindole) solution for 24 h in the dark. Microscopical observations and photomicrography were carried out using a Leica DMR microscope equipped with a Leica DFC500 digital camera. Coleoptile epidermal cells' length was measured using the Zeiss software (AxioVision Rel. 4.8). Lengths of fifteen epidermal cells were measured in five replicates of each genotype. The percentage of cells in mitotic phase was determined on the coleoptile (C) and leaf primordium (LP) regions of five replicates per genotype.

2.7. Statistical analysis

Mean values were compared by the Student's *t* test ($p \leq 0.05$) using the SPSS Base 21.0 for Windows (SPSS Inc., USA).

3. Results and discussion

3.1. Overview of cold-tolerant and cold-sensitive cDNA library sequencing

Rice genotypes IRGA 959-1-2-2F-4-1-4-A and IRGA 959-1-2-2F-4-1-4-D-1-CA-1 previously characterized, respectively, as tolerant and sensitive to low temperature stress, were used to identify differentially expressed genes in germinating seeds of plants under cold treatment (13 °C) for 7 days, using the Illumina Platform. Deep sequencing generated approximately 20 million reads in each library (tolerant and sensitive genotypes). From these, 25.81% (4,974,030 reads) and 26.12% (6,033,254 reads) were mapped, respectively, in genomic regions. The reads not mapped to genomic regions correspond to those presenting more than two mismatches with the rice reference genome.

As these *indica* rice genotypes are sister lines, most of the genes showed similar normalized number of reads. Overall, there were clear linear relationships in the gene expression levels between the two rice genotypes (R^2 = 0.9605; Fig. 1A). For this reason, we preferred to use an extremely stringent adjusted *p*-value (<1.0 e^{-12}, according to EdgeR) in detriment to the use of fold-change as criteria for differential gene expression. It is already known that even small changes on the gene expression can have biological meaning, and that a gene can be considered differentially expressed if its expression level changes systematically between two treatment conditions, regardless of how small the difference might be. These parameters yielded 1,361 differentially expressed genes, which were included in our analysis (Supplementary Table 2). From these, 758 genes (56%) were up-regulated and 603 genes (44%) were down-regulated in the cold-tolerant seedlings, in relation to the cold-sensitive ones (Fig. 1B).

3.2. Enrichment of GO terms comparing tolerant and sensitive transcriptomes

In order to compare the transcriptomes of germinating seeds from both genotypes, we used GO terms to find enriched categories comparing each dataset (Fig. 2). Seventy-six terms were differentially enriched, with 22 being enriched in the cold-tolerant seedlings and 54 enriched in the cold-sensitive. Interestingly, among terms enriched in the cold-tolerant dataset, we found "carbohydrate metabolic process", "lipid metabolic process", "transport", "ion transport", and "antioxidant activity". These terms could be related to known cold-induced processes, such as

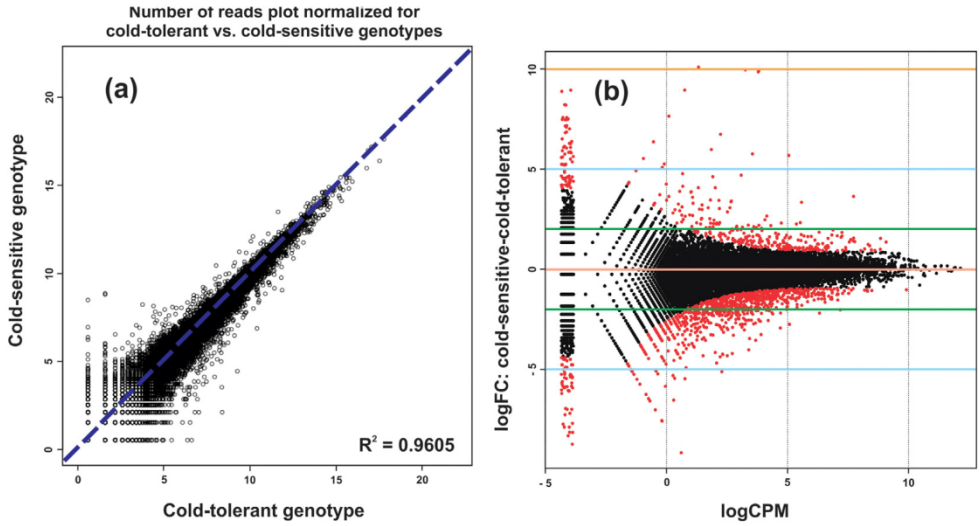

Fig. 1. Comparison of RNAseq results between libraries from cold-tolerant and cold-sensitive *indica* rice genotypes. (a) Scatter plot comparing the gene expression levels between the two genotypes. (b) Genes identified as differentially expressed by EdgeR (red dots). FC: fold change; CPM: counts per million. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

increased cellulose synthesis, accumulation of unsaturated membrane lipids, osmoregulation, and antioxidant defense activation, respectively. Thus, these mechanisms are potentially involved in tolerance to cold observed in the tolerant genotype. Among the terms enriched in the cold-sensitive dataset, we observed "response to abiotic stimulus". The same term is not represented in the cold-tolerant dataset, which could indicate that the cold-sensitive seedlings respond "normally" to abiotic stimulus, while the cold-tolerant ones trigger other mechanisms, which are more efficient to confer cold tolerance. The data obtained here provide indications of processes that might be involved in the cold-tolerance mechanism of IRGA 959-1-2-2F-4-1-4-A seedlings.

3.3. Differential gene expression determination

Using RNAseq method, we detected 1361 differentially expressed genes in rice germinating seeds from the cold-tolerant genotype in relation to the cold-sensitive one (Supplementary Table 2). All the 1,361 differentially expressed genes were carefully analyzed and, based on the predicted molecular function and differences in gene expression, we selected 109 sequences which we considered most likely to be related to the cold-tolerance mechanism found in IRGA 959-1-2-2F-4-1-4-A seedlings (Table 1). Several expressed proteins with unknown function were detected in our experiment (Supplementary Table 2), including the most differentially expressed gene between cold-tolerant and sensitive seedlings (Table 1). Even though none of these genes have been functionally characterized until now, it will be interesting to study if in fact these genes are related to cold tolerance in rice germinating seeds.

It is known for years that low temperatures can impair and delay germination, and maintenance of cell division under cold stress seems to be an efficient approach used by cold-tolerant rice plants. In fact, the rice genotypes used in our experiment show different coleoptile lengths when germinated under low temperature, with shorter coleoptiles on the cold-sensitive germinating seeds (Supplementary Fig. 1). As seen in Table 1, genes related to cell division and cell growth have been detected with higher expression levels in the cold-tolerant seedlings. One of these genes encodes the membrane-localized phytosulfokine receptor that functions binding to phytosulfokine (PSK), a sulfate pentapeptide growth factor which strongly promotes proliferation of plant cells [21]. PSK controls hypocotyl length and cell expansion in *Arabidopsis thaliana* [22], probably acting together with brassinosteroids [23].

Cell proliferation in plants is mainly controlled by a family of cyclin-dependent kinases (CDKs), whose activity is directly regulated by binding and activation of cyclins [24]. We found cyclin and cyclin-dependent kinase with higher expression in the cold-tolerant seedlings (Table 1). Reduced cyclin-dependent kinase activity results in slower cell division as well as inhibition of growth under water deficit condition [25], and it is known that drought and low temperature stresses share several molecular responses, as both stresses ultimately result in dehydration of the cell and osmotic imbalance. Ma et al. [26] over-expressed the transcription factor *OsMYB3R-2* and enhanced tolerance to chilling stress by altering expression of cell cycle-related genes, including cyclin genes, suggesting that a cold resistance mechanism in rice could be mediated by regulating the cell cycle. Recently, Endo et al. [27] showed that *OsCDKB2;1* (the same gene we identified) is involved in mitosis and DNA damage response in rice, which prevents delay mitotic cell-cycle progression in response to DNA damage checkpoints. Yet, Chen et al. [28] over-expressed *OsRAN2* gene, which is essential for mitosis and can promote intranuclear tubulin export at the end of mitosis. The transgenic rice plants maintained cell division under cold condition and enhanced cold tolerance (up to 80% survival, compared to 14% survival in WT plants).

To check if the cold-tolerant seedlings present higher levels of cell expansion and division than the cold-sensitive ones, longitudinal sections of seedlings were stained with toluidine blue and DAPI, respectively. Seedlings from the cold-tolerant genotype in fact have larger epidermal cells and higher percentage of cells in mitotic phase than the cold-sensitive seedlings, under cold stress (Fig. 3). Under control conditions, coleoptiles from the tolerant genotype still have larger epidermal cells than the sensitive ones, although the difference is lower compared to cold stressed seedlings. Together with gene expression analysis, these data indicate that control of cell division and expansion is related to cold tolerance differences between these genotypes.

Cell growth is an irreversible increase in cell volume and is achieved in plants by an increase in cell wall extensibility, by the cells' osmotic potential that manifests itself as turgor pressure, and through water uptake driven by the increased turgor pressure. Three of the main solutes involved in osmoregulation are K^+,

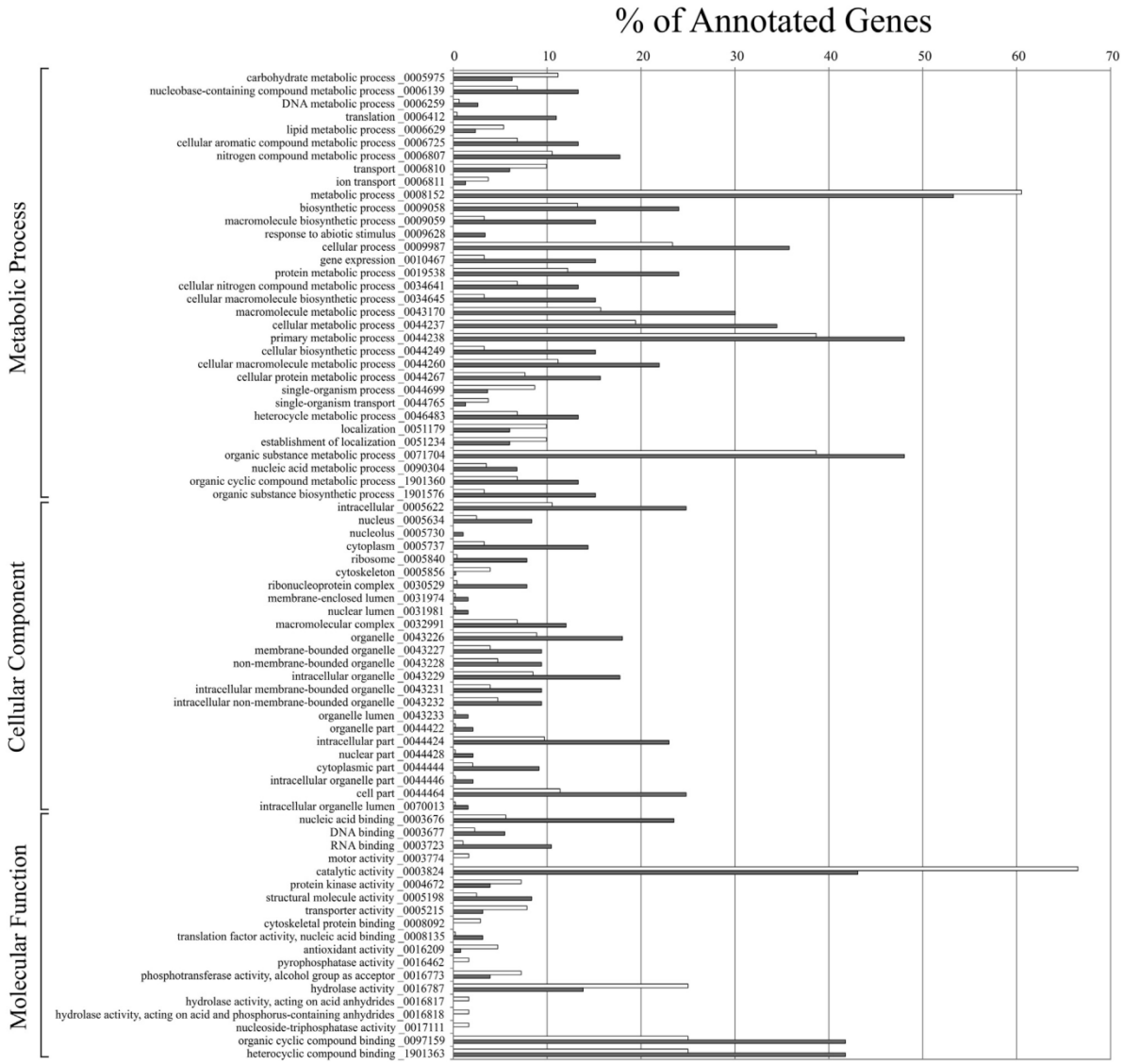

Fig. 2. Gene Ontology (GO) analysis of differentially expressed genes from transcriptomes of cold-tolerant and cold-sensitive genotypes. Terms enriched in either cold-tolerant or cold-sensitive datasets are shown as percentage of annotated genes in the dataset. All GO terms shown are differentially enriched in either groups using Fisher's Exact Test ($p \leq 0.05$). White columns refer to the cold-tolerant and gray columns to the cold-sensitive genotype.

Cl^- and sucrose [22]. One of the genes detected as more expressed in the cold-tolerant seedlings encodes an expansin protein, which facilitates cell wall loosening via a nonenzymatic mechanism, leading to cell expansion [29]. Low expansin expression levels have already been linked to low temperature sensitivity in cotton plants [30], and high expression levels seem to be important in abiotic stress tolerance conditions in *A. thaliana* [31]. We also found high expression of aquaporin encoding genes in the cold-tolerant seedlings, suggesting that water uptake is more efficient in this genotype. Seed germination is completely dependent of appropriate water uptake to restore the metabolic activity of the seed, and the developmental delay seen in the cold-sensitive seedlings could be related to an inefficient water uptake process. The higher expression of small hydrophilic plant seed proteins in the cold-sensitive seedlings (Table 1) could be related to this inefficient water transport. As cold ultimately results in low water availability within the cell, an efficient water transport is crucial to maintain cell viability. Aquaporins have already been suggested as responsible for cold stress-induced acclimation [32] and chilling tolerance in rice plants [33].

Seedlings from the cold-tolerant genotype also presented high expression levels of several ion transporters, including K^+ transporter and Cl^- channel protein, along with boron, sulfate, sodium, calcium, amino acid, peptide and citrate transporters (Table 1). It seems that transport capacity is, in general, more active in seedlings of the cold-tolerant genotype than in the cold-sensitive ones, being able to preserve osmotic as well as ionic equilibrium of the cell and maintain cellular homeostasis under the condition of stress. It is widely accepted that low temperatures activate ion channels [25], and over-expression of ion transporters can result in enhanced tolerance to abiotic stresses, such as the expression of *AtHKT1;1* (a sodium transporter), which improved salinity tolerance in rice plants [34]. As mentioned before, accumulation of sucrose and other simple sugars that occur with cold acclimation also

Table 1
List of selected differentially expressed genes identified by RNAseq in rice germinating seeds of cold-tolerant and cold-sensitive genotypes, after 7 days at 13 °C.

Functional category	Description	Normalized number of reads			
		Location	Tolerant	Sensitive	*p*-value
Unknown	Expressed protein (*OsEXD*)	**LOC_Os07g20164**	**1**	**1,295**	**0**
	Expressed protein	LOC_Os04g52750	61	3,731	0
	Expressed protein	LOC_Os11g09710	481	1	4.1 e^{-151}
Cell division and growth	Growth regulator-related protein	LOC_Os04g47520	558	248	8.2 e^{-39}
	Phytosulfokine receptor precursor	LOC_Os02g06090	122	0	3.2 e^{-37}
	Cyclin (*OsCYC*)	**LOC_Os01g59120**	**390**	**182**	**5.7 e^{-25}**
	Cyclin	LOC_Os06g51110	195	75	7.7 e^{-17}
	Cyclin-dependent kinase B2-1 (*OsCDKB2;1*)	**LOC_Os08g40170**	**430**	**223**	**5.3 e^{-23}**
Cell wall-related	WIP5 – wound-induced protein precursor	LOC_Os11g37970	3854	1128	0
	Glycine-rich cell wall structural protein 2 precursor	LOC_Os10g31660	379	13	2.4 e^{-106}
	Fasciclin-like arabinogalactan protein 8 precursor	LOC_Os08g23180	993	574	3.4 e^{-42}
	Glycine-rich cell wall protein	LOC_Os10g31530	1,016	601	7.5 e^{-41}
	CESA8 – cellulose synthase	LOC_Os07g10770	3,425	2,443	1.4 e^{-80}
	CESA6 – cellulose synthase	LOC_Os07g14850	1593	981	7.0 e^{-58}
	CESA1 – cellulose synthase	LOC_Os05g08370	3,844	3,098	2.3 e^{-55}
	CSLE6 – cellulose synthase-like family E	LOC_Os09g30130	429	111	2.9 e^{-55}
	CSLF6 – cellulose synthase-like family F	LOC_Os08g06380	471	136	3.0 e^{-55}
	CSLA1 – cellulose synthase-like family A; mannan synthase	LOC_Os02g09930	1069	608	4.1 e^{-47}
	CSLE1 - cellulose synthase-like family E (*OsCSLE1*)	**LOC_Os09g30120**	**354**	**100**	**3.4 e^{-42}**
	Expansin precursor	LOC_Os10g40730	921	372	7.0 e^{-74}
	Os3bglu7 – beta-glucosidase, exo-beta-glucanase	LOC_Os03g49600	5,959	3,431	5.8 e^{-256}
	Periplasmic beta-glucosidase precursor	LOC_Os03g53800	2,709	1,332	4.7 e^{-160}
	Glucan endo-1,3-beta-glucosidase precursor	LOC_Os11g47820	3,857	2,362	1.0 e^{-142}
	Glycosyl hydrolase	LOC_Os06g46284	1,728	915	2.1 e^{-88}
	Cellulase	LOC_Os10g22520	372	131	1.6 e^{-35}
	COBRA	LOC_Os05g32110	1,901	1,488	3.2 e^{-31}
	Caffeoyl-CoA O-methyltransferase	LOC_Os08g38900	606	332	5.1 e^{-29}
Transport	**Aquaporin protein (*OsAQU*)**	**LOC_Os07g26690**	**2,523**	**1,072**	**8.7 e^{-189}**
	Boron transporter protein	LOC_Os12g37840	2,030	1,218	7.4 e^{-79}
	Sulfate transporter	LOC_Os03g09940	747	285	1.3 e^{-64}
	Sodium/calcium exchanger protein	LOC_Os01g11414	902	397	1.1 e^{-63}
	Metal transporter Nramp6	LOC_Os07g15370	158	1	7.7 e^{-49}
	Amino acid transporter	LOC_Os02g09810	1,164	746	4.0 e^{-38}
	Transmembrane amino acid transporter protein	LOC_Os02g01100	511	246	3.6 e^{-31}
	Oligopeptide transporter	LOC_Os08g38400	165	52	1.9 e^{-17}
	Potassium transporter	LOC_Os04g32920	1,171	754	8.2 e^{-38}
	Transporter family protein	LOC_Os07g01560	470	192	3.0 e^{-37}
	Peptide transporter PTR2	LOC_Os06g15370	1,313	924	2.2 e^{-32}
	Major facilitator superfamily domain-containing protein 5	LOC_Os03g02380	776	472	3.7 e^{-29}
	MDR-like ABC transporter	LOC_Os01g50160	1,476	1,129	8.9 e^{-27}
	Citrate transporter protein	LOC_Os03g05390	1,889	1,632	5.6 e^{-19}
	Chloride channel protein	LOC_Os08g20570	75	7	7.9 e^{-17}
	OsHKT2;1 – Na^+ transporter	LOC_Os06g48810	104	21	6.4 e^{-16}
Lipid metabolism	Desaturase/cytochrome b5 protein	LOC_Os09g16920	1,028	534	1.1 e^{-54}
	3-ketoacyl-CoA synthase	LOC_Os05g49290	988	595	5.2 e^{-38}
	3-ketoacyl-CoA synthase (*OsKET*)	**LOC_Os11g37900**	**496**	**330**	**1.3 e^{-14}**
	3-ketoacyl-CoA thiolase	LOC_Os02g57260	6,692	6,543	2.0 e^{-26}
	Acyl-desaturase, chloroplast precursor	LOC_Os01g69080	649	1,412	1.4 e^{-37}
	Fatty acid desaturase	LOC_Os07g23410	217	53	3.9 e^{-29}
	Fatty acid hydroxylase	LOC_Os07g01150	630	372	1.4 e^{-25}
	WAX2	LOC_Os09g25850	210	53	2.2 e^{-27}
Detoxification	Peroxidase precursor	LOC_Os07g48020	2,253	588	2.2 e^{-290}
	Peroxidase precursor (*OsPRX*)	**LOC_Os01g19020**	**1,160**	**205**	**1.1 e^{-195}**
	OsAPx1 – Cytosolic Ascorbate Peroxidase	LOC_Os03g17690	8,885	6,839	5.9 e^{-157}
	OsAPx2 – Cytosolic Ascorbate Peroxidase	LOC_Os07g49400	1,957	1,581	5.3 e^{-28}
	Metallothionein	LOC_Os12g38270	1,022	359	6.4 e^{-98}
	Glutathione S-transferase	LOC_Os01g27210	942	479	3.5 e^{-52}
	Monodehydroascorbate reductase	LOC_Os08g44340	1,260	999	1.1 e^{-19}
Protein structure maintenance	Chaperone protein dnaJ	LOC_Os03g44620	7,204	13,328	6.5 e^{-208}
	Heat shock protein	LOC_Os09g30412	11,729	19,185	6.2 e^{-167}
	Heat shock protein 101	LOC_Os05g44340	328	822	5.9 e^{-31}
	Hsp20/alpha crystallin family protein	LOC_Os01g08860	26	320	8.2 e^{-53}
	Chaperone protein clpB 1	LOC_Os03g31300	953	1,617	2.8 e^{-17}
	Early-responsive to dehydration protein-related	LOC_Os01g72210	664	1,428	6.9 e^{-37}

Table 1 (*Continued*)

Functional category	Description	Normalized number of reads			
		Location	Tolerant	Sensitive	*p*-value
Protein degradation	**F-box domain containing protein (*OsFBX221*)**	**LOC_Os07g09814**	**21**	**204**	**2.8 e^{-30}**
	OsFBX114 – F-box domain containing protein	LOC_Os04g02280	0	91	1.4 e^{-22}
	OsFBX335 – F-box domain containing protein	LOC_Os09g32860	0	80	1.1 e^{-19}
	OsFBDUF53 – F-box and DUF domain containing protein	LOC_Os11g37300	0	83	1.5 e^{-20}
	Ubiquitin family protein	LOC_Os06g46770	38,810	51,667	3.2 e^{-65}
	OsFBL22 – F-box domain and LRR containing protein	LOC_Os05g35110	969	1,879	5.8 e^{-35}
	OsFBL7 – F-box domain and LRR containing protein	LOC_Os02g10700	939	1,820	9.0 e^{-34}
	Proteasome-related	LOC_Os05g48340	1,254	2,177	8.0 e^{-26}
	Ubiquitin-conjugating enzyme	LOC_Os03g57790	1,135	1,947	6.8 e^{-22}
	Ubiquitin carboxyl-terminal hydrolase domain containing protein	LOC_Os11g34270	83	263	2.5 e^{-15}
Transcription factor	Zinc finger/CCCH transcription factor	LOC_Os01g09620	259	907	3.5 e^{-60}
	Zinc finger A20 and AN1 domain-containing stress-associated protein	LOC_Os06g41010	1,633	3,068	9.2 e^{-51}
	Zinc finger, ZZ type domain containing protein	LOC_Os09g33740	128	0	2.7 e^{-39}
	LSD1 zinc finger domain containing protein	LOC_Os08g06280	587	277	5.0 e^{-37}
	OsWRKY30	LOC_Os08g38990	235	703	7.4 e^{-37}
	MYB family transcription factor	LOC_Os01g12860	339	121	1.2 e^{-31}
	bZIP transcription factor family protein	LOC_Os02g14910	182	34	3.0 e^{-29}
	E2F family transcription factor protein	LOC_Os02g50630	166	40	9.9 e^{-23}
Hormone signaling	1-aminocyclopropane-1-carboxylate oxidase protein	LOC_Os02g53180	281	34	6.5 e^{-57}
	Ethylene-insensitive protein	LOC_Os03g49400	514	1,270	2.4 e^{-46}
	Ethylene-responsive transcription factor	LOC_Os02g51670	319	189	6.0 e^{-13}
	***OsIAA13* - Auxin-responsive Aux/IAA gene family member (*OsAUX*)**	**LOC_Os03g53150**	**677**	**277**	**3.5 e^{-53}**
	OsIAA31 – Auxin-responsive Aux/IAA gene family member	LOC_Os12g40900	445	215	3.5 e^{-27}
	Auxin efflux carrier component	LOC_Os09g38130	188	49	3.0 e^{-24}
	Auxin response factor	LOC_Os01g70270	1,312	2,584	1.0 e^{-50}
	Auxin-responsive protein	LOC_Os01g48850	463	257	1.3 e^{-21}
	Gibberellin 20 oxidase 1	LOC_Os03g63970	204	72	1.9 e^{-19}
	Cytokinin-O-glucosyltransferase 3	LOC_Os04g44240	620	451	8.1 e^{-14}
	OsCML1 – Calmodulin-related calcium sensor protein	LOC_Os01g59530	617	326	1.1 e^{-31}
Ca^{2+}-signaling	Calcium/calmodulin-depedent protein kinase	LOC_Os05g26820	460	210	9.6 e^{-31}
	IQ calmodulin-binding motif domain containing protein	LOC_Os05g03190	658	379	2.6 e^{-28}
	OsCam1-1 – Calmodulin	LOC_Os03g20370	906	658	4.3 e^{-20}
	OsCML7 – Calmodulin-related calcium sensor protein	LOC_Os08g02420	282	158	5.9 e^{-13}
Others	Small hydrophilic plant seed protein	LOC_Os05g28210	198	2,389	0
	Small hydrophilic plant seed protein	LOC_Os01g06630	6	761	4.2 e^{-186}
	Cysteine synthase	LOC_Os03g53650	2,188	1,028	9.3 e^{-140}
	Sucrose synthase	LOC_Os03g28330	11,360	10,770	2.7 e^{-59}
	Dehydrin (*OsDHN*)	**LOC_Os11g26570**	**1,795**	**5,557**	**1.5 e^{-309}**
	Late embryogenesis abundant group 1	LOC_Os04g49980	984	3,721	1.6 e^{-273}
	Late embryogenesis abundant protein, group 3 (*OsLEA*)	**LOC_Os01g50910**	**791**	**2,733**	**1.7 e^{-178}**
	Phytochrome A	LOC_Os03g51030	1,475	3,383	9.8 e^{-104}
	S-adenosyl-L-methionine decarboxylase leader peptide	LOC_Os02g39795	4,468	6,562	7.5 e^{-27}
	S-adenosylmethionine synthetase	LOC_Os01g18860	1,094	1,957	1.5 e^{-26}
	HVA22	LOC_Os08g36440	102	392	4.0 e^{-29}
	Spermidine synthase	LOC_Os06g33710	364	791	4.7 e^{-21}

A list of 1,361 most differentially expressed genes obtained from the RNAseq experiments is shown in Supplementary Table 2. Differential expression of transcripts marked in bold was confirmed by RT-qPCR.

contributes to the stabilization of membrane as these molecules can protect membranes against low temperature damages [25]. Several sucrose synthases, β-glucosidases, glycosil hydrolases and cellulases were detected with higher expression on the cold-tolerant seedlings, suggesting that these processes (synthesis of sucrose and breakdown of polysaccharides to generate simple sugars, as glucose) operate in higher levels when compared to the cold-sensitive seedlings.

As previously observed by Gothandam et al. [35], who overexpressed a cell wall-related gene (*OsPRP3*), one of the mechanisms to acquire low temperature tolerance in rice plants is cell wall reinforcement. Several cell wall structural proteins, including cellulose and lignin metabolism-related genes, were identified in our experiment, with higher expression in the cold-tolerant seedlings. Expression levels of cellulose synthase genes have already been correlated with abiotic stress tolerance in rice plants [36]. Consistently, preliminary data from our group has indicated higher cellulose staining on the mesophyll cell walls of the leaves from the cold-tolerant genotype, when compared to the cold-sensitive ones, after plants at the vegetative growth stage were maintained for 10 days at 10 °C. Recently, Li et al. [37] reported that downregulation of *caffeoyl-CoA O-methyltransferase* (one of our identified genes) by RNA interference leads to reduced lignin production in maize straw. Another gene identified (*WIP5 – Wound-induced protein*) is involved with the reinforcement of cell wall composition, as previously reported by Yen et al. [38]. All these data suggest that the cold-tolerant seedlings can maintain cell wall integrity under low temperature more efficiently than the cold-sensitive ones.

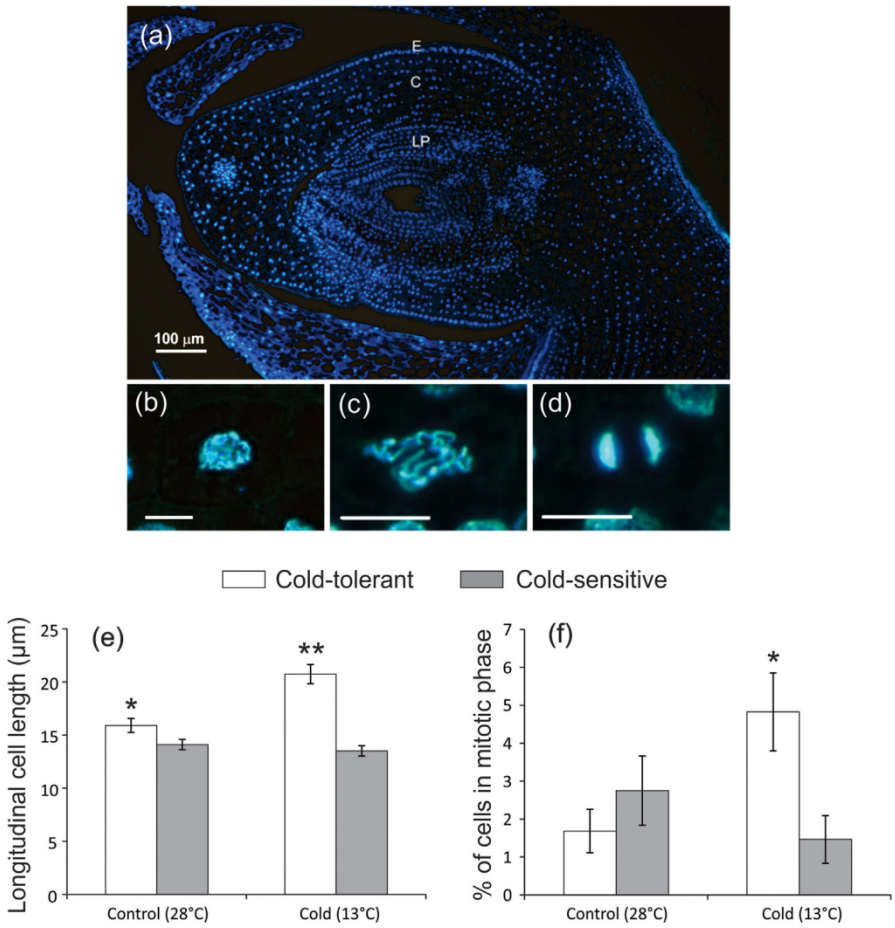

Fig. 3. Cell expansion and division levels in rice seedlings at the S1 stage of the two *indica* rice genotypes. (a) Longitudinal section of germinating seeds. (b)–(d) Mitotic cells in prophase, metaphase and telophase, respectively. (e) Epidermal (E) cell length (μm) of seeds germinating at 28 °C for one day (control) and at 13 °C for 10 days (cold). (f) Percentage of coleoptile (C) and leaf primordium (LP) cells in mitotic phase analyzed in seeds germinating at 28 °C for one day (control) and at 13 °C for 10 days (cold). Values are the averages of at least five samples ± SE. Mean values with one or two asterisks are different by Student's *t* test ($p \leq 0.05$ and 0.01, respectively). Bars in figures (b), (c), and (d) indicate 10 μm.

One of the major negative effects of cold stress is the induction of severe membrane damage. It is known that lipids containing saturated fatty acids solidify at temperatures higher than those containing unsaturated fatty acids. Therefore, the relative proportion of unsaturated fatty acids in the membrane strongly influences membrane fluidity [25,39]. Several fatty acid desaturases with higher expression in the cold-tolerant seedlings were detected (Table 1 and Supplementary Table 2), suggesting that these germinating seeds present higher levels of unsaturated fatty acids when submitted to cold stress. Kargiotidou et al. [40] showed an enhanced production of unsaturated fatty acids in cotton plants submitted to low temperature conditions. Other two genes related with lipid metabolism which showed different expression levels between the two tested genotypes are 3-ketoacyl-CoA synthase and WAX2, both involved in wax biosynthesis. Recently, two different groups established a connection between wax biosynthesis in rice and drought tolerance via stress-induced wax accumulation [41,42]. Apparently, wax biosynthesis can also be stimulated by low temperature stress in rice, and our results suggest that the cold-tolerant seedlings synthesize more wax than the cold-sensitive ones under cold conditions.

We found several genes related to antioxidant capacity with higher expression in the cold-tolerant seedlings, including peroxidase, ascorbate peroxidase, metallothionein, glutathione S-transferase and monodehydroascorbate reductase. Many reports have linked the activity of antioxidant enzymes with cold stress response and tolerance in rice plants [43,44]. Sato et al. [45] over-expressed *OsAPXa* (an ascorbate peroxidase-coding gene) and obtained cold-tolerant rice plants.

Metallothioneins are low molecular weight, cysteine-rich metal chelators with an ability to bind heavy metal ions and are also capable of reactive oxygen species (ROS) scavenging. Several reports have shown that metallothioneins have a role on abiotic stress response, and recently Kumar et al. [46] showed that ectopic expression of *OsMT1e-P* confers multiple abiotic stress tolerance in tobacco plants via ROS scavenging. In accordance with this finding, we also detected higher expression of the cysteine synthase gene in the cold-tolerant seedlings.

Glutathione S-transferase, which presents ROS scavenger activity, has been related to cold tolerance in rice plants. According to Kim et al. [47], a SNP which results in amino acid substitution (I99V – Ile to Val) leads to reduced enzyme activity, explaining the differential response observed between cold-tolerant *japonica* and cold-sensitive *indica* rice genotypes. Also, expression of a rice glutathione S-transferase (*OsGSTL2*) in Arabidopsis provides tolerance to heavy metal and other abiotic stresses, including low

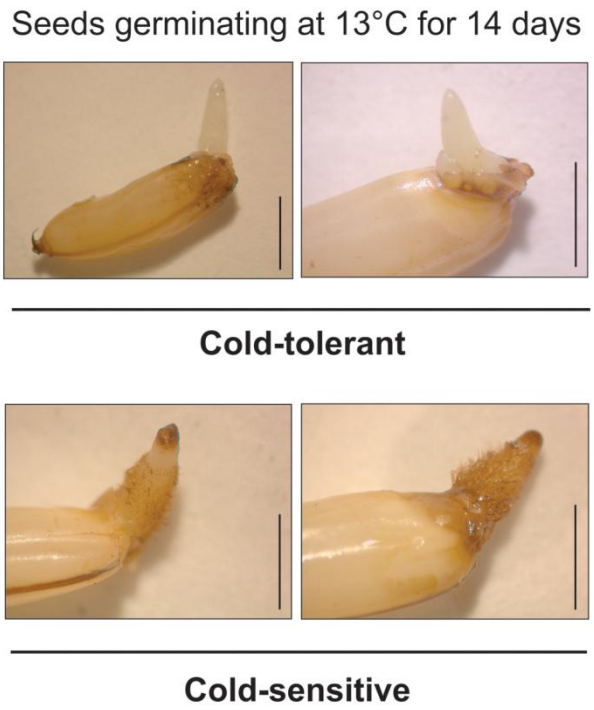

Fig. 4. Histochemical staining assay of H_2O_2 by diaminobenzidine (DAB) in rice germinating seeds of the both genotypes after 14 days at 13 °C. The positive staining (detected in higher levels on the cold-sensitive genotype) in the photomicrographs shows as bright brown images. The images shown are representative of the other 47 analyzed seeds. No difference in H_2O_2 staining was detected in seeds germinating at 28 °C. Bars in figures indicate 0.5 cm.

temperature [48]. Additionally, over-expression of monodehydroascorbate reductase from the mangrove plant *Acanthus ebracteatus* generates transgenic rice plants with enhanced salt tolerance [49], suggesting that this class of antioxidant could be used to generate stress-tolerant rice plants.

To check if the seedlings from the cold-tolerant genotype present lower levels of H_2O_2 when compared to the cold-sensitive one, due to the higher expression of several peroxidases, we detected *in situ* accumulation of H_2O_2 by histochemical staining with diaminobenzidine (DAB). In fact, as seen in Fig. 4, coleoptiles of cold-tolerant germinating seeds present lower levels of H_2O_2 staining when seeds are germinated at 13 °C for 14 days, suggesting that high activity levels of peroxidases are present when seeds from this genotype are subjected to low temperature germination.

Several families of transcription factors (TFs) were detected in our experiments as differentially expressed, including zinc finger proteins, WRKY, MYB, bZIP and E2F. Some of these genes are more expressed in the cold-tolerant and others in the cold-sensitive seedlings (Table 1). A number of TFs have been related to cold stress response in *japonica* rice genotypes, and several of these lead to cold-tolerance in rice plants when over-expressed (for a comprehensive review see [3]). Transcription factors are excellent targets to manipulate complex pathways, and a list of putative cold-related TFs is provided here, even though more in-depth studies will be needed to elucidate their functions.

By modification on the production and distribution of phytohormones, plants are able to grow under stress conditions [50]. We detected hormone-related genes with different expression levels (Table 1), which could be related to the cold-stress response of both genotypes and cold-tolerance of IRGA 959-1-2-2F-4-1-4-A seedlings. From the 10 genes listed in Table 1, five are related to auxin, three to ethylene, one to gibberellin and one to cytokinin. Zhang et al. [44] suggested that a close interaction between auxin signaling and low temperature stress response occurs in rice plants. Ethylene signaling has been already linked to cold tolerance in rice seedlings, when the response factor *LeTERF2* was over-expressed [51]. Moreover, plant growth restriction after exposure to stress conditions (including cold) is at least partially caused by a reduction of gibberellin levels and signaling [50]. Our findings suggest that a hormonal crosstalk regulates the germinating seeds response and growth under low temperature stress, probably with auxin gradient playing a central role [52].

The proteins which sense cytoplasmic Ca^{2+} perturbations and relay this information to downstream molecules serve as an important component of cold signaling [53]. We detected several Ca^{2+}-signaling related genes differentially expressed in the seedlings of the cold-tolerant genotype, mostly related to Calmodulin (CaM) (Table 1), evidencing a highly active Ca^{2+}-signaling pathway in this genotype. According to Chinpongpanich et al. [54], CaM-related genes can play differential roles as sensor relays in regulating Ca^{2+}-mediated responses to abiotic stresses, including cold. Yet, the Ca^{2+}-dependent protein kinase 13 (*OsCDPK13*) play an important role in the cold signal transduction, since cold-tolerant rice varieties exhibited higher expression of *OsCDPK13* than the cold-sensitive ones, the protein accumulates in response to cold, and the overexpressing transgenic lines are cold-tolerant [55,56].

Several genes with higher expression in the cold-sensitive seedlings (heat shock proteins, early-responsive to dehydration, ubiquitin/proteasome-related proteins, dehydrin, late embryogenesis abundant proteins, S-adenosyl-L-methionine decarboxylase, S-adenosylmethionine synthetase and spermidine synthase) seem to show that these seedlings present more stressful responses to the low temperature condition. Accumulation of several cryoprotectants, including polyamines, can preserve the intracellular proteins by inducing molecular chaperones-encoding genes [25]. Extensive evidence indicates that heat shock proteins in plants are involved in abiotic stress response, especially to extreme environmental temperature [57]. The *A. thaliana* ERD10 gene can play roles in plant protection from various stresses, including cold [58], and also in seed development and germination, acting as chaperone-like proteins [59]. Three genes, S-adenosylmethionine synthetase, S-adenosyl-L-methionine decarboxylase (SAMDC) and spermidine synthase are involved in polyamine biosynthesis, and according to Pillai and Akiyama [60], SAMDC could be used as a molecular marker in the identification of rice tolerance to low temperature. Yet, Wi et al. [61] reported that SAMDC over-expression enhanced cold tolerance in transgenic tobacco plants. As seen above, some genes identified in our study as more expressed in the cold-sensitive seedlings have been previously shown to confer cold tolerance when over-expressed in plants. This apparent contradiction may be related to different expression levels (much higher in over-expressing plants than in WT plants under cold stress) and to different plant genetic backgrounds (Arabidopsis genes [58,59]; carnation gene expressed in tobacco plants [61]; higher expression in cold-tolerant japonica rice than in sensitive indica rice [60], which is the background of both lines used here). Moreover, cold tolerance is a complex trait, which may depend on the proper combination of changes in expression of several genes. Increased expression of only some of them may not be enough to confer tolerance.

Several genes related to protein degradation via ubiquitin/proteasome pathway suggest that a highly active ubiquitination/degradation occurs in the cold-sensitive seedlings, probably evidencing a high protein turnover stimulated by cold stress injuries. Yan et al. [62] showed that over-expression of an F-box protein gene (*OsMAIF1*) resulted in low abiotic stress tolerance in rice plants, suggesting that these proteins can play negative roles in

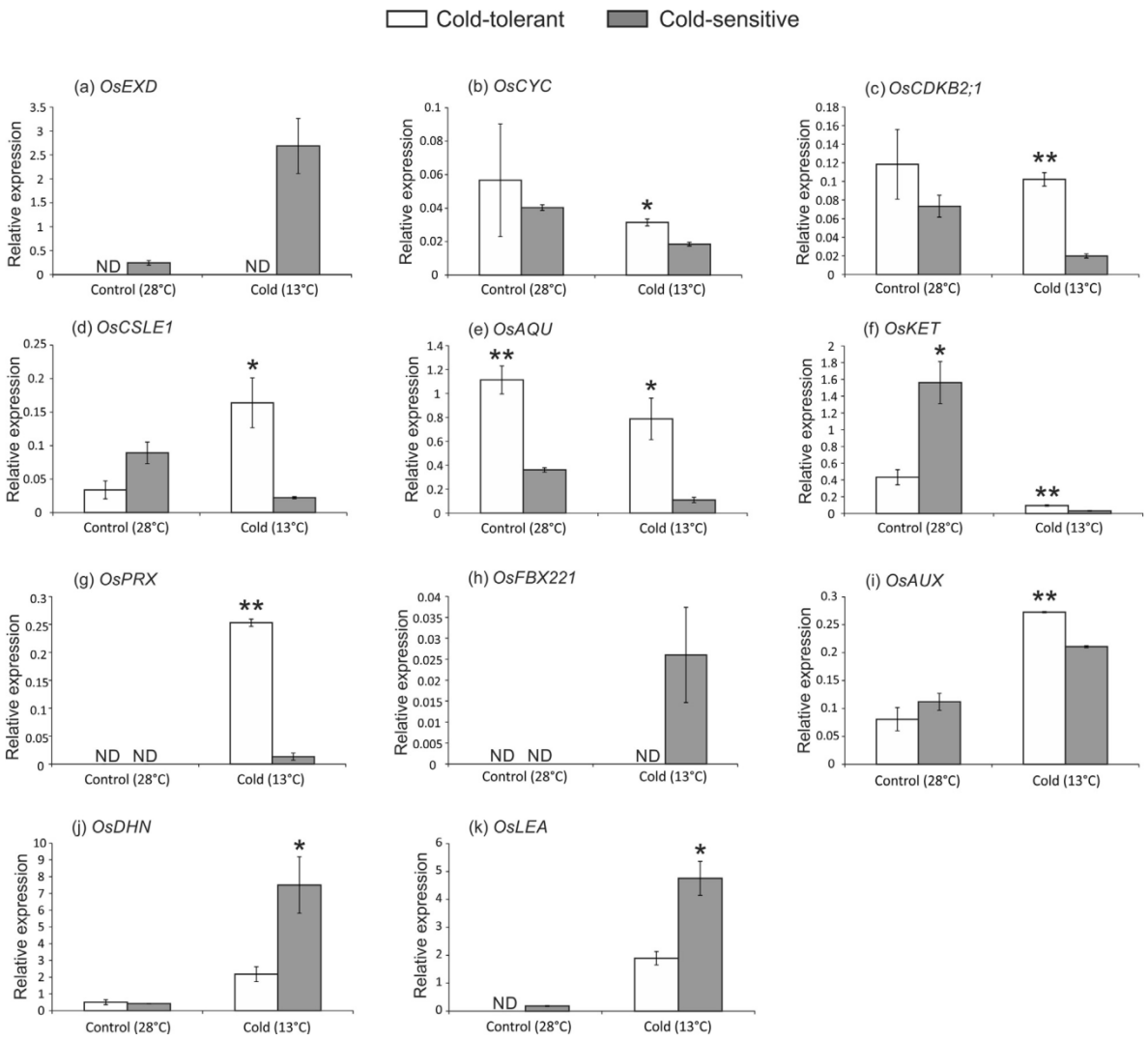

Fig. 5. Relative expression levels (RT-qPCR, relative to *OsUBQ5* expression) of selected genes identified by deep sequencing under control temperature (28 °C for 2 days) and after 7 days of cold (13 °C). Coleoptiles were about the same length on both treatments at the time of RNA extraction. *OsEXD*, expressed protein; *OsCYC*, cyclin; *OsCDKB2;1*, cyclin-dependent kinase *B2-1*; *OsCSLE1*, cellulose synthase-like family *E*; *OsAQU*, aquaporin protein; *OsKET*, 3-ketoacyl-CoA synthase; *OsPRX*, peroxidase precursor; *OsFBX221*, F-box domain containing protein; *OsAUX*, OsIAA13 – Auxin-responsive Aux/IAA gene family member; *OsDHN*, Dehydrin; OsLEA, late embryogenesis abundant protein, *group 3*. Values are the averages of three samples ± SE. Mean values with one asterisk are different by Student's *t* test ($p \leq 0.05$). ND = not detected.

response to abiotic stress. It is widely accepted that cold and other stressful treatments induce the synthesis of dehydrins and late embryogenesis abundant protein (LEAs), and many reports showed that these genes are related to abiotic stress response and/or tolerance [63,64]. It is important to note that these genes with higher expression in the cold-sensitive seedlings may represent part of the cold response of this genotype, and should not be related to the cold-tolerance phenotype of the other genotype.

Two other genes, more expressed in the cold-sensitive seedlings, could be responsible for delayed seed germination. The *HVA22* gene, first identified in *Hordeum vulgare*, can be induced by ABA and environmental stresses, such as cold and drought [65]. It may play an important role in protecting cells from damage under stress conditions, and seems to be part of the seed germination process regulation, through the inhibition of vesicular trafficking involved in nutrient mobilization, delaying coalescence of protein storage vacuoles [66]. We also detected Phytochrome A gene as differentially expressed (Table 1). According to Xie et al. [67], coleoptile growth in rice can be photo-inhibited by a process mediated by phytochromes. Therefore, the higher expression of this gene in the cold-sensitive seedlings could be partially responsible for delayed seed germination and coleoptile elongation.

3.4. Confirmation of RNAseq expression patterns using RT-qPCR

To validate the RNAseq results and to confirm the differential expression of the isolated genes, *OsEXD* (expressed protein); *OsCYC* (cyclin); *OsCDKB2;1* (Cyclin-dependent kinase B2-1); *OsCSLE1* (cellulose synthase-like family E); *OsAQU* (aquaporin protein); *OsKET* (3-ketoacyl-CoA synthase); *OsPRX* (peroxidase precursor); *OsFBX221* (F-box domain containing protein); *OsAUX* (*OsIAA13* – Auxin-responsive Aux/IAA gene family member); *OsDHN* (dehydrin); *OsLEA* (late embryogenesis abundant protein, group 3), all listed in Supplementary Table 1, were further evaluated in germinating seeds of both genotypes by RT-qPCR. These genes were chosen based on *p* value and/or fold-change. The differences in

expression found by the RNAseq methodology were confirmed for the 11 tested genes (Fig. 5). Most of the tested genes (*OsCYC*, *OsCDKB2;1*, *OsCSLE1*, *OsAQU*, *OsKET*, *OsPRX* and *OsAUX*) showed higher expression in the cold-tolerant seedlings after 7 days of cold. From these, only one (*OsAQU*) is also more expressed in the cold-tolerant seedlings under control growth conditions (48 h at 28 °C), which could be representative of a constitutive tolerance mechanism, as previously suggested [30]. Of the 11 tested genes, only four (*OsEXD*, *OsFBX221*, *OsDHN* and *OsLEA*) showed higher expression in the cold-sensitive seedlings after 7 days of cold, and gene expression of two (*OsEXD* and *OsFBX221*) were not detected in the cold-tolerant seedlings.

4. Conclusion

In summary, this study suggests different molecular and physiological changes in response to low temperature stress which can probably be related to the contrasting levels of cold-tolerance presented by the two *indica* rice genotypes evaluated. High throughput RNAseq showed that several genes are differentially expressed in these genotypes, and some of these genes are probably involved in cold stress tolerance during germination. Several processes are more active in the cold-tolerant than in the cold-sensitive genotype: cell division and growth rates, cell wall integrity and extensibility, water uptake and membrane transport capacity, sucrose synthesis, generation of simple sugars, proportion of unsaturated fatty acids, wax biosynthesis, antioxidant activity, and hormone and Ca^{2+}-signaling, which ultimately lead to cold adaptation and tolerance. On the other hand, the cold-sensitive genotype respond to low temperature stress increasing the synthesis of heat shock proteins, dehydrins, LEAs, along with enhanced ubiquitin/proteasome protein degradation pathway and polyamine biosynthesis. It is important to highlight that only a small portion (109 out of 1,361) of the differentially expressed genes were selected as probably involved in cold response (Table 1 and Supplementary Table 2). We believe that additional genes, especially the ones which encode unknown expressed proteins and transcription factors, are related to the contrasting level of cold-tolerance observed in the analyzed *indica* rice genotypes. However, further studies are needed to establish this connection between gene expression and cold response. Even though the level of mRNAs can be different from the protein level or activity, our findings can be useful starting points for more in-depth analyses of the cold-regulated mechanisms in rice germinating seeds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.05.009

References

[1] K. Datta, S.K. Datta, *Indica* rice (*Oryza sativa*, BR29 and IR64), in: K. Wang (Ed.), Methods in Molecular Biology, vol. 343: Agrobacterium Protocols, Humana Press, Totowa, NJ, 2006, pp. 201–212.
[2] H. Ye, H. Du, N. Tang, X. Li, L. Xiong, Identification and expression profiling analysis of TIFY family genes involved in stress and phytohormone responses in rice, Plant Mol. Biol. 71 (2009) 291–305.
[3] R.P. Cruz, R.A. Sperotto, D. Cargnelutti, J.M. Adamski, T.F. Terra, J.P. Fett, Avoiding damage and achieving cold tolerance in rice plants, Food Energy Secur. 2 (2013) 96–119.
[4] Q. Zhang, Q. Chen, S. Wang, Y. Hong, Z. Wang, Rice and cold stress: methods for its evaluation and summary of cold tolerance-related quantitative trait loci, Rice 7 (2014) 24.
[5] P. Yang, X. Li, X. Wang, H. Chen, F. Chen, S. Shen, Proteomic analysis of rice (*Oryza sativa*) seeds during germination, Proteomics 7 (2007) 3358–3368.
[6] S.C.K. Milach, R.P. Cruz, Cold tolerance at the germination stage of rice: methods of evaluation and characterization of genotypes, Sci. Agric. 61 (2004) 1–8.
[7] M.G.S. Lima, N.F. Lopes, P.D. Zimmer, G.E. Meneghello, C.R. Mendes, L. Amarante, Enzyme expression in *indica* and *japonica* rice genotypes under saline stress, Acta Sci. Biol. Sci. 34 (2012) 473–481.
[8] K. Fujino, H. Sekiguchi, T. Sato, H. Kiuchi, Y. Nonoue, Y. Takeuchi, T. Ando, S.Y. Lin, M. Yano, Mapping of quantitative trait loci controlling low-temperature germinability in rice (*Oryza sativa* L.), Theor. Appl. Genet. 108 (2004) 794–799.
[9] L.J. Xue, J.J. Zhang, H.W. Xue, Genome-wide analysis of the complex transcriptional networks of rice developing seeds, PLoS ONE 7 (2012) e31081.
[10] D. He, C. Han, P. Yang, Gene expression profile changes in germinating rice, J. Integr. Plant Biol. 53 (2011) 835–844.
[11] S.J. Liu, H.H. Xu, W.Q. Wang, N. Li, W.P. Wang, I.M. Moller, S.Q. Song, A proteomic analysis of rice seed germination as affected by high temperature and ABA treatment, Physiol. Plant. (2014), http://dx.doi.org/10.1111/ppl.12292
[12] W. Wang, S. Liu, S. Song, I.M. Moller, Proteomics of seed development, desiccation tolerance, germination and vigor, Plant Physiol. Biochem. 86C (2015) 1–15.
[13] M.D. Robinson, A. Oshlack, A scaling normalization method for differential expression analysis of RNA-seq data, Genome Biol. 11 (2010) R25.
[14] M.D. Robinson, D.J. McCarthy, G.K. Smyth, EdgeR: a bioconductor package for differential expression analysis of digital gene expression data, Bioinformatics 26 (2010) 139–140.
[15] A. Conesa, S. Gotz, Blast2GO: a comprehensive suite for functional analysis in plant genomics, Int. J. Plant Genomics 2008 (2008) 619832.
[16] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat. Prot. 3 (2008) 1101–1108.
[17] M. Jain, A. Nijhawan, A.K. Tyagi, J.P. Khurana, Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR, Biochem. Biophys. Res. Commun. 345 (2006) 646–651.
[18] J. Shi, X.Z. Fu, T. Peng, X.S. Huang, Q.J. Fan, J.H. Liu, Spermine pretreatment confers dehydration tolerance of citrus *in vitro* plants via modulation of antioxidative capacity and stomatal response, Tree Physiol. 30 (2010) 914–922.
[19] P.A. Counce, T.C. Kiesling, A.J. Mitchell, A uniform, objective, and adaptive system for expressing rice development, Crop Sci. 40 (2000) 436–443.
[20] P.O. Gerrits, L. Smid, A new, less toxic polymerization system for the embedding of soft tissues in glycol methacrylate and subsequent preparing of serial sections, J. Microsc. 132 (1983) 81–85.
[21] H. Yang, Y. Matsubayashi, H. Hanai, Y. Sakagami, Phytosulfokine-alpha, a peptide growth factor found in higher plants: its structure, functions, precursor and receptors, Plant Cell Physiol. 41 (2000) 825–830.
[22] N. Stuhrwohldt, R.I. Dahlke, B. Steffens, A. Johnson, M. Sauter, Phytosulfokine-alpha controls hypocotyl length and cell expansion in *Arabidopsis thaliana* through phytosulfokine receptor 1, PLoS ONE 6 (2011) e21054.
[23] J. Hartmann, N. Stuhrwohldt, R.I. Dahlke, M. Sauter, Phytosulfokine control of growth occurs in the epidermis, is likely to be non-cell autonomous and is dependent on brassinosteroids, Plant J. 73 (2013) 579–590.
[24] H. La, J. Li, Z. Ji, Y. Cheng, X. Li, S. Jiang, P.N. Venkatesh, S. Ramachandran, Genome-wide analysis of cyclin family in rice (*Oryza sativa* L.), Mol. Genet. Genom. 275 (2006) 374–386.
[25] S. Mahajan, N. Tuteja, Cold, salinity and drought stresses: an overview, Arch. Biochem. Biophys. 444 (2005) 139–158.
[26] Q. Ma, X. Dai, Y. Xu, J. Guo, Y. Liu, N. Chen, J. Xiao, D. Zhang, Z. Xu, X. Zhang, K. Chong, Enhanced tolerance to chilling stress in OsMYB3R-2 transgenic rice is mediated by alteration in cell cycle and ectopic expression of stress genes, Plant Physiol. 150 (2009) 244–256.
[27] M. Endo, S. Nakayama, C. Umeda-Hara, N. Ohtsuki, H. Saika, M. Umeda, S. Toki, CDKB2 is involved in mitosis and DNA damage response in rice, Plant J. 69 (2012) 967–977.
[28] N. Chen, Y. Xu, X. Wang, C. Du, J. Du, M. Yuan, Z. Xu, K. Chong, OsRAN2, essential for mitosis, enhances cold tolerance in rice by promoting export of intranuclear tubulin and maintaining cell division under cold stress, Plant Cell Environ. 34 (2011) 52–64.
[29] J. Sampedro, D.J. Cosgrove, The expansin superfamily, Genome Biol. 6 (2005) 242.
[30] M. Zheng, Y. Wang, K. Liu, H. Shu, Z. Zhou, Protein expression changes during cotton fiber elongation in response to low temperature stress, J. Plant Physiol. 169 (2012) 399–409.
[31] S. Abuqamar, S. Ajeb, A. Sham, M.R. Enan, R. Iratni, A mutation in the *expansin-like A2* gene enhances resistance to necrotrophic fungi and hypersensitivity to abiotic stress in *Arabidopsis thaliana*, Mol. Plant Pathol. 14 (2013) 813–827.

[32] A. Ahamed, M. Murai-Hatano, J. Ishikawa-Sakurai, H. Hayashi, Y. Kawamura, M. Uemura, Cold stress-induced acclimation in rice is mediated by root-specific aquaporins, Plant Cell Physiol. 53 (2012) 1445–1456.
[33] T. Matsumoto, H.L. Lian, W.A. Su, D. Tanaka, C. Liu, I. Iwasaki, Y. Kitagawa, Role of the aquaporin PIP1 subfamily in the chilling tolerance of rice, Plant Cell Physiol. 50 (2009) 216–229.
[34] D. Plett, G. Safwat, M. Gilliham, I. Skrumsager Moller, S. Roy, N. Shirley, A. Jacobs, A. Johnson, M. Tester, Improved salinity tolerance of rice through cell type-specific expression of AtHKT1;1, PLoS ONE 5 (2010) e12571.
[35] K.M. Gothandam, E. Nalini, S. Karthikeyan, J.S. Shin, OsPRP3, a flower specific proline-rich protein of rice, determines extracellular matrix structure of floral organs and its overexpression confers cold-tolerance, Plant Mol. Biol. 72 (2010) 125–135.
[36] C.W. Liu, Y.K. Hsu, Y.H. Cheng, H.C. Yen, Y.P. Wu, C.S. Wang, C.C. Lai, Proteomic analysis of salt-responsive ubiquitin-related proteins in rice roots, Rapid Commun. Mass Spectro. 26 (2012) 1649–1660.
[37] X. Li, W. Chen, Y. Zhao, Y. Xiang, H. Jiang, S. Zhu, B. Cheng, Downregulation of caffeoyl-CoA O-methyltransferase (CCoAOMT) by RNA interference leads to reduced lignin production in maize straw, Genet. Mol. Biol. 36 (2013) 540–546.
[38] S.K. Yen, M.C. Chung, P.C. Chen, H.E. Yen, Environmental and developmental regulation of the wound-induced cell wall protein WI12 in the halophyte ice plant, Plant Physiol. 127 (2001) 517–528.
[39] K. Iba, Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance, Annu. Rev. Plant Biol. 53 (2002) 225–245.
[40] A. Kargiotidou, D. Deli, D. Galanopoulou, A. Tsaftaris, T. Farmaki, Low temperature and light regulate delta 12 fatty acid desaturases (FAD2) at a transcriptional level in cotton (*Gossypium hirsutum*), J. Exp. Bot. 59 (2008) 2043–2056.
[41] M.A. Islam, H. Du, J. Ning, H. Ye, L. Xiong, Characterization of Glossy1-homologous genes in rice involved in leaf wax accumulation and drought resistance, Plant Mol. Biol. 70 (2009) 443–456.
[42] L. Zhou, E. Ni, J. Yang, H. Zhou, H. Liang, J. Li, D. Jiang, Z. Wang, Z. Liu, C. Zhuang, Rice OsGL1-6 is involved in leaf cuticular wax accumulation and drought resistance, PLoS ONE 8 (2013) e65139.
[43] V. Bonnecarrere, O. Borsani, P. Diaz, F. Capdevielle, P. Blanco, J. Monza, Response to photoxidative stress induced by cold in *japonica* rice is genotype dependent, Plant Sci. 180 (2011) 726–732.
[44] T. Zhang, X. Zhao, W. Wang, Y. Pan, L. Huang, X. Liu, Y. Zong, L. Zhu, D. Yang, B. Fu, Comparative transcriptome profiling of chilling stress responsiveness in two contrasting rice genotypes, PLoS ONE 7 (2012) e43274.
[45] Y. Sato, Y. Masuta, K. Saito, S. Murayama, K. Ozawa, Enhanced chilling tolerance at the booting stage in rice by transgenic overexpression of the ascorbate peroxidase gene, *OsAPXa*, Plant Cell Rep. 30 (2011) 399–406.
[46] G. Kumar, H.R. Kushwaha, V. Panjabi-Sabharwal, S. Kumari, R. Joshi, R. Karan, S. Mittal, S.L. Pareek, A. Pareek, Clustered *metallothionein* genes are co-regulated in rice and ectopic expression of *OsMT1e-P* confers multiple abiotic stress tolerance in tobacco via ROS scavenging, BMC Plant Biol. 12 (2012) 107.
[47] S.I. Kim, V.C. Andaya, T.H. Tai, Cold sensitivity in rice (*Oryza sativa* L.) is strongly correlated with a naturally occurring I99V mutation in the multifunctional glutathione transferase isoenzyme GSTZ2, Biochem. J. 435 (2011) 373–380.
[48] S. Kumar, M.H. Asif, D. Chakrabarty, R.D. Tripathi, R.S. Dubey, P.K. Trivedi, Expression of a rice Lambda class of glutathione S-transferase, *OsGSTL2*, in *Arabidopsis* provides tolerance to heavy metal and other abiotic stresses, J. Hazard. Mater. 248–249 (2013) 228–237.
[49] S. Sultana, C.Y. Khew, M.M. Morshed, P. Namasivayam, S. Napis, C.L. Ho, Overexpression of monodehydroascorbate reductase from a mangrove plant (*AeMDHAR*) confers salt tolerance on rice, J. Plant Physiol. 169 (2012) 311–318.
[50] E.H. Colebrook, S.G. Thomas, A.L. Phillips, P. Hedden, The role of gibberellin signalling in plant responses to abiotic stress, J. Exp. Biol. 217 (2014) 67–75.
[51] Y. Tian, H. Zhang, X. Pan, X. Chen, Z. Zhang, X. Lu, R. Huang, Overexpression of ethylene response factor *TERF2* confers cold tolerance in rice seedlings, Transg. Res. 20 (2011) 857–866.
[52] A. Rahman, Auxin: a regulator of cold stress response, Physiol. Plant. 147 (2013) 28–35.
[53] J.N. Zhang, G.L. Wang, Q. Hong, Y. Wang Z, Advances in understanding cold sensing and the cold-responsive network in rice, Adv. Crop Sci. Technol. 1 (2013) 1.
[54] A. Chinpongpanich, K. Limruengroj, O.P.S. Phean, T. Limpaseni, T. Buaboocha, Expression analysis of *calmodulin* and *calmodulin-like* genes from rice, *Oryza sativa* L, BMC Res. Notes 5 (2012) 625.
[55] F. Abbasi, H. Onodera, S. Toki, H. Tanaka, S. Komatsu, *OsCDPK13*, a calcium-dependent protein kinase gene from rice, is induced by cold and gibberellin in rice leaf sheath, Plant Mol. Biol. 55 (2004) 541–552.
[56] S. Komatsu, G. Yang, M. Khan, H. Onodera, S. Toki, M. Yamaguchi, Over-expression of calcium-dependent protein kinase 13 and calreticulin interacting protein 1 confers cold tolerance on rice plants, Mol. Genet. Genom. 277 (2007) 713–723.
[57] H. Chauhan, N. Khurana, P. Agarwal, P. Khurana, Heat shock factors in rice (*Oryza sativa* L.): genome-wide expression analysis during reproductive development and abiotic stress, Mol. Genet. Genom. 286 (2011) 171–187.
[58] S.Y. Kim, K.H. Nam, Physiological roles of ERD10 in abiotic stresses and seed germination of *Arabidopsis*, Plant Cell Rep. 29 (2010) 203–209.
[59] D. Kovacs, E. Kalmar, Z. Torok, P. Tompa, Chaperone activity of ERD10 and ERD14, two disordered stress-related plant proteins, Plant Physiol. 147 (2008) 381–390.
[60] M.A. Pillai, T. Akiyama, Differential expression of an *S-adenosyl-L-methionine decarboxylase* gene involved in polyamine biosynthesis under low temperature stress in *japonica* and *indica* rice genotypes, Mol. Genet. Genom. 271 (2004) 141–149.
[61] S.J. Wi, W.T. Kim, K.Y. Park, Overexpression of carnation *S-adenosylmethionine decarboxylase* gene generates a broad-spectrum tolerance to abiotic stresses in transgenic tobacco plants, Plant Cell Rep. 25 (2006) 1111–1121.
[62] Y.S. Yan, X.Y. Chen, K. Yang, Z.X. Sun, Y.P. Fu, Y.M. Zhang, R.X. Fang, Overexpression of an F-box protein gene reduces abiotic stress tolerance and promotes root growth in rice, Mol. Plant 4 (2011) 190–197.
[63] B.F. Hanin, M. Ebel, C. Toda, Y. Takeda, S. Masmoudi K, Plant dehydrins and stress tolerance: versatile proteins for complex mechanisms, Plant Signal. Behav. 6 (2011) 1503–1509.
[64] J. Duan, W. Cai, *OsLEA3-2*, an abiotic stress induced gene of rice plays a key role in salt and drought tolerance, PLoS ONE 7 (2012) e45117.
[65] Q. Shen, C.N. Chen, A. Brands, S.M. Pan, T.H. Ho, The stress- and abscisic acid-induced barley gene *HVA22*: developmental regulation and homologues in diverse organisms, Plant Mol. Biol. 45 (2001) 327–340.
[66] W.J. Guo, T.H. Ho, An abscisic acid-induced protein, HVA22, inhibits gibberellin-mediated programmed cell death in cereal aleurone cells, Plant Physiol. 147 (2008) 1710–1722.
[67] X. Xie, T. Shinomura, N. Inagaki, S. Kiyota, M. Takano, Phytochrome-mediated inhibition of coleoptile growth in rice: age-dependency and action spectra, Photochem. Photobiol. 83 (2007) 131–138.

CAPÍTULO III

Artigo a ser submetido para publicação:

Plant Science

Cold tolerance mechanisms of *indica* rice plants during early vegetative stage revealed by deep RNAseq

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Abstract

Incidence of low temperature during early vegetative stage is one of the major limiting factors to the development of rice plants. Genes related to cold tolerance have been previously identified, mostly in genotypes from the *japonica* subspecies, which naturally have higher cold tolerance than *indica* genotypes. In a previous work, our group evaluated 90 genotypes according to the percentage of plant survival after 10 days at 10 ^{o}C, allowing

the identification of cold tolerant and sensitive genotypes from the *indica* subspecies. To help further clarify the physiological and molecular mechanisms that regulate cold tolerance of *indica* rice plants during early vegetative stage, comparative transcriptome analysis of 6 h cold-treated leaves was performed using RNAseq. Differential expression was observed for 1,305 genes, coding for proteins related to various metabolic pathways. Quantitative RT-PCR was used to confirm the high-quality of RNAseq. This study revealed that several processes are more active in the cold-tolerant than in the cold-sensitive plants, including photosynthetic efficiency, fatty acids unsaturation, antioxidant capacity, reinforcement of cell wall composition and Ca^{2+} and hormone-signaling. For the first time, cold tolerance in *indica* plants was related to high expression of the genes *OsERD15* (NAC domain-containing transcription factor), *OsTIL-2* (Temperature-induced lipocalin-2) and *Cellulose Synthase*. Physiological analyses of cold-treated plants indicate higher contents of cellulose and of the unsaturated fatty acid linoleic acid in leaves of the tolerant genotype. Our data uncovered numerous molecular and physiological changes that occur during low temperature stress, which could be important for cold tolerance in *indica* rice.

Keywords

cellulose; cold tolerance; deep sequencing; fatty acid; rice

Abbreviations

OsRBC, ribulose bisphosphate carboxylase small chain; *OsGAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *OsFAD*, omega-3 fatty acid desaturase; *OsCAT*, catalase domain-containing protein; *OsCES*, CESA1 - cellulose synthase; *OsEXP*, expansin precursor; *OsWIP*, wound-induced protein Wi12; *OsCBP*, calmodulin-binding protein; *OsAXN*, annexin.

Introduction

Rice is one of the world's most important staple foods; however, several abiotic stresses affect the physiological status of rice plants, leading to the reduction on grain yield. Low temperature is one of the major environmental factors limiting rice production, mostly in temperate and high altitude areas, due to the tropical origin of the rice species [1]. Cold temperature has the potential to affect growth and development of rice plants during any developmental stage, from germination to grain filling [2]. During early growth

stages, it can severely affect seedling establishment, leading to yellowing of the leaves, growth retardation, and decreased tillering [1]. Improved cold tolerance is, therefore, an effective way to increase rice production.

The degree of injury in rice plants depends of several factors, such as growth stage, duration of stress condition and severity. Some rice cultivars can tolerate stressful conditions and are able to grow under low temperature [3]. According to several studies with large number of cultivars belonging to *japonica* and *indica* subspecies, cold tolerant cultivars normally belong to the *japonica* subspecies [4, 5, 6], which are more adapted to temperate climates such as the ones in Japan, Korea, and Java [7]. The *indica* subspecies includes cultivars better adapted to tropical and subtropical environments such as India, China, Indonesia, and Brazil. In these locations, low temperatures can be a problem and almost the entire rice production is based on varieties from the *indica* subspecies, evidencing the need for studies which ultimately lead to cold tolerance in *indica* genotypes. Fortunately, although less common, *indica* cold tolerant genotypes were already described, and appropriate genotypes are available for breeding.

Low temperature affects chlorophyll content and fluorescence, interfering with photosynthesis in rice leaves [8]. Also, cold stress increases reactive oxygen species (ROS) and malondialdehyde (MDA) levels, which accumulate and impair metabolism via cellular oxidative damage [9]. Nevertheless, rice plants have several strategies to respond to cold stress, which is not always effective, depending on the intensity. To cope with or adapt to cold stress, rice plants accumulate compatible osmolytes such as free proline, an amino acid that maintains the function of rice cells under low temperature through the stabilization of protein synthesis [10]. Yet, antioxidant protection is also increased to scavenge ROS and protect rice plants against oxidative damage [11, 12].

Genetic analysis indicates that cold tolerance in rice is a quantitative trait, since several QTLs for cold tolerance have been mapped in the rice genome [1, 13]. Therefore, it seems that several genes are involved in biochemical and physiological modifications which ultimately lead to rice cold acclimation and tolerance. A large number of genes has been isolated and characterized as responsive to cold stress. Some of them lead to cold tolerance when over-expressed in rice plants (for a comprehensive review see [1]). These cold-responsive genes encode proteins involved with biosynthesis of osmoprotectants [10], signaling components [14-18], chaperones [19], and transcription factors [8, 2, 20], especially from the CBF/DREB1 family (C-repeat binding factor/dehydration responsive element binding; [21-24]). Another family of proteins, which play central roles in rice

responses to cold stress, is MYB transcription factors. Overexpression of *OsMYBS3* in rice plants repressed the CBF/DREB-dependent cold signaling pathway. Also, it was shown that *OsMYBS3* responds slowly to cold stress, which suggests that this gene acts in long-term cold adaptation in rice [25].

It is important to note that most of the mentioned studies have used *japonica* genotypes. Therefore, the challenge still remains to develop cold tolerant *indica* genotypes suitable for high-latitude regions. In order to identify and characterize novel genes and metabolic pathways involved in rice cold tolerance, we used two *indica* rice genotypes (previously identified as cold-tolerant and cold-sensitive) in parallel transcriptomic analysis under cold treatment (10 ^{o}C for 6 h). Our findings should contribute to a better understanding of the molecular and physiological mechanisms involved in cold acclimation and tolerance in *indica* rice plants, and could be useful for future biotechnological and molecular breeding efforts.

Material and methods

Plant materials and cold treatment

Previously, we identified low temperature tolerant and sensitive genotypes from the *indica* subspecies. At the vegetative stage, 90 genotypes were evaluated according to the percentage of three leaf-stage plant survival after ten days at 10 ^{o}C (climatic chamber) and seven days of recovery under normal temperature (in greenhouse conditions). In this screening, the sister lines (derived from the same cross) IRGA 959-1-2-2F-4-1-4-A and IRGA 959-1-2-2F-4-1-4-D-1-CA-1 were characterized, respectively, as tolerant and sensitive to low temperature stress. Percentage of plant survival after ten days of cold treatment was about 90% and 15%, respectively, for the cold-tolerant and cold-sensitive genotypes [26]. In addition, photosynthetic performance was heavily affected by cold in both genotypes. However, the cold-tolerant genotype was able to recover photosynthetic performance after cold exposure [26] .

These two genotypes were developed by the IRGA (Rio Grande do Sul Rice Institute) breeding program. They present the same genealogy, which is a triple cross "Lemont / IRGA117-72-1P-3-2A // P1790". The first cross "Lemont / IRGA117-72-1P-3-2A" was performed in 1988. One year later, the F1 of this cross was crossed to P1790. In the following years, the segregating generations were field cultivated and individual plants were selected according to agronomic traits of interest (including cold-response) using the

pedigree method. After six generations of selection, many F7 lines were obtained, all designated IRGA 959. The two lines evaluated in this study were chosen among them.

Both selected genotypes were germinated for five days in distilled water and then planted into soil and grown in a greenhouse (temperature range from 20 to 30 °C, full daylight). At the three-leaf stage (approximately 20 days), plants were transferred to a climatic chamber and maintained at 10 °C for 6 h (light intensity of 40 μMol m^{-2} s^{-1}). Three biological replicates (each one containing leaves of at least three plants) were collected and immediately frozen in liquid nitrogen. Samples were then maintained at - 80 ^{o}C until RNA extraction.

RNA extraction and comparative transcriptomic profiling by RNAseq

Total RNA was extracted from rice leaves using Plant RNA Purification Reagent (Invitrogen) and treated with DNase I (Invitrogen). Approximately 20 μg of total RNA was used in high-throughput cDNA sequencing by Illumina HiSeq 2000 technology (Fasteris SA, Plan-les-Ouates, Switzerland - http://www.fasteris.com/). We constructed one individual single-end cDNA library for each rice genotype. The cDNA libraries were prepared according to Illumina's protocols. Briefly, RNAseq was performed using the following successive steps: poly-A purification; cDNA synthesis using a poly-T primer, shotgun method to generate inserts of approximately 500 bp; 3p and 5p adapter ligations; pre-amplification; colony generation; and Illumina single-end 100 bp sequencing.

All low quality reads (FASTq value <13) were removed, and 3p and 5p adapter sequences were trimmed using Genome Analyzer Pipeline (Fasteris). The remaining low quality reads with 'n' were removed using Python script. After cleaning the data (low quality reads, adapter sequences), the mRNAseq data from the two libraries were aligned to the rice genome using the software Bowtie version 0.12.7. Only sequences with up to two mismatches to the rice reference genome (ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_6.1/all.dir/all.seq) were used. The SAM files from Bowtie were then processed using Python scripts to assign the frequencies of each read and map them onto references. For data normalization, we used the scaling normalization method proposed by Robinson and Oshlack [27]. To assess whether the sequences were differentially expressed, we used the R package EdgeR [28]. We considered that the sequences were differentially expressed if they had an adjusted *p*-value < 0.000001, according to EdgeR.

Gene Ontology (GO) terms enrichment analysis

The loci from differentially expressed genes with increased expression in plants from the cold-tolerant or cold-sensitive genotypes (Supplementary Table 2) were used to search for enriched Gene Ontology (GO) terms comparing both datasets. The rice proteome was downloaded from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) and GO categories of all sequences in the rice genome were annotated using Blast2GO (http://www.blast2go.com; [29]). Then, we compared category enrichment between the two datasets. Enrichment analysis was performed using Blast2GO built-in Fisher's Exact Test, using Two Tailed and Remove Double IDs options ($p \leq 0.05$). From a total of 938 (cold-tolerant) and 367 (cold-sensitive) differentially expressed genes, 595 and 226 genes could be assigned a GO term, respectively, and thus considered in the enrichment analysis.

Gene expression analysis by quantitative RT-PCR

To confirm the high-quality of deep sequencing results, RT-qPCR was used to check the gene expression of putative cold tolerance-related genes. Total RNA was extracted from rice leaves of plants exposed to 10 °C for 6h using Concert Plant RNA Reagent (Invitrogen) and treated with DNase I (Invitrogen). First-strand cDNA synthesis was performed with reverse transcriptase (M-MLV, Invitrogen) using 1 μg of RNA. RT-qPCRs were carried out in a StepOne Real-Time Cycler (Applied Biosystems). All primers (listed in Supplementary Table 1) were designed to amplify 100-150 bp of the 3'-UTR of the genes and to have similar Tm values (60 ± 2 °C). Reaction settings were composed of an initial denaturation step of 5 min at 94 ^{o}C, followed by 40 cycles of 10 s at 94 ^{o}C, 15 s at 60 ^{o}C (fluorescence data collection) and 15 s at 72 ^{o}C; samples were held for 2 min at 40 ^{o}C for annealing of the amplified products and then heated from 55 to 99 ^{o}C with a ramp of 0.1 ^{o}C /s to produce the denaturing curve of the amplified products. RT-qPCRs were carried out in 20 μl final volume composed of 10 μl of each reverse transcription sample diluted 100 times, 2 μl of 10X PCR buffer, 1.2 μl of 50 mM $MgCl_2$, 0.1 μl of 5 mM dNTPs, 0.4 μl of 10 μM primer pairs, 4.25 μl of water, 2.0 μl of SYBR green (1:10,000, Molecular Probe), and 0.05 μl of Platinum Taq DNA Polymerase (5 U/μl, Invitrogen, Carlsbad, CA, USA). Gene expression was evaluated using a modified $2^{-\Delta CT}$ method [30], which takes into account the PCR efficiencies of each primer pair (Relative expression $_{\text{TESTED GENE / CONTROL GENE}} = (PCR_{eff}\ CG)^{Ct\ CG} / (PCR_{eff}\ TG)^{Ct\ TG}$). Experiments were

performed with three biological and four technical replicates, collected in a second experiment (i.e. distinct from the one for RNAseq).

Cellulose staining in rice leaves

Rice plants of cold-tolerant and cold-sensitive genotypes were grown for 20 days (three-leaf stage) and submitted to cold treatment (10 ^{o}C) for 10 days in climatic chamber. Leaves were collected before exposure to cold (control) and after 10 days of cold and subjected to cellulose staining with 0.01% Calcofluor White (Fluorescent Brightener 28; Sigma-Aldrich, St. Louis, MO, USA). All sections (5 μm) were observed using optical microscopy DMRB (Leica Microsystems, Wetzlar, Germany), with UV filter A, excitation filter BP 340-380 nm, suppression filter LP 425, and an exposure time of 270 ms.

Lipid extraction and fatty acid determination

Rice plants of both genotypes were grown for 20 days (three-leaf stage) and subjected to cold treatment (10 ^{o}C) for 10 days in a climatic chamber (light intensity of 40 μMol m^{-2} s^{-1}, 16/8 hs light/dark) or maintained at greenhouse conditions (control treatment). After grinding the leaves (approximately 2 g of dry matter), lipids were extracted as described by Bligh and Dyer [31]. Fatty acid composition was determined by Gas Cromatography, with lipids being saponified and metanolized with KOH solution and then sterefied and metanolized with H_2SO_4 solution [32]. Methyl-esters fatty acids were analyzed with a Gas Cromatographer (Agilent Technologies - HP 6890) adjusted with a capillary column HP-INNOWax (60m x 0.25mm x 0.25μm) with flame ionization detector. Injection and detection temperature was around 250°C and conduction was made by Nitrogen gas. Standardization of methyl-esters fatty acids and the subsequent retention times were used for fatty acid identification. Linoleic acid levels were expressed as the percentage of total fatty acids contained in the pattern.

Statistical analysis

Mean values of linoleic acid and gene expression (RT-qPCR) were compared by the Student's *t* test ($p \leq 0.05$) using the SPSS Base 21.0 for Windows (SPSS Inc., USA).

Results and discussion

Overview of cold-tolerant and cold-sensitive cDNA library sequencing

The rice genotypes IRGA 959-1-2-2F-4-1-4-A and IRGA 959-1-2-2F-4-1-4-D-1-CA-1 were previously characterized, respectively, as tolerant and sensitive to low temperature stress [26] (Supplementary Figure 1). Plants from both genotypes under cold treatment (10 ^{o}C for 6 h) were used to identify differentially expressed mRNAs using the Illumina Platform. We chose to use 6 h of cold treatment in order to detect cold-specific and not general stress-related transcripts. Deep sequencing generated approximately 20 million reads in each library (tolerant and sensitive genotypes). From these, 23.28% (4,910,832 reads) and 24.49% (4,964,985 reads) were mapped, respectively, in genomic regions.

Most of the sequences showed similar normalized number of reads. This may be partially explained by the fact that the rice genotypes used in this study are sister lines. Overall, there were clear linear relationships in the gene expression levels among the two rice genotypes (R^2 = 0.9629; Fig. 1A). For this reason, we preferred to use an extremely rigorous adjusted *p*-value (< 0.000001, according to EdgeR) as criteria for assigning a gene as differentially expressed, instead of the use of fold-change as criteria for differential gene expression. It is already known that even small changes on the gene expression can have biological meaning, and according to McCarthy and Smyth [33], a gene is differentially expressed if its expression level changes systematically between two treatment conditions, regardless of how small the difference might be. Using these parameters, we found 1,305 differentially expressed sequences, which were included in our analysis (Supplementary Table 2). From these, 938 sequences (71.8%) were more expressed in the tolerant genotype, while 367 sequences (28.2%) were more expressed in the cold-sensitive one (Fig. 1B).

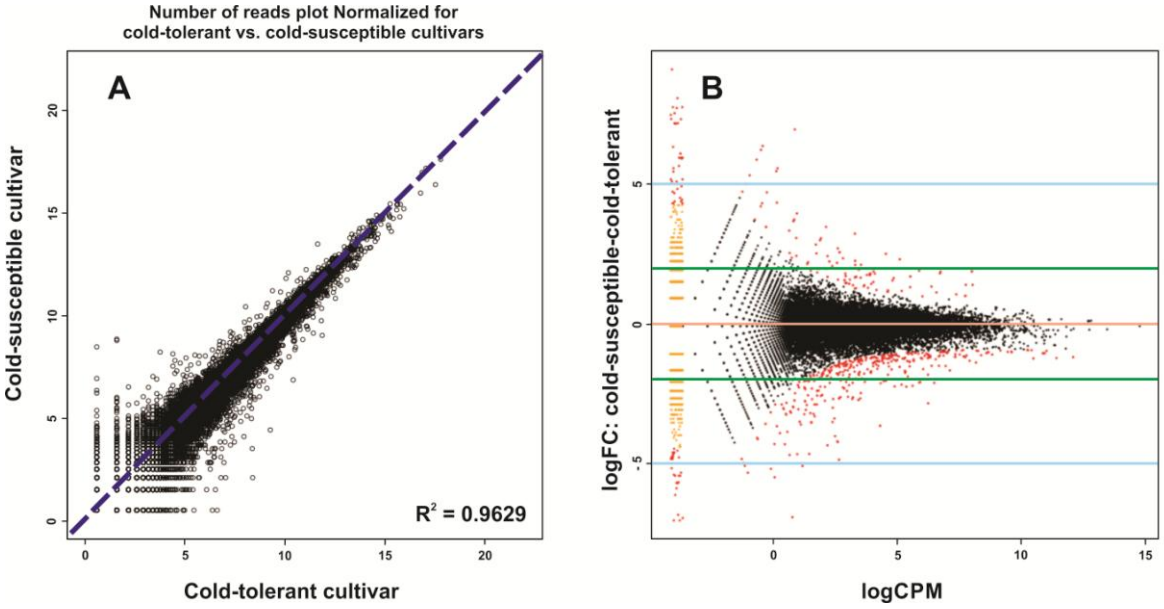

Figure 1. Analysis of differentially expressed genes between leaves from cold-tolerant and cold-sensitive *indica* rice genotypes. (A) Scatter plot comparing the gene expression levels between cold-tolerant and cold-sensitive genotypes. (B) Genes identified as differentially expressed by EdgeR (red dots).

Enrichment of GO terms comparing cold-tolerant and cold-sensitive datasets

We performed GO term enrichment analysis comparing differentially expressed genes of each dataset. Loci from genes with increased expression in leaves from the cold-tolerant or the cold-sensitive genotype were collected, listed and compared using Blast2GO (http://www.blast2go.com) [29] to search for enriched terms in each group. Functional analysis indicated that the differentially expressed genes were involved in various protein categories. Twenty-eight terms were significantly different, with 25 enriched in the cold-sensitive and only three in the cold-tolerant dataset (Fig. 2). Among the GO terms enriched in the cold-tolerant dataset, we found "carbohydrate metabolic process". The carbohydrates serve as energy sources and as structural elements in living cells. Interestingly, we observed that cold-tolerant plants have higher cellulose content (see below), corroborating the term enrichment. We also found "thylakoid" cellular component term enriched in the cold-sensitive dataset, which is in line with our previous observations that photosynthesis is severely affected in the cold-sensitive genotype, but not in the cold-tolerant one, upon cold treatment [26].

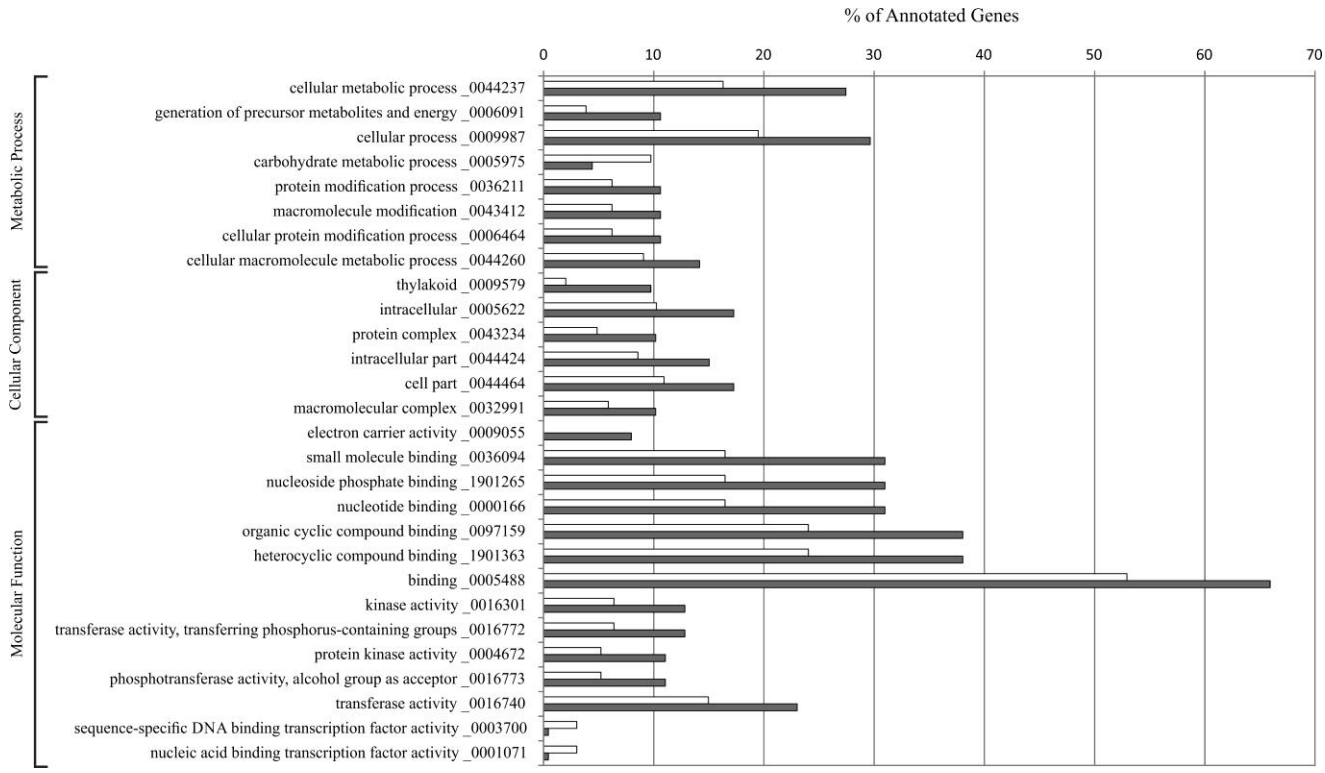

Figure 2. Gene Ontology (GO) analysis of differentially expressed genes from leaf transcriptomes of cold-tolerant and cold-sensitive *indica* rice genotypes. Terms enriched in either tolerant or sensitive datasets are shown as percentage of annotated genes in the dataset. All GO terms shown are differentially enriched in either groups using Fisher's Exact Test ($p \leq 0.05$).

Genes with differential expression

Using the RNAseq method, we detected, after 6 h of cold treatment, 1,305 differentially expressed sequences in leaves of rice plants from the cold-tolerant genotype in relation to the cold-sensitive one (Supplementary Table 2). All the 1,305 differentially expressed sequences were carefully analyzed. Based on the predicted molecular function and differences in gene expression, we selected 61 sequences which might be related to the cold-tolerance mechanism in plants from the IRGA 959-1-2-2F-4-1-4-A genotype (Table 1).

Table 1. List of differentially expressed sequences identified by RNAseq in rice leaves of cold-tolerant and cold-sensitive genotypes, after 6 hs at 10 ^{o}C

			Normalized number of reads		
Functional category	**Description**	**Location**	**Tolerant**	**Sensitive**	**p-value**
Photosynthesis	Chlorophyll A-B binding protein	LOC_Os11g13890	13,548	7,074	0
	Ribulose bisphosphate carboxylase small chain (*OsRBC*)	**LOC_Os12g17600**	**50,959**	**23,043**	**0**
	Carbonic anhydrase	LOC_Os01g45274	32,772	16,643	0
	Photosystem I reaction center subunit II	LOC_Os08g44680	13,312	7,077	0
	Plastocyanin	LOC_Os06g01210	19,701	12,013	0
	Ribulose bisphosphate carboxylase large chain precursor	LOC_Os10g21268	51,470	39,623	0
	Low photochemical bleaching 1 protein	LOC_Os05g39230	981	529	$3.6\ e^{-35}$
	Protochlorophyllide reductase A	LOC_Os04g58200	461	248	$1.8\ e^{-16}$
Carbohydrate metabolism and energy production	**Glyceraldehyde-3-phosphate dehydrogenase (*OsGAPDH*)**	**LOC_Os04g38600**	**41,746**	**25,914**	**0**
	ATP synthase gamma chain	LOC_Os07g32880	9,812	6,666	$3.6\ e^{-171}$
	ATP synthase B chain	LOC_Os03g17070	3,685	2,798	$7.5\ e^{-38}$
Lipid metabolism	**Omega-3 fatty acid desaturase (*OsFAD*)**	**LOC_Os03g18070**	**9,646**	**6,175**	**$1.8\ e^{-210}$**
	Lipoxygenase	LOC_Os02g10120	1,713	639	$5.2\ e^{-123}$
	Fatty acid desaturase	LOC_Os02g48560	2,346	1,760	$1.1\ e^{-25}$
	Omega-3 fatty acid desaturase	LOC_Os07g49310	582	337	$3.6\ e^{-17}$
Transcription factor	Dehydration-responsive element-binding protein (*OsDREB1A*)	LOC_Os09g35030	3,986	1,928	$7.4\ e^{-184}$
	Dehydration-responsive element-binding protein (*OsDREB1B*)	LOC_Os09g35010	1,885	991	$5.7\ e^{-72}$
	NAC domain-containing protein 67	LOC_Os03g60080	3,766	2,532	$2.5\ e^{-68}$
	OsWRKY71	LOC_Os02g08440	2,317	1,770	$5.9\ e^{-23}$
	Early responsive to dehydration 15 (*ERD15*)	LOC_Os07g46670	1,148	770	$6.6\ e^{-21}$

	OsWRKY45	LOC_Os05g25770	488	319	9.6 e^{-10}
	OsWRKY53	LOC_Os05g27730	3,571	3,221	3.1 e^{-9}
	OsMYBS3	LOC_Os10g41200	2,325	2,025	9.3 e^{-9}
Detoxification	**Catalase domain-containing protein (*OsCAT*)**	**LOC_Os03g03910**	**26,655**	**21,682**	**1.2 e^{-178}**
	Peroxidase precursor	LOC_Os01g19020	862	284	1.1 e^{-72}
	Glutathione peroxidase domain containing protein	LOC_Os04g46960	5,387	3,933	5.0 e^{-68}
	OsAPx8 - Thylakoid-bound Ascorbate Peroxidase	LOC_Os02g34810	4,617	3,405	1.8 e^{-55}
	Copper/Zinc superoxide dismutase	LOC_Os08g44770	1,303	707	4.3 e^{-46}
	OsAPx2 - Cytosolic Ascorbate Peroxidase	LOC_Os07g49400	12,978	11,573	3.4 e^{-38}
	Glutathione peroxidase	LOC_Os06g08670	6,491	5,745	9.3 e^{-21}
Cell wall-related	**CESA1 - Cellulose synthase (*OsCES*)**	**LOC_Os05g08370**	**2,290**	**1,275**	**7.9 e^{-76}**
	Expansin precursor (*OsEXP*)	**LOC_Os10g40720**	**543**	**139**	**1.1 e^{-59}**
	CSLE6 - Cellulose synthase-like family E	LOC_Os09g30130	413	15	4.9 e^{-58}
	CESA8 - Cellulose synthase	LOC_Os07g10770	1,788	1,008	3.2 e^{-57}
	CSLE1 - Cellulose synthase-like family E	LOC_Os09g30120	216	20	2.6 e^{-43}
	COBRA (cell expansion protein)	LOC_Os05g32110	1,258	738	6.2 e^{-36}
	CESA6 - Cellulose synthase	LOC_Os07g14850	611	288	2.0 e^{-29}
	CSLF6 - Cellulose synthase-like family F	LOC_Os08g06380	253	75	7.2 e^{-24}
	CESA5 - Cellulose synthase	LOC_Os03g62090	512	269	2.1 e^{-19}
	Wound-induced protein WI12 (*OsWIP*)	**LOC_Os07g49114**	**159**	**59**	**2.8 e^{-11}**
	CSLD2 - cellulose synthase-like family D	LOC_Os06g02180	503	361	6.6 e^{-7}
Hormone-signaling	Gibberellin receptor GID1L2	LOC_Os09g28620	182	24	2.3 e^{-31}
	Ethylene-insensitive 3	LOC_Os07g48630	4,314	3,633	4.9 e^{-22}
	Auxin efflux carrier component	LOC_Os09g38130	568	336	8.5 e^{-16}
	Cytokinin-N-glucosyltransferase 1	LOC_Os07g13634	123	30	8.5 e^{-14}
	1-aminocyclopropane-1-carboxylate oxidase protein	LOC_Os09g27820	377	204	2.6 e^{-13}
	Gibberellin response modulator protein	LOC_Os07g39470	4,884	4,435	1.3 e^{-11}
	Cytokinin dehydrogenase precursor	LOC_Os01g71310	205	97	1.2 e^{-9}

	OsCDPK7	LOC_Os04g49510	4,081	3,152	4.8 e^{-38}
	OsCML16 - Calmodulin-related calcium sensor protein	LOC_Os01g04330	821	422	7.5 e^{-33}
	Calmodulin-binding protein (*OsCBP*)	**LOC_Os01g38980**	**2,155**	**1,533**	**1.2 e^{-30}**
Ca^{2+}-signaling	IQ calmodulin-binding motif domain containing protein	LOC_Os05g46350	198	34	3.2 e^{-29}
	Annexin (*OsAXN*)	**LOC_Os09g23160**	**1,512**	**1,188**	**1.1 e^{-12}**
	OsCML27 - Calmodulin-related calcium sensor protein	LOC_Os03g21380	489	328	7.6 e^{-9}
	Calcium-transporting ATPase, plasma membrane-type	LOC_Os04g51610	666	501	3.4 e^{-7}
Others	Phenylalanine ammonia-lyase	LOC_Os02g41630	4,339	2,658	2.7 e^{-109}
	Dehydrin (*OsLIP9*)	LOC_Os02g44870	1,019	514	8.6 e^{-43}
	Potassium transporter	LOC_Os07g47350	3,957	3,104	1.4 e^{-33}
	Cold acclimation protein WCOR413	LOC_Os03g55850	731	444	1.0 e^{-18}
	Dehydrin (*OsLIP5*)	LOC_Os03g45280	3,943	3,383	3.8 e^{-17}
	OsTIL-2 - Temperature-induced lipocalin-2	LOC_Os08g34150	340	170	2.0 e^{-14}

A list of 1,305 most differentially expressed genes obtained from the RNAseq experiments is shown in Supplementary Table 2. Bold sequences were confirmed by qRT-PCR.

We found several genes related to photosynthesis with higher expression in the leaves of cold-tolerant genotype after only six hours of cold treatment (Table 1), showing that higher expression levels of photosynthesis-related genes probably account for the previously reported better photosynthetic efficiency of these tolerant plants after five days of recovery previously submitted to five days of cold treatment (results to be submitted to publication). It is known that photosynthesis is highly affected by low temperatures. Studies using chlorophyll fluorescence suggest that the reaction center of photosystem II (PSII) is one of the main targets of cold stress [34]. Our group analyzed several chlorophyll fluorescence parameters on these two contrasting genotypes, and found that the photosynthetic performance indexes on the basis of light absorption were heavily affected by cold in both genotypes, however, the tolerant genotype was able to recover these performances after cold exposure [26]. It is known that when the rice seedlings are chilled, the superstructure of chloroplasts is altered and the efficiency of electron transfer function decreases [35]. In fact, rice transgenic plants over-expressing the zinc finger protein *AlSAP* (Stress-Associated Protein) have enhanced cold tolerance, probably through the maintenance of the photosynthetic apparatus integrity [36].

In our study, we found several fatty acid desaturases with higher expression levels in leaves from the cold-tolerant genotype (Table 1) and an increase in the linoleic acid (C18:2n6) level on the cold-tolerant genotype under low temperature stress (10 ^{o}C for 10 days - Figure 3). These data suggest that the tolerance mechanism developed by this genotype includes higher level of unsaturated fatty acid production. As far as we know, this is the first time that fatty acid unsaturation level is related to cold tolerance in an *indica* rice genotype. In the cold-sensitive one, no increase in linoleic acid level was detected under low temperature stress, when compared to control conditions. The adaptability of plants to low temperature stress depends directly on their capacity for developing mechanisms of temperature adaptation, and membranes are major targets for temperature acclimation strategies. Membrane lipids provide a dynamic and fluid environment essential for living organisms [37], and there is strong association between temperature and fatty acid content of plant membranes [38]. For example, increasing levels of saturated fatty acids were correlated to higher sensitivity to cold temperatures in Arabidopsis plants [39], while lower temperatures are associated with an increase in the production of unsaturated fatty acids in cotton plants [40]. Such maintenance of membrane fluidity by unsaturated fatty acids is probably due to their lower melting temperatures [38].

Also, most studies indicate that mainly linolenic acid (C18:3n3) is responsible for cold-tolerant phenotypes [40- 41]. However, in our study, the levels of linolenic acid did not change in both tested genotypes, under cold or control conditions (data not shown), unlike the levels of linoleic acid.

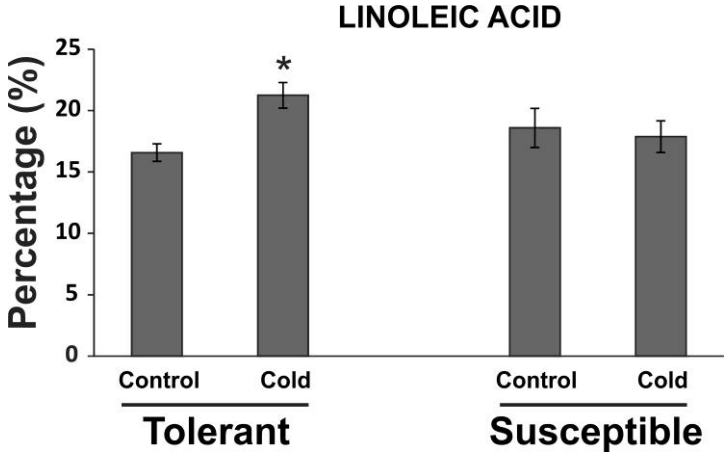

Figure 3. Linoleic acid percentage in leaves of cold-tolerant and cold-sensitive genotypes under control conditions (greenhouse, with temperature range from 20 to 30 °C) or cold-treatment (10 °C) for 10 days. Values are the averages of four samples ± SE. Mean values with one asterisk are different by Student's *t* test ($p \leq 0.05$).

Several transcription factors were detected in our experiments with higher expression in leaves from the cold-tolerant genotype, including the cold-related *OsDREB1A*, *OsDREB1B* and *OsMYBS3* genes, along with a NAC domain-containing protein and *OsERD15* (Table 1). *DREB1* genes represent one of the most important discoveries in the field of cold adaptation [3]. Manipulation of *DREB1* rice genes expression contributes to increased cold tolerance in rice plants (for a comprehensive review see Cruz et al. [1]). Another cold-response signaling pathway was described recently, which is regulated by the *OsMYBS3* gene [25]. The OsMYBS3-mediated cold adaptation response occurs after the DREB1-mediated one, as it has a slow response, implying that there is an additional pathway controlling cold adaptation in rice. Actually, MYBS3 can repress the expression of DREB1 genes [25]. Interestingly, we found high expression of *OsMYBS3* after only 6 h of cold treatment in this study. It is important to note that we used 10 ^{o}C instead of chilling stress (4 ^{o}C). More studies are needed to investigate whether DREB1 and MYBS3 act together under 10 ^{o}C and sequentially under 4 ^{o}C. Zhang et al. [42] also found higher expression of *OsMYBS3* in a cold-tolerant rice

cultivar, and suggested that *OsMYBS3* plays key roles in the regulatory pathway of chilling stress tolerance. One NAC domain-containing protein (OsNAC5) has been previously over-expressed in rice, generating cold-tolerant plants [43]. The authors suggest that *OsNAC5* is important for cold tolerance in rice, acting associated with an ABA-related transcriptional regulation network. ERD15 was identified as a central component in several stress responses in *Arabidopsis thaliana*. The biological role of all ERD15 family members studied so far appears to be associated with stress responses and stress adaptation, including drought and freezing, functioning as a negative regulator of ABA signaling [44]. To the best of our knowledge, this is the first work which shows a relation between *OsERD15* expression and cold-tolerance. In this way, one more transcription factor should be added to the list of cold tolerance-related genes which could be used in future biotechnological applications.

Antioxidant capacity has been extensively related to different stress responses in plants. We found several related sequences with higher expression in the cold-tolerant genotype, including catalase, peroxidase, glutathione peroxidase, ascorbate peroxidase and superoxide dismutase (Table 1). Bonnecarrère et al. [45] analyzed two cold-tolerant *japonica* rice cultivars, and concluded that the strategy for cold tolerance differed between the two genotypes, and that increased cold tolerance of L2825CA relative to INIA Tacuarí was related to higher constitutive superoxide dismutase, ascorbate peroxidase and catalase activities. Zhang et al. [42] also found differences in antioxidant activities when two contrasting genotypes were tested, and concluded that certain antioxidants were involved in chilling stress response and tolerance. Transgenic rice overexpressing *OsAPXa* (ascorbate peroxidase) exhibits elevated cold tolerance, which is negatively correlated to the levels of H_2O_2 and lipid peroxidation. It is assumed that H_2O_2 is scavenged by increased ascorbate peroxidase activity during cold treatment, which results in lower lipid damage [11]. However, Yun et al. [46] showed that H_2O_2 levels are particularly high during the initial 12 hours of cold treatment in *japonica* rice plants, being the primary signal that integrates the cold-response regulatory network, particularly in relation to the execution of 'early response' mechanisms. In a previous study using the same contrasting cultivars, we showed that plants from the cold-tolerant genotype have higher SOD activity after 48 h and higher catalase activity after 72 h of cold treatment, when compared to the cold-sensitive one [26]. In this way, a higher antioxidant capacity may be part of the tolerance mechanisms in this *indica* rice genotype.

Maintenance of cell wall integrity under low temperature is an efficient approach to generate transgenic cold-tolerant rice plants, as previously seen by Gothandam et al. [47], which over-expressed a cell wall protein (OsPRP3). A number of cell wall-related sequences (including several cellulose synthase genes) were found as differentially expressed in the tolerant genotype (Table 1), suggesting that one of the tolerance mechanisms involves the reinforcement of cell wall composition after cold exposure. In fact, we detected higher cellulose staining on the mesophyll cell walls of the cold-tolerant genotype (Figure 4c), when compared to the cold-sensitive one (Figure 4d), after ten days at 10 ^{o}C. Under control temperature, no difference was detected on the cellulose staining (Figures 4a and 4b). This is the first time that cold tolerance in rice is related to higher expression of cellulose synthase genes, although a relationship between high expression of cellulose synthase genes with tolerance to biotic [48] and other abiotic [49] stresses in rice plants have been reported.

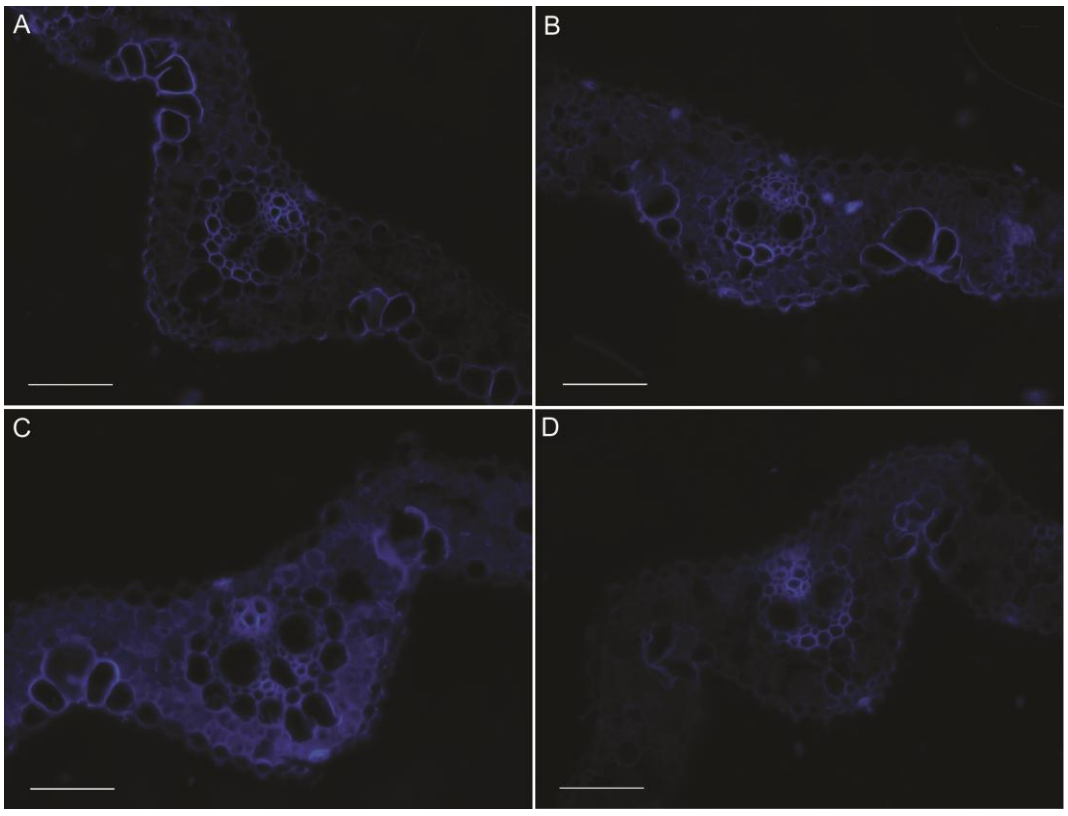

Figure 4. Cellulose staining using Calcofluor White and UV visualization. Rice leaves of cold-tolerant (A and C) and cold-sensitive (B and D) genotypes, after 10 days of control condition (room temperature in the greenhouse - A and B) or cold treatment (10 ^{o}C - C and D). Bars in figures indicate 50 μm. All experiments were performed at least twice with similar results.

We also detected higher expression of the genes encoding expansin and the wound-induced protein WI12 in the cold-tolerant genotype (Table 1). Expansins loosen cell walls via a nonenzymatic mechanism that induces slippage of cellulose microfibrils in the plant cell wall [50], facilitating cell expansion and other developmental events during which cell wall modification occurs. According to Zheng et al. [51], low levels of expansin proteins may potentially lead to the low temperature sensitivity of cotton Sumian 15 cultivar. Yet, the expansin-like A2 gene appears to be important in the tolerance to abiotic stress conditions in *Arabidopsis thaliana* [52]. Using immunogold labeling, Yen et al. [53] showed that wound-induced protein WI12 preferentially accumulates in the cell wall, suggesting its role in the reinforcement of cell wall composition after wounding, and, as suggested by our results, also after low temperature exposure. The higher expression levels of expansin proteins identified in this study shows the capacity of the tolerant rice genotype to continue growing under low temperature, what is extremely important to guarantee a good crop establishment under adverse field conditions.

We detected several Ca^{2+}-signaling related genes differentially expressed in the tolerant genotype, including Calmodulin-related calcium sensor proteins (*OsCML16* and *OsCML27*), Calmodulin-binding proteins, Calcium-transporting ATPase (plasma membrane type), *OsCDPK7* (a Calcium-dependent protein kinase) and Annexin gene (Table 1). The important roles of calcium in cold sensing and signaling during rice vegetative stage have already been revealed (for a comprehensive review see Zhang et al. [54]). Briefly, membrane rigidification-activated mechano-sensitive or ligand-activated Ca^{2+} channels promote Ca^{2+} influx into the cystol, an important and early event caused by cold stress [54, 55]. Subsequently, Ca^{2+} signatures are interpreted and amplified by calcium sensors, such as Calmodulin (CaM), Calmodulin-like proteins (CMLs) and Calcium-dependent Protein Kinases (CDPKs) [54]. According to Saijo et al. [14], over-expression of *OsCDPK7* confers cold tolerance to transgenic rice plants, and the extent of cold tolerance correlates well with the level of *OsCDPK7* expression. Yet, over-expression of the *AtbZIP60* gene in rice plants leads to cold tolerance through the increased expression of Ca^{2+}-dependent protein kinase genes [56]. Annexins are proteins able to interact with membrane phospholipids in a Ca^{2+}-dependent manner and probably may function in the process of membrane resealing [57]. Some reports have linked annexin signaling to stress response and also with abiotic stress tolerance [58, 59], including low temperatures in wheat and rice [59, 60].

Based on our findings (Table 1), the complex cross talk between various hormones in response to cold stress seems to be more active in the cold-tolerant genotype, as we found several hormone signaling-related genes (Gibberellin receptor GID1L2, Ethylene-insensitive 3, Auxin efflux carrier protein, Cytokinin-N-glucosyltransferase 1, ACC oxidase, Gibberellin response modulator, Cytokinin dehydrogenase precursor) with higher expression in this genotype. Plant hormones regulate plant growth and development, as well as responses to changing environmental conditions. By modifying the production, distribution or signal transduction of these hormones, plants are able to coordinate both growth and stress tolerance [61]. Auxin plays an important role in plant responses to cold stress, since intracellular auxin gradients are tightly linked to plant growth and development under stress conditions [62]. A number of auxin-related genes were regulated by late phase chilling stress in both *indica* and *japonica* rice, indicating an interaction between auxin signaling and low temperature stress response [42]. Reduction of gibberellin levels and signaling has been shown to contribute to Arabidopsis plant growth restriction on exposure to several stresses, including cold [61]. Over-expression of ethylene response factor *LeTERF2* confers cold tolerance in *japonica* rice seedlings [63]. According to Werner and Schmülling [64], cytokinin mediates the responses to variable extrinsic factors, and has a role in the response to biotic and abiotic stress. Recently, O'Brien and Benková [65] established that cytokinin plays an important role in stress responses and adaptation, but does not act alone, being part of a complex network of multiple synergistic and antagonistic interactions between various hormones, such as abscisic acid, jasmonate, salicylic acid, ethylene, and auxin.

Four other genes that could be part of the cold-tolerant mechanism are two Dehydrins (*OsLIP9* and *OsLIP5*), Cold acclimation protein (*OsWCOR413*) and Temperature-induced Lipocalin-2 (*OsTIL-2*) (Table 1). The *OsLIP9* gene, firstly described by Aguan et al. [66], is up-regulated in *japonica* rice plants over-expressing *OsDREB1B* (Dehydration-responsive element-binding protein), and these transgenic plants also show improved cold-tolerance [23]. *OsLIP9* expression was analyzed by our group in a previous work and we suggested that a cold-tolerance mechanism involving *OsLIP9* and *OsDREB1B* could be present in two out of the three cold-tolerant genotypes tested (unpublished results).

The cold acclimation *OsWCOR413* gene expression was also previously analyzed by our group. Higher expression of *OsWCOR413* was seen before the onset of low temperature stress in the tolerant genotype, which indicates that this gene may maintain a

higher basal expression level in this genotype than in the sensitive one [26]. The higher expression of *OsWCOR413* in the cold-tolerant genotype in relation to the cold-sensitive one before cold treatment at 10 ^{o}C may be involved with the intrinsic tolerance to chilling stress, but additional studies (such as analyses of Arabidopsis over-expressing lines, which are currently being grown in our lab) are necessary to understand its function.

Very little is known about plant Lipocalins, except that they function during plant development under environmental stresses, including low temperature exposure in wheat [67]. Lipocalins generally bind small hydrophobic ligands such as retinoids, fatty acids, steroids, odorants, and pheromones, and interact with cell surface receptors [68]. Low expression levels are found in oat and barley, two plant species which are less cold tolerant. This difference in accumulation indicates that Lipocalin expression is correlated with the plant's capacity to develop freezing tolerance [68]. In rice, this is the first time that such gene is related to cold stress response and tolerance.

Confirmation of RNAseq expression patterns using RT-qPCR

To validate the RNAseq results and to confirm the differential expression of the isolated genes, *OsRBC* (ribulose bisphosphate carboxylase small chain); *OsGAPDH* (glyceraldehyde-3-phosphate dehydrogenase); *OsFAD* (omega-3 fatty acid desaturase); *OsCAT* (catalase domain-containing protein); *OsCES* (CESA1 - cellulose synthase); *OsEXP* (expansin precursor); *OsWIP* (wound-induced protein Wi12); *OsCBP* (calmodulin-binding protein) and *OsAXN* (annexin), all listed in Supplementary Table 1, were further evaluated in leaves of both genotypes by RT-qPCR, using an Ubiquitin gene (*OsUBQ5*) as control. The differences in expression found by the RNAseq methodology were confirmed for the nine tested genes (Figure 5). Expression of most of the genes (*OsGAPDH*, *OsFAD*, *OsCAT*, *OsWIP*, *OsCBP*) was up-regulated after 6 h of cold treatment, with higher expression in the cold-tolerant genotype. There was higher expression of three out of the nine tested genes (*OsCES*, *OsEXP*, *OsAXN*) in the cold-tolerant genotype after 6 h of cold and also under normal growth conditions (T0, before cold treatment). Previous studies have suggested that the highly constitutive gene expression prior to abiotic stress treatment might represent a constitutive tolerance mechanism in *indica* and *japonica* rice tolerant genotypes [69, 42]. Of the nine tested genes, only two (*OsRBC* and *OsAXN*) were down-regulated in plants from the cold-tolerant genotype after 6 h of cold. Even though, their expression was higher in the cold-tolerant plants than in the cold-sensitive ones.

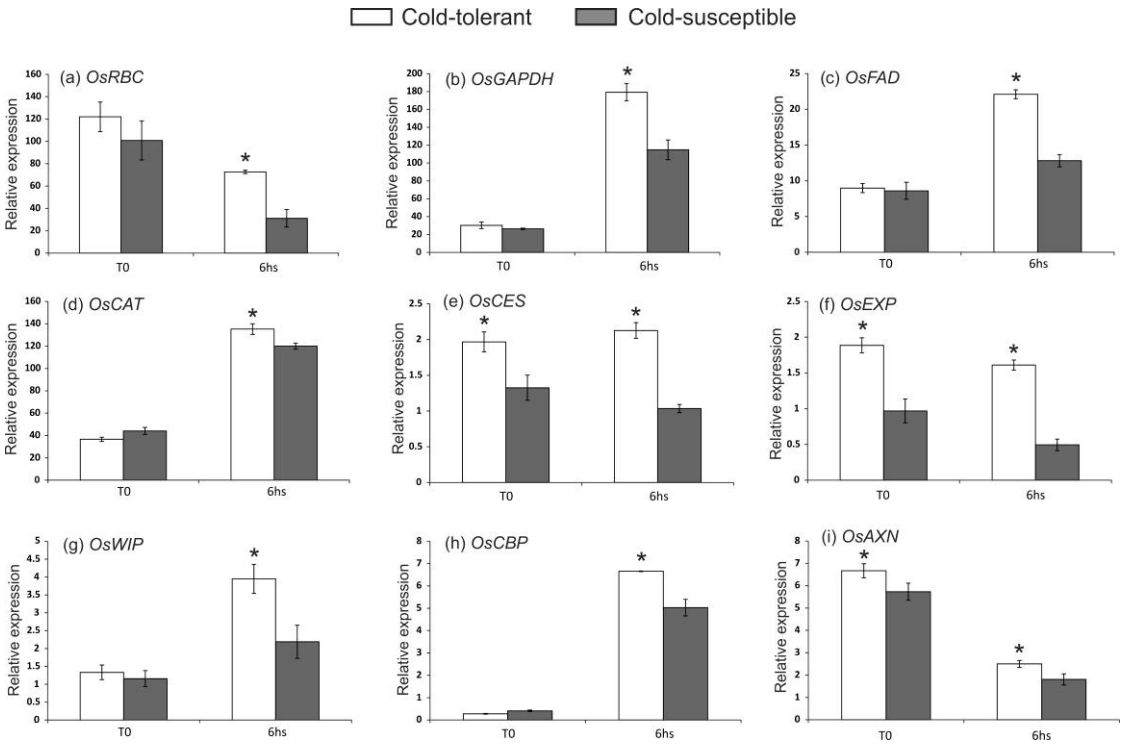

Figure 5. Relative expression levels (qRT-PCR, relative to *OsUBQ5* expression) of selected genes identified by deep sequencing in leaves of *indica* rice plants before (T0) and after 6 h of cold (10 ^{o}C). *OsRBC, ribulose bisphosphate carboxylase small chain*; *OsGAPDH*, *glyceraldehyde-3-phosphate dehydrogenase*; *OsFAD*, *omega-3 fatty acid desaturase*; *OsCAT*, *catalase domain-containing protein*; *OsCES*, *CESA1 - cellulose synthase*; *OsEXP*, *expansin precursor*; *OsWIP*, *wound-induced protein* Wi12; *OsCBP*, *calmodulin-binding protein*; *OsAXN*, *annexin*. Values are the averages of three samples ± SE. Mean values with one asterisk are different by Student's *t* test ($p \leq 0.05$). ND = not detected.

Conclusion

In summary, this study revealed different molecular and physiological mechanisms in response to low temperature stress in two *indica* rice genotypes with contrasting levels of cold-tolerance. Comprehensive gene expression using high throughput RNAseq revealed several differentially expressed genes, and some of them are probably involved in cold stress tolerance. Through the analysis of these sequences, in relation to the cold-sensitive genotype, the cold-tolerant one seems to have better photosynthetic efficiency, higher level of unsaturated fatty acids, higher antioxidant capacity, higher reinforcement of cell wall composition, more active Ca^{2+} and hormone-signaling, which ultimately lead to

more active cold response signaling and cold-tolerance. Also, it was the first time that cold-tolerance in rice was related with high expression of cellulose synthase genes, transcription factor *OsERD15* and *OsTIL-2* (Temperature-induced lipocalin-2). Even considering that mRNAs can be subject of post-transcriptional regulation, the differentially expressed sequences defined in this study do provide useful starting points for more in-depth analyses of the molecular and physiological mechanisms behind cold tolerance in rice plants, and therefore, could be used in future biotechnological applications.

Authors' contribution

RAS, JMA, DC, RPC and JPF conceived and designed research. RAS, JMA, DC, AD and FKR conducted experiments. LFVO, FKR, RPS, LP and RM contributed with analytical tools. RAS, JMA, LFVO, AD and FKR analyzed data. RAS, JMA, FKR and JPF wrote the manuscript. All authors read and approved the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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References

[1] R.P. Cruz, R.A. Sperotto, D. Cargnelutti, J.M. Adamski, T.F. Terra, J.P. Fett, Avoiding damage and achieving cold tolerance in rice plants. Food Energy Secur. 2 (2013) 96-119.

[2] H. Ye, H. Du, N. Tang, X. Li, L. Xiong, Identification and expression profiling analysis of TIFY family genes involved in stress and phytohormone responses in rice. Plant Mol. Biol. 71 (2009) 291-305.

[3] G.S. Sanghera, S.H. Wani, W. Hussain, N.B. Singh, Engineering cold stress tolerance in crop plants. Curr Genom. 12 (2011) 30-43.

[4] T.G. Li, R.M. Visperas, B.S.Vergara, Correlation of cold tolerance at different growth stages in rice. Acta Bot. Sin. 23 (1981) 203-207.

[5] D.J. Mackill, X. Lei, Genetic variation for traits related to temperate adaptation of rice cultivars. Crop Sci. 37 (1997) 1340-1346.

[6] L.M. Mertz, F.A. Henning, R.C. Soares, R.F. Baldiga, F.B. Peske, D.M. Moraes, Physiological changes in rice seeds exposed to cold in the germination phase. Rev. Bras. Sem. 31 (2009) 254-262.

[7] N. Takahashi, Differentiation of ecotypes in *Oryza sativa* L. *In*: Takahashi N and Tsunoda S, eds. Biology of rice. Japan Science Society, Tokyo, Japan, (1984) pp. 31-67.

[8] S.J. Kim, S.C. Lee, S.K. Hong, K. An, G. An, S.R. Kim, Ectopic expression of a cold-responsive *OsAsr1* cDNA gives enhanced cold tolerance in transgenic rice plants. Mol. Cells 27(2009) 449-458.

[9] K. Nakashima, L.S. Tran, D. Van Nguyen, M. Fujita, K. Maruyama, D. Todaka, Y. Ito, N. Hayashi, K. Shinozaki, K. Yamaguchi-Shinozaki, Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. Plant J. 51 (2007) 617-630.

[10] J. Huang, S. Sun, D. Xu, H. Lan, H. Sun, Z. Wang, Y. Bao, J. Wang, H. Tang, H. Zhang, A TFIIIA-type zinc finger protein confers multiple abiotic stress tolerances in transgenic rice (*Oryza sativa* L.). Plant Mol. Biol. 80 (2012) 337-350.

[11] Y. Sato, Y. Masuta, K. Saito, S. Murayama, K. Ozawa, Enhanced chilling tolerance at the booting stage in rice by transgenic overexpression of the ascorbate peroxidase gene, *OsAPXa*. Plant Cell Rep. 30 (2011) 399-406.

[12] H. Fang, Q. Meng, J. Xu, H. Tang, S. Tang, H. Zhang, J. Huang, Knock-down of stress inducible *OsSRFP1* encoding an E3 ubiquitin ligase with transcriptional activation activity confers abiotic stress tolerance through enhancing antioxidant protection in rice. Plant Mol Biol. 87 (2015) 441-458.

[13] Q. Zhang, Q. Chen, S. Wang, Y. Hong, Z. Wang, Rice and cold stress: methods for its evaluation and summary of cold tolerance-related quantitative trait loci. Rice (NY) (2014) 7:24.

[14] Y. Saijo, S. Hata, J. Kyozuka, K. Shimamoto, K. Izui, Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J. 23 (2000) 319-327.

[15] J.Q. Wen, K. Oono, R. Imai, Two novel mitogen-activated protein signaling components, OsMEK1 and OsMAP1, are involved in a moderate low-temperature signaling pathway in rice. Plant Physiol. 129 (2002) 1880-1891.

[16] L. Xiong, Y. Yang, Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. Plant Cell 15 (2003) 745-759.

[17] N.A. Chen, Y. Xu, X. Wang, C. Du, J. Du, M. Yuan, Z. Xu, K. Chong, OsRAN2, essential for mitosis, enhances cold tolerance in rice by promoting export of intranuclear tubulin and maintaining cell division under cold stress. Plant Cell Environ. 34 (2011) 52-64.

[18] G. Xie, H. Kato, R. Imai, Biochemical identification of the OsMKK6-OsMPK3 signaling pathway for chilling stress tolerance in rice. Biochem. J. 443 (2012) 95-102.

[19] S.C. Lee, M.Y. Lee, S.J. Kim, S.H. Jun, G. An, S.R. Kim, Characterization of an abiotic stress-inducibledehydrin gene, *OsDhn1*, in rice (*Oryza sativa* L.). Mol. Cells 19 (2005) 212-218.

[20] Z.Tao, Y. Kou, H. Liu, X. Li, J. Xiao, S. Wang, OsWRKY45 alleles play different roles in abscisic acid signaling and salt stress tolerance but similar roles in drought and cold tolerance in rice. J. Exp. Bot. 62 (2011) 4863-4874.

[21] J.G. Dubouzet, Y. Sakuma, Y. Ito, M. Kasuga, E.G. Dubouzet, S. Miura, M. Seki, K. Shinozaki, K. Yamaguchi-Shinozaki, *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought, high-salt- and cold-responsive gene expression. Plant J. 33 (2003) 751-763.

[22] S.C. Lee, K.W. Huh, K. An, G. An, S.R. Kim, Ectopic expression of a cold-inducible transcription factor, CBF1/DREB1b, in transgenic rice (*Oryza sativa* L.). Mol. Cells 18: (2004a) 107-114.

[23] Y. Ito, K. Katsura, K. Maruyama, T. Taji, M. Kobayashi, M. Seki, K. Shinozaki, K. Yamaguchi-Shinozaki, Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant Cell Physiol. 47 (2006) 141-153.

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

[25] C.F. Su, Y.C .Wang, T.H. Hsieh, C.A. Lu, T.H. Tseng, S.M. Yu, A novel MYBS3-dependent pathway confers cold tolerance in rice. Plant Physiol. 153 (2010) 145-158.

[26] J.M. Adamski, D. Cargnelutti, R.A. Sperotto, T.F. Terra, L.M.G .Rosa, Cruz R.P., J.P. Fett, Identification and physiological characterization of two sister lines of *indica* rice (*Oryza sativa* L.) with contrasting levels of cold tolerance. Can. J. Plant Sci. (2015) (submitted).

[27] M.D. Robinson, A. Oshlack, A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol 11 (2010) R25.

[28] M.D. Robinson, D.J. McCarthy, G.K. Smyth, EdgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26 (2010) 139-140.

[29] A. Conesa, A. Götz, Blast2GO: a comprehensive suite for functional analysis in plant genomics. Int J Plant Genom 2008 (2008) 619832.

[30] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative CT method. Nature Prot. 3 (2008) 1101-1108.

[31] E.C. Bligh, W.J. Dyer, A rapid method of total lipid. Extraction and purification. Can J Biochem Physiol. 37 (1959) 911-917.

[32] L. Hartman, B.C.A. Lago, Rapid preparation of fatty, methyl esters from lipids. Lab. Pract . 22 (1973) 457-477.

[33] D.J. McCarthy, G.K. Smyth, Testing significance relative to a fold-change threshold is a TREAT. Bioinformatics 25 (2009) 765-771.

[34] R.J. Strasser, M. Tsimilli-Michael, A. Srivastava, Chlorophyll a fluorescence: a signature of photosynthesis, analysis of the chlorophyll a fluorescence transient. Chapter 12, Springer, The Netherlands, v. 14, (2004) 321-362.

[35] W. Guo-Li, G. Zhen-Fei, Effects of chilling stress on photosynthetic rate and chlorophyll fluorescence parameter in seedlings of two rice cultivars differing in cold tolerance. Rice Sci. 12 (2005) 187-191.

[36] B.R. Saad, D. Fabre, D. Mieulet, D. Meynard, M. Dingkuhn, A. Al-Doss, et al., Expression of the *Aeluropus littoralis AlSAP* gene in rice confers broad tolerance to abiotic stresses through maintenance of photosynthesis. Plant Cell Environ. 35 (2012) 626-643.

[37] A. Román, V. Andreu, M.L. Hernández, B. Lagunas, R. Picorel, J.M. Martínez-Rivas, M. Alfonso, Contribution of the different omega-3 fatty acid desaturase genes to the cold response in soybean. J. Exp. Bot. 63 (2012) 4973-4982.

[38] K. Iba, Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. Annu. Rev. Plant Biol. 53 (2002) 225-245.

[39] N. Murata, O. Ishizaki-Nishizawa, S. Higashi, H. Hayashi, Y. Tasaka, I. Nishida, Genetically engineered alteration in the chilling sensitivity of plants. Nature 356 (1992) 710-713.

[40] A. Kargiotidou, D. Deli, D. Galanopolou, A. Tsaftaris, T. Farmaki, Low temperature and light regulate delta 12 fatty acid desaturases (FAD2) at a transcriptional level in cotton (*Gossypium hirsutum*). J. Exp. Bot. 59 (2008) 2043-2056.

[41] R.P. Cruz, J.I. Golombieski, M.T. Bazana, C. Cabreira, T.F. Silveira, L.P. da Silva, Alterations in fatty acid composition due to cold exposure at the vegetative stage in rice. Braz. J. Plant Physiol. 22 (2010) 199-207.

[42] T. Zhang, X. Zhao, W. Wang, Y. Pan, L. Huang, X. Liu, et al., Comparative transcriptome profiling of chilling stress responsiveness in two contrasting rice genotypes. PLoS ONE 7 (2012) e43274.

[43] S.Y. Song, Y. Chen, J. Chen, X.Y. Dai, W.H. Zhang, Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress. Planta 234 (2011) 331-345.

[44] M.K. Aalto, E. Helenius, T. Kariola, V. Pennanen, P. Heino, H. Hõrak, I. Puzõrjova, H. Kollist, E.T. Palva, ERD15 - An attenuator of plant ABA responses and stomatal aperture. Plant Sci. 182 (2012) 19-28.

[45] V. Bonnecarrère, O. Borsani, P. Díaz, F. Capdevielle, P. Blanco, J. Monza, Response to photoxidative stress induced by cold in *japonica* rice is genotype dependent. Plant Sci. 180 (2011) 726-732.

[46] K.Y. Yun, M.R. Park, B. Mohanty, V. Herath, F. Xu, R. Mauleon et al., Transcriptional regulatory network triggered by oxidative signals configures the early response mechanisms of *japonica* rice to chilling stress. BMC Plant Biol. 10 (2010) 16.

[47] K.M. Gothandam, E. Nalini, S. Karthikeyan, J.S. Shin, OsPRP3, a flower specific proline-rich protein of rice, determines extracellular matrix structure of floral organs and its overexpression confers cold-tolerance. Plant Mol. Biol. 72 (2010) 125-135.

[48] W. Zheng, L. Ma, J. Zhao, Z. Li, F. Sun, X. Lu, Comparative transcriptome analysis of two rice varieties in response to rice stripe virus and small brown planthoppers during early interaction. PLoS ONE 8 (2013) e82126.

[49] C.W. Liu, Y.K. Hsu, Y.H. Cheng, H.C. Yen, Y.P. Wu, C.S. Wang, C.C. Lai, Proteomic analysis of salt-responsive ubiquitin-related proteins in rice roots. Rapid Commun. Mass Sp. 26 (2012) 1649-1660.

[50] J. Sampedro, D.J. Cosgrove, The expansin superfamily. Genome Biol. 6 (2005) 242.

[51] M. Zheng, Y. Wang, K. Liu, H. Shu, Z. Zhou, Protein expression changes during cotton fiber elongation in response to low temperature stress. J. Plant Physiol. 169 (2012) 399-409.

[52] S. Abuqamar, S. Ajeb, A. Sham, M.R. Enan, R. Iratni, A mutation in the expansin-like A2 gene enhances resistance to necrotrophic fungi and hypersensitivity to abiotic stress in *Arabidopsis thaliana*. Mol. Plant Pathol. 14 (2013) 813-827.

[53] S.K. Yen, M.C. Chung, P.C. Chen, H.E. Yen, Environmental and developmental regulation of the wound-induced cell wall protein WI12 in the halophyte ice plant. Plant Physiol. 127 (2001) 517-528.

[54] Q. Zhang, N. Jiang, G.L. Wang, Y. Hong, Z .Wang, Advances in understanding cold sensing and the cold-responsive network in rice. Adv. Crop Sci. Technol. (2013) 1:1.

[55] V. Chinnus amy, J. Zhu, J.K. Zhu, Gene regulation during cold acclimation in plants. Physiol. Plant 126 (2006) 52-61.

[56] W. Tang, M. Page, Transcription factor AtbZIP60 regulates expression of Ca^{2+}-dependent protein kinase genes in transgenic cells. Mol. Biol. Rep. 40 (2013) 2723-2732.

[57] D. Konopka-Postupolska, G. Clark, A. Hofmann, Structure, function and membrane interactions of plant annexins: an update. Plant Sci. 181 (2011) 230-241.

[58] S. Lee, E.J. Lee, E.J. Yang, J.E. Lee, A.R. Park, W.H. Song, O.K. Park, Proteomic identification of annexins, calcium-dependent membrane binding proteins that mediate osmotic stress and abscisic acid signal transduction in *Arabidopsis*. Plant Cell 16 (2004b) 1378-1391.

[59] S.K. Jami, G.B. Clark, B.T. Ayele, S.J. Roux, P.B. Kirti, Identification and characterization of annexin gene family in rice. Plant Cell Rep. 31 (2012) 813-825.

[60] M.O. Winfield, C. Lu, I.D. Wilson, J.A. Coghill, K.J. Edwards, Plant responses to cold: transcriptome analysis of wheat. Plant Biotech. J. 8 (2010) 749-771.

[61] E.H. Colebrook, S.G. Thomas, A.L. Phillips, P. Hedden, The role of gibberellin signaling in plant responses to abiotic stress. J. Exp. Biol. 217 (2014) 67-75.

[62] A. Rahman, Auxin: a regulator of cold stress response. Physiol. Plant 147 (2013) 28-35.

[63] Y. Tian, H. Zhang, X. Pan, X. Chen, Z. Zhang, X. Lu, et al., Overexpression of ethylene response factor TERF2 confers cold tolerance in rice seedlings. Transg. Res. 20 (2011) 857-866.

[64] T. Werner, T. Schmülling, Cytokinin action in plant development. Curr. Opin. Plant Biol. 12 (2009) 527-538.

[65] J.A. O'Brien, E. Benková, Cytokinin cross-talking during biotic and abiotic stress responses. Front Plant Sci. 4 (2013) 451.

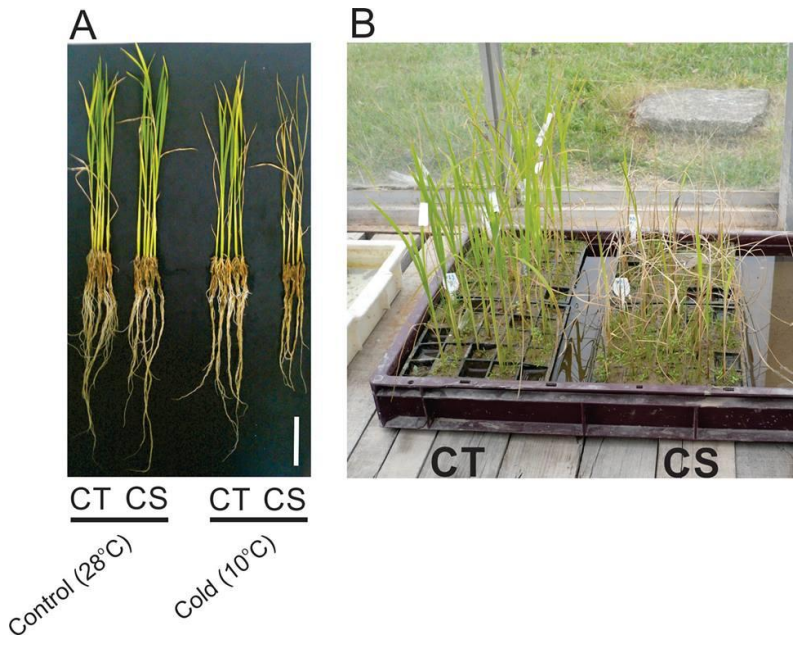
[66] K. Aguan, K. Sugawara, N. Susuki, T. Kusano, Isolation of genes for low-temperature-induced proteins in rice by a simple subtractive method. Plant Cell Physiol. 32 (1991) 1285-1289.

[67] F.J.B. Charron, G. Breton, M. Badawi, F. Sarhan, Molecular and structural analyses of a novel temperature stress-induced lipocalin from wheat and Arabidopsis. FEBS Lett 517 (2002) 129-132.

[68] J-B. F. Charron, F. Ouellet, M. Pelletier, J. Danyluk, C. Chauve, F. Sarhan, Identification, expression, and evolutionary analyses of plant lipocalins. Plant Physiol. 139 (2005) 2017-2028.

[69] S. Kumari, V.P. Sabharwal, H.R. Kushwaha, S.K. Sopory, S.L. Singla-Pareek, A. Pareek, Transcriptome map for seedling stage specific salinity stress response indicates a specific set of genes as candidate for saline tolerance in *Oryza sativa* L. Funct Integrat Genom. 9 (2009) 109-123.

Supplementary Material



Supplementary Figure 1. Visual symptoms of rice plants from the cold-tolerant (CT) and cold-sensitive (CS) genotypes after 10 days under control (28 ^{o}C) or cold (10 ^{o}C) treatment, followed by 7 days of recovery at 28 ^{o}C. Bar in figure (A) indicates 7 cm. All experiments were performed at least twice with similar results.

Supplementary Table 1. Gene-specific PCR primers used for qRT-PCR.

Gene	Forward primer 5' → 3'	Reverse primer 5' → 3'
OsRBC	TTTCGTGTGTTCCCAGTTTG	CACATATGCCTCACCCAACA
OsGAPDH	GCTGCTGCCTTTGTAACTCC	TGGGGAAGAAAAGCAAAGAA
OsFAD	GAGCCTGGGCTTAACATCTG	GGTGATCTCCGTCTTCAATCA
OsCAT	GCCTCAGCCTCACTCTATCG	ACACGAATTGTGCGGTGATA
OsCES	CGCTAGCTGCTGATACGATG	TGTGTAACAACGACCCCTCA
OsEXP	ATGGATTTGCATGGATGGTT	GCCTCCTCCTTATCCCACTC
OsWIP	CCATCAATCAGCTTCAACCA	CGCAGACGCAGTAAAAGTGA
OsCBP	GTTCATGCAGCGGTCTCAT	TGGCAGCACACTTATGAACA
OsAXN	CGCCATCATTGTGCTCTCTA	TTGTCAGATGGAACCACCAA
OsUBQ5	AACCAGCTGAGGCCCAAGA	ACGATTGATTTAACCAGTCCATGA

Supplementary Table 2. Differentially expressed genes revealed by RNAseq in leaves of cold-tolerant (IRGA 959-1-2-2F-4-1-4-A) and cold-sensitive (IRGA 959-1-2-2F-4-1-4-D-1-CA-1) genotypes, after 6 h at 10 ^{o}C. **Enviado aos membros da banca.**

CAPÍTULO IV

Artigo a ser submetido para publicação:

Photosynthetica

Photosynthetic activity of two sister lines of *indica* rice (*Oryza sativa* L.) with contrasting levels of cold tolerance

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Abstract

Incidence of low temperature during early vegetative stage is one of the major limiting factors to the development of rice plants. Cold stress has strong negative effects on photosynthetic activity. Previously, our group evaluated plant survival after cold treatment in 90 *indica* rice genotypes. The genotypes IRGA 959-1-2-2F-4-1-4-A and IRGA 959-1-2-2F-4-1-4-D-1-CA-1, derived from the same cross, were characterized, respectively, as tolerant and sensitive to low temperature. This work investigated the involvement of photosynthetic activity in cold tolerance of *indica* rice plants during early vegetative growth. Chlorophyll *a* fluorescence and gas exchange analyses were performed during low temperature stress and after cold exposure, during a recovery period. The photosynthetic performance was more efficient in the tolerant genotype than in the sensitive one, during the cold treatment and during the recovery period. The higher efficiency of the photosynthetic performance during the cold treatment appears to be related to a lower fraction of the reduced Q_A^-, as well as to lower stomatal conductance and transpiration rates. After the recovery period, the higher efficiency in the cold tolerant genotype seems to relate to a lower fraction of the reduced Q_A^- and with a larger pool of end electron acceptors at the PSI. This work uncovered changes in photosynthetic performance that occur during low temperature stress, which may be important components of cold tolerance mechanisms in *indica* rice.

Additional keywords: chlorophyll *a* fluorescence, cold stress, photosynthesis.

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Abbreviations: F_0 = F (160μs) – minimal fluorescence, when all PSII RCs are open; F_1 = F_K (300μs), $F_2 = F_J$ (2ms) and $F_3 = F_I$ (30ms) – fluorescence intensity at the K, J and the I step, respectively; $F_P = F_m$ (300 ms) – maximal fluorescence, when all PSII RCs are closed; F_t – fluorescence intensity at any time; PI_{total} – performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors.

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Introduction

Rice (*Oryza sativa* L.) is a staple food for half of the world population; however, the physiological status of rice plants is affected by a variety of abiotic stresses, leading to reduction on grain yield. Cold temperature is one major environmental factor limiting rice production, especially in high altitude areas or temperate regions, due to the tropical origin of the species (Cruz *et al.* 2013).

The rice subspecies *indica* and *japonica* differ widely in their responses to cold temperatures. Several studies have demonstrated that cold tolerance is higher among the *japonica* genotypes, which are better adapted to temperate climates such as the ones in Japan, Korea, and Java (Cruz *et al.* 2013). In Brazil, the largest rice producer outside Asia, cold occurrence is common in the southernmost state, Rio Grande do Sul (RS), where over 68.5% of the Brazilian rice grain is produced in about one million hectares of irrigated rice cultivated annually (IBGE 2015). Losses due to cold temperatures during the vegetative growth stage are increased because the varieties cultivated in this region belong to the *indica* variety, mostly sensitive to cold stress (Cruz *et al.* 2006).

Although the *indica* subspecies of *O. sativa* is classified as sensitive to cold temperatures, there is large variability within this group in response to cold. In a previous study, we identified low temperature tolerant and sensitive *indica* genotypes. Ninety genotypes were evaluated according to the percentage of survival of three leaf-stage plants after ten days at 10°C (climatic chamber) and seven days of recovery in greenhouse conditions (20–30°C). The genotypes IRGA 959-1-2-2F-4-1-4-A and IRGA 959-1-2-2F-4-1-4-D-1-CA-1 were characterized, respectively, as tolerant and sensitive to low

temperature both during early vegetative development and during germination (Adamski *et al.* 2015, Dametto *et al*. 2015).

Rice plants are damaged by chilling (low temperatures between 10 and 13°C) during early stages of development (Yoshida 1981). One of the major effects is the inhibition of photosynthetic activity through decrease in electron transport rate in photosystem II (PS II) (Jeong *et al.* 2002). Due to the decreased activity of electron transport proteins, the photosystem II (P680) is overloaded with excess energy, leading to damage in PSII proteins and compromising ATP and NADPH production.

Cold stress can induce profound changes in plant metabolism and its effect can be assessed by analyzing different parameters. Chlorophyll *a* fluorescence is an important parameter used to evaluate plant photosynthetic efficiency, especially the of PS II behavior (Yusuf *et al*. 2010). The kinetics of fluorescence emission corresponds to an increase in the polyphasic or transient fluorescence from the initial to the maximum, which include the steps O-J-I-P. This analysis also allows *in vitro* and *in vivo* evaluations of plant vitality in terms of biophysical parameters and the quantification of photosynthetic energy conservation (Tsimilli-Michael and Strasser 2008).

This study aimed to evaluate the effect of cold temperature (10°C) on the photosynthetic activity in plants of two sister lines of *indica* rice (*Tolerant* × *Sensitive*) to identify parameters that may serve as markers of cold tolerance for this stress.

Material and methods

Plant material and growth conditions: Seeds were germinated for five days in distilled water, planted in commercial soil and grown in a greenhouse under ambient temperature (30 ± 5 °C), under full daylight. After 20 days of growth in the greenhouse, plants at the three leaf stage were transferred to climatic chambers with light intensity of 150 [µmol (photon) m^{-2} s^{-1}] and maintained at 10 °C (cold treatment) or at 28 °C (control treatment) for five days. After this period, all plants were transferred to the greenhouse for a recovery period of five days.

Chlorophyll *a* fluorescence transient: The chlorophyll fluorescence transients were evaluated on the youngest fully expanded leaves of ten plants from each genotype (sensitive and tolerant) and treatment (cold or control), using a portable pulse modulated fluorometer (OS30p, Optisciences, UK). Before the measurements, leaves were dark adapted for 20 minutes. The fluorescence intensity was measured by applying a saturating

pulse of 3,000 [µmol (photon) m^{-2} s^{-1}] and the resulting fluorescence of the chlorophyll *a* measured from 160µs to 1s. The intensities at the time points 160µs (minimal fluorescence), 300µs (F_K), 2ms (F_J), 30ms (F_I) and maximal fluorescence 300ms (F_m) were collected and used to calculate PI_{total} according to the JIP-Test (Strasser and Strasser 1995).

Gas exchange: Measurements were performed using a portable infrared gas analyzer (Licor 6400; LiCor Inc., Lincoln, NE, USA). The youngest fully expanded leaves were used for these measurements. Photosynthetically active radiation (PAR) inside the leaf chamber was fixed in 300 [µmol (photon) m^{-2} s^{-1}]. The CO_2 concentration inside the chamber was controlled using 12g CO_2 bottles and fixed on 400ppm. Temperature and relative humidity fluctuated naturally, according to the environmental conditions. The readings were taken on ten plants per genotype and per treatment.

Statistical analysis: Mean values were compared by the Student's *t* test ($p \leq 0.05$) using the GraphPad Software (http://graphpad.com/quickcalcs/ttest2/).

Results

Normalizations and subtraction of Chlorophyll *a* fluorescence transients: The transient fluorescence intensity of chlorophyll *a* from O to P in leaves from the cold tolerant (IRGA 959-1-2-2F-4-1-4-A) and sensitive (IRGA 959-1-2-2F-4-1-4-D-1-CA-1) *indica* rice genotypes showed a typical polyphasic rise (Fig. 1). The fluorescence intensity (F_t) was lower in leaves from plants maintained at 10°C for five days than under the control treatment, in both genotypes, in the entire curve. This effect was more pronounced in the sensitive genotype (Fig. 1*A*). After a recovery period of five days, fluorescence levels were similar to control levels in both genotypes, although with Ft values a little lower than the control in the I–P phases. This recovery was more efficient in the tolerant genotype (Fig. 1*B*).

The fluorescence data was normalized between the O and P phases as V_{OP} ($V_{OP} = F_t\text{-}F_o/F_m\text{-}F_o$). The main differences between the tolerant and sensitive genotypes in the normalized curves are visible at 2ms (J phase) (Fig. 1*C*). The normalized fluorescence V_{OP} values are lower for the tolerant genotype than for the sensitive, both after five days of cold treatment and after the recovery period (Fig. 1*C–D*).

The fluorescence data was also normalized as V_{OI} ($V_{OI} = F_t\text{-}F_o/F_I\text{-}F_o$). Higher values are observed for both genotypes compared to the control after five days of cold

treatment between the steps I (30ms) and P (300ms) (Fig. 2*A*). However, no difference between the two genotypes was observed (Fig. 2*A*). The differences of the V_{OI} values between the tolerant and the sensitive genotypes in the I-P phase [$\Delta V_{OI} = V_{OI(tolerant)} - V_{OI(sensitive)}$] remained near to zero, emphasizing the similar responses to cold in both genotypes (Fig. 2*C*). However, after five days of recovery at control temperatures, the V_{OI} values obtained for the tolerant genotype were similar to the ones from plants not subjected to cold stress (control), while vales from the sensitive genotype were lower (Fig. 2*B*). The diverse responses between the two genotypes are also clear at the ΔV_{OI} plots, where positive values are observed in the I–P phase (Fig. 2*D*).

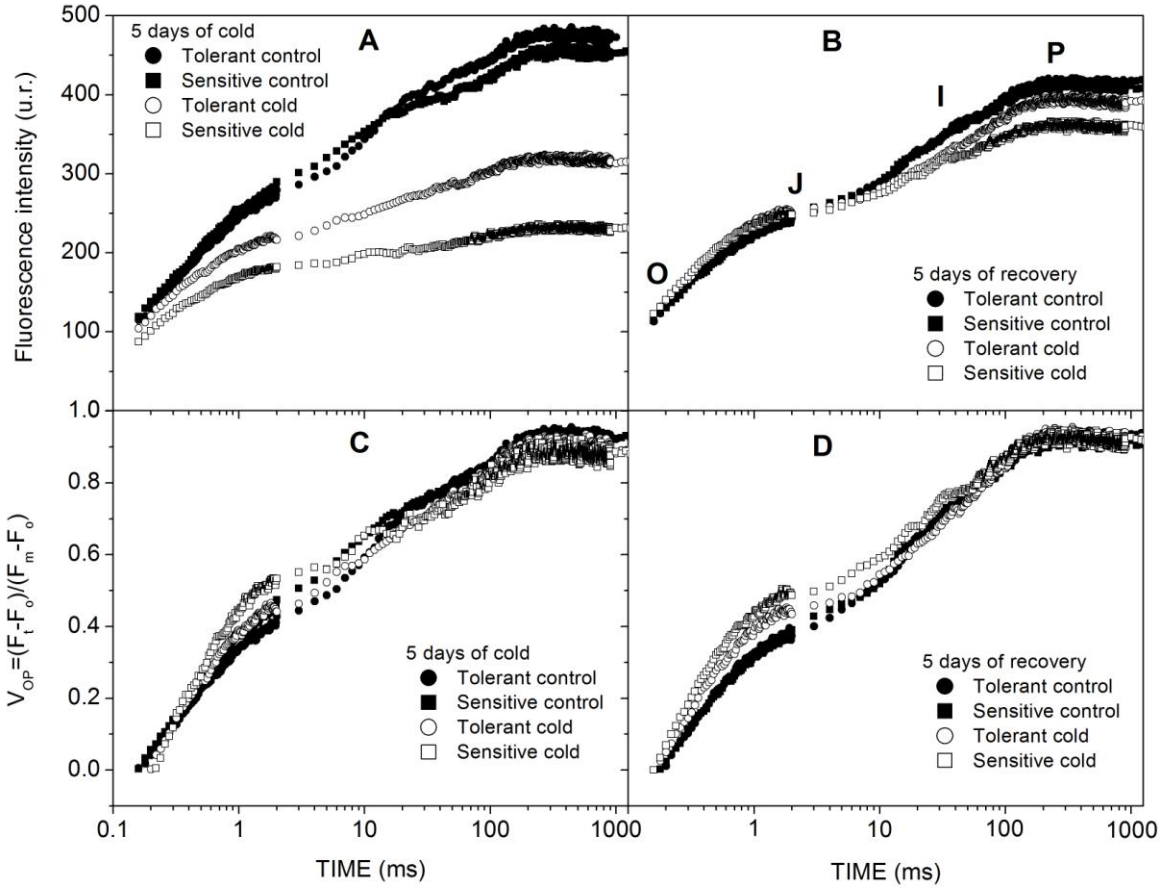

Fig. 1. Fluorescence intensity (*A* and *B*) and relative variable fluorescence [V_{OP} ($V_{OP} = F_t-F_o/F_m-F_o$)] (*C* and *D*) at transient fluorescence of chlorophyll *a* from O to P of *indica* rice genotypes tolerant (IRGA 959-1-2-2F-4-1-4-A) and sensitive (IRGA 959-1-2-2F-4-1-4-D-1-CA-1) after 5 days of cold and after restoring at room temperature for 5 days.

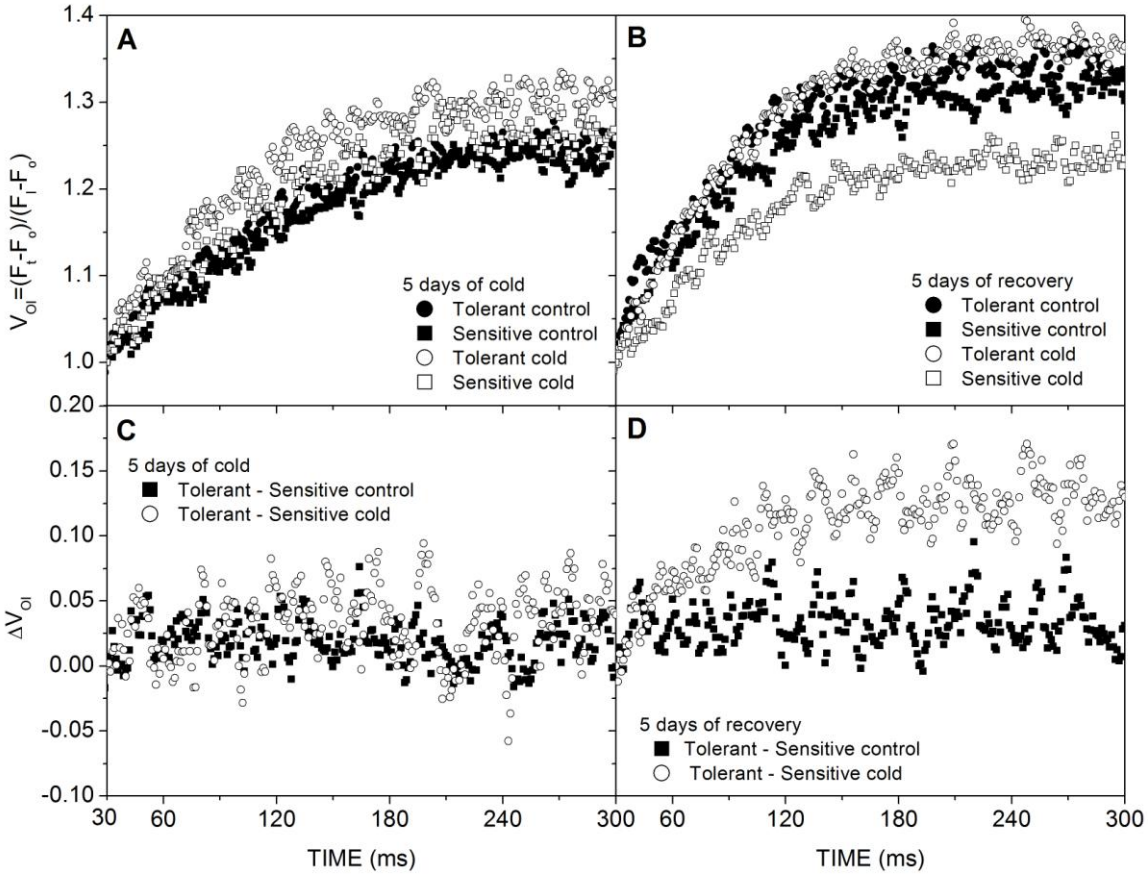

Fig. 2. Relative variable fluorescence [$V_{OI}=(F_t-F_o)/(F_I-F_o)$] (*A* and *B*) and kinetic difference of V_{OI} [$\Delta V_{OI}= V_{OI(tolerant)}- V_{OI(sensitive)}$] (*C* and *D*) at transient fluorescence of chlorophyll *a* from I to P of *indica* rice genotypes tolerant (IRGA 959-1-2-2F-4-1-4-A) and sensitive (IRGA 959-1-2-2F-4-1-4-D-1-CA-1) after 5 days of cold and after recovery at room temperature for 5 days.

Total performance index (PI_{total}) parameter derived by the JIP-Test equations: The biophysical parameter PI_{total} was calculated from the transient fluorescence curves according to JIP–Test (Strasser and Strasser 1995) (Fig. 3). PI_{total} values were higher in the tolerant genotype than in the sensitive one, both in the cold treated and in the untreated (control) samples (Figure 3*A*).

After five days of recovery, the PI_{total} value was significantly higher in the tolerant genotype than in cold sensitive plants. No difference in PI_{total} values was observed between plants from the two genotypes not subjected to cold treatment (Figure 3*B*).

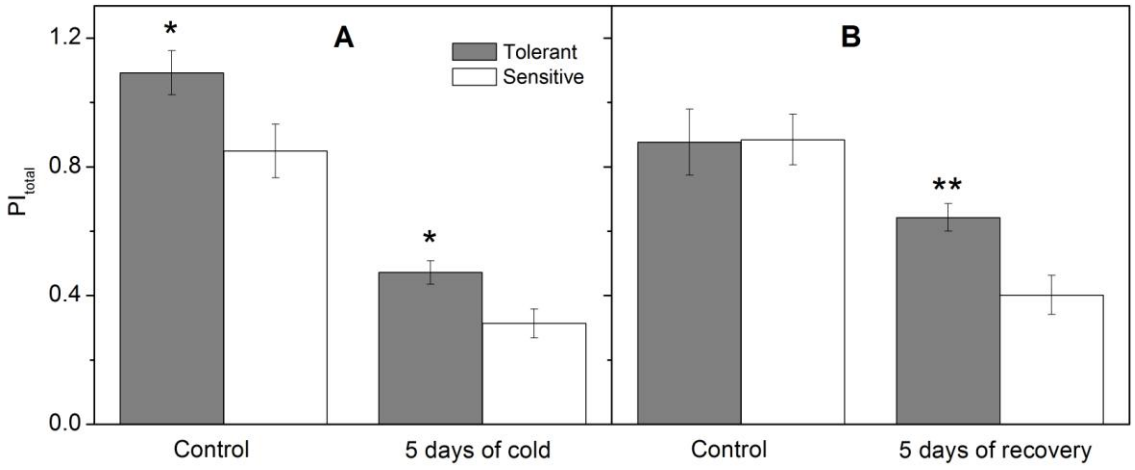

Fig. 3. Performance index total (PI_{Total}) of plants of *indica* rice genotypes tolerant (IRGA 959-1-2-2F-4-1-4-A) and sensitive (IRGA 959-1-2-2F-4-1-4-D-1-CA-1) after 5 days of cold (A) and after restoring at room temperature for 5 days (B). Mean values with one or two asterisks are different by Student's *t* test ($p \leq 0.05$ and $p \leq 0.01$, respectively; $n = 10$).

Gas exchange: The effects of cold stress (10°C), followed by recovery at room temperature on net photosynthesis, stomatal conductance, transpiration rate and internal CO_2 concentration are shown in Table 1. There were no significant differences in these parameters between the two genotypes when plants were maintained in the control treatment, during the whole period of the experiment. After cold treatment, there was a large increase in net photosynthesis, stomatal conductance and transpiration rate in plants from both genotypes. However, the values of these parameters in the tolerant genotype were significantly smaller than in the sensitive one. The values of net photosynthesis, stomatal conductance and transpiration rate of both genotypes increased after five days of recovery at room temperature, but the tolerant genotype showed significantly higher values than the sensitive one. The internal CO_2 concentration was not affected by cold stress in both genotypes.

Table 1. Net assimilation rate (μmol CO_2 m^{-2} s^{-1}), stomatal conductance (mol H_2O m^{-2} s^{-1}), internal CO_2 concentration (μmol CO_2 mol^{-1}) and transpiration rate (mmol H_2O m^{-2} s^{-1}) of *indica* rice genotypes tolerant (T) and sensitive (S) after five days of cold and after recovery at room temperature for five days

Treatments			Net assimilation rate	Stomatal conductance	Transpiration rate	Internal CO_2 concentration
Stress	Control	T	9.54 ± 0.42	0.260 ± 0.018	3.60 ± 0.21	321.70 ± 3.74
		S	10.37 ± 0.65	0.304 ± 0.027	3.96 ± 0.26	323.82 ± 4.90
	Cold	T	1.36 ± 0.12	0.010 ± 0.003	0.17 ± 0.06	238.91 ± 7.92
		S	1.87 ± 0.17*	0.025 ± 0.003**	0.45 ± 0.06**	252.66 ± 21.18
Recovery	Control	T	7.20 ± 0.62	0.125 ± 0.028	2.03 ± 0.38	255.59 ± 14.30
		S	8.27 ± 0.89	0.172 ± 0.031	2.61 ± 0.37	283.30 ± 9.70
	Cold	T	7.36 ± 0.58*	0.116 ± 0.017*	1.84 ± 0.24*	271.20 ± 5.85
		S	4.85 ± 0.73	0.060 ± 0.011	1.05 ± 0.18	253.79 ± 9.03

(n = 10, means ± standard error); mean values of the two genotypes in each treatment with one or two asterisks are different by Student's t test ($p \leq 0.05$ and $p \leq 0.01$, respectively).

Discussion

Previously, the *indica* genotypes IRGA 959-1-2-2F-4-1-4-A and IRGA 959-1-2-2F-4-1-4-D-1-CA-1 were characterized, respectively, as tolerant and sensitive to low temperature stress by our research group (Adamski *et al.* 2015, Dametto *et al*., 2015). After ten days of cold treatment (10°C), the sensitive genotype was unable to recover from the stress (Adamski *et al.* 2015). These two genotypes originated from the same cross, and therefore are sister lines, closely related. The identification of genetically similar genotypes with contrasting levels of cold tolerance may be useful for the characterization of cold stress responses related to tolerance in *indica* rice, which may rely on distinct mechanisms compared to *japonica* rice. Here we report the effect of cold (10°C) on the photosynthetic activity in plants of two sister lines of *indica* rice (*Tolerant* × *Sensitive*).

The decrease in the photosynthetic performance of rice plants under cold treatment and the recovery to a certain degree after removal from the cold chamber was expected (Guo-Li and Hen-Fei, 2005, Saad *et al.* 2012). Both expected results were obtained, being well demonstrated by the fluorescence intensity curves (Fig. 1) and the net assimilation rate (Table 1) of the treated plants. However, the subject of this investigation is the

comparison between the two genotypes, focusing on the differences between their responses to the cold treatment, which will be mostly discussed.

Illumination of a dark-adapted leaf induces characteristic changes in the intensity of chlorophyll *a* fluorescence, known as the Kautsky effect. When the Kautsky transient is plotted on a logarithmic time scale, the kinetics reveals an increase of fluorescence, consisting of a sequence of phases (O–J and I–P phases) (Strasser *et al.* 2000). In the present work, the fluorescence intensity curves indicate that the tolerant genotype had a higher photosynthetic activity during the cold treatment (Fig. 1*A*), as well as a higher photosynthetic activity at the recovery period, compared to the sensitive one (Fig 1*B*).

The data obtained by the calculation of the PI_{total} values (Fig. 3) is in agreement with that of the fluorescence intensity curves. Values of PI_{total} are higher in the tolerant genotype than in the sensitive genotype, both after the cold treatment (Fig. 3*A*) and after the recovery period (Fig. 3*B*). The PI_{total} is an indication of the photosynthetic performance, on the basis of light absorption, characterizing the efficiency of the electron transport flux until the PSI acceptors. It is a combination of four partial components that affect photosynthetic performance: the abundance of photosynthetic reaction centers (RCs); the maximum energy flux that reaches the RC of PSII; the electron transport at the beginning of illumination; and the oxy-reduction reactions occurring on the side of the electron acceptor of PSI (Strasser *et al.* 2004). Among other parameters, photosynthetic performance index (PI) was used to evaluate the cold tolerance of transgenic rice expressing the *AlSAP* gene (Saad *et al.* 2012). Therefore, in the present study, the analysis of the PI_{total} values indicate a better photosynthetic performance, considering all this steps of the photosynthetic process, of the tolerant genotype, both in the cold treatment and in the recovery period.

The net assimilation rate values (Table 1) are in agreement with the results obtained from the fluorescence curves and PI_{total} analyses, considering the decrease in photosynthetic activity under the cold treatment and recovery after five days at room temperature. Interestingly, there was an inversion in the effect of the cold treatment between the two genotypes. The net assimilation rate after cold exposure was lower in plants from the tolerant genotype than in the sensitive ones, indicating a higher influence of the treatment in the former. However, after five days of recovery, the net assimilation values were higher in the tolerant than in the sensitive genotype (Table 1). This result is in agreement with the fluorescence analyses (Fig 1*A* and 1*B*), indicating better photosynthetic activity in plants from the cold tolerant genotype.

The use of OJIP curves with normalized parameters is a potential tool for in depth analyses of the influence of stress treatments on different stages of the photosynthetic process (Yusuf *et al.* 2010). In this study, it was possible to discriminate the cold effects on the two rice genotypes by comparing the fluorescence data normalized as V_{OP} and the V_{OI} (on logarithmic time scale).

Considering normalized V_{OP} data, an increase in fluorescence intensity at 2ms (J phase) is usually interpreted as evidence of accumulation of the fraction of reduced Q_A^- pool, possibly due to a decrease in electron transport beyond Q_A^- (Strasser *et al.* 1995; Haldimann and Strasser 1999). In the present study, lower V_{OP} values were obtained for the tolerant genotype (Fig. 1*C–D*), indicating lower fraction of the reduced Q_A^-, compared to the sensitive, at the end of both periods (cold treatment and recovery). Although this behavior was also observed in the untreated plants (control), it may be a factor that contributes to the better total performance of the photosynthesis in the tolerant genotype.

The differences observed between the two genotypes in the I–P phase of the V_{OI} curves (Fig. 2) can be used to explain their different photosynthetic performances. An increase of the normalized values of V_{OI} in this phase indicates that the size of the pool of end electron acceptors at the PSI has increased (Yusuf *et al.* 2010). In the present study, five days of cold exposure resulted in similar values for the two genotypes, indicating similar responses in these steps of the photosynthetic process (Fig. 2*A*). This was confirmed by the ΔV_{OI} around zero, obtained at this period for the two genotypes (Fig. 2*C*). However, after five days of recovery, higher values of V_{OI} were obtained for the tolerant than for the sensitive genotype (Fig. 2*B*). This is highlighted by the ΔV_{OI} curves, where positive values of ΔV_{OI} are observed for the cold treatment at the end of the recovery period (Fig. 2*D*). This result is an indication that the pool size of the end electron acceptors at the PSI was probably larger in the cold tolerant than in the sensitive plants after five days of recovery.

The higher size of the pool of the end electron acceptors at the PSI in the tolerant genotype may also be related with the smaller accumulation of the fraction of reduced Q_A^- pool, indicated by the normalized fluorescence data as V_{OP} at J phase (Fig. 1*C–D*), as discussed before. These two differences between the genotypes may explain the better total photosynthetic performance of the tolerant genotype (Fig. 3 and Table 1).

The lower values of net assimilation rates in plants from the tolerant genotype after cold treatment, compared with the sensitive genotype, seem to disagree with its general trend of higher photosynthetic performance. This apparent contradiction may be explained

by another endogenous adaptive mechanism to the cold treatment in the tolerant plants, which was not effectively accomplished in the sensitive genotype. One of these mechanisms may be stomatal closure, which is related to the stomatal conductance (Table 1). Stomatal conductance and transpiration rate values were lower in plants from the tolerant genotype than in the sensitive ones. According to Murai-Hatano *et al.* (2008), cold stress reduces hydraulic conductivity of roots and causes dehydration in rice plants even when the soil is abundant in water. Stomatal closure, with consequent decrease in transpiration rate, may be another protection mechanism against cold exposure present in the tolerant genotype.

Conclusion

The photosynthetic performance in the tolerant genotype is more efficient than in the sensitive one, after five days of cold treatment and after the recovery period. This better performance is probably related to a lower fraction of the reduced Q_A^- during the cold treatment and to lower transpiration rates. At the recovery period, the better performance seems to be related to a lower fraction of the reduced Q_A^- and to a larger pool size of the final electron acceptors at the PSI.

References

Adamski J.M., Cargnelutti D., Sperotto R.A. *et al*: Identification and physiological characterization of two sister lines of *indica* rice (*Oryza sativa* L.) with contrasting levels of cold tolerance. – Can J Plant Sci (artigo submetido para publicação, em fase de revisão).

Cruz R.P., Milach S.C.K., Federizzi L.C.: (Rice cold tolerance at the reproductive stage in a controlled environment. – Sci Agr **63**: 255-261, 2006.

Cruz R.P., Sperotto R.A., Cargnelutti D. *et al*.: Avoiding damage and achieving cold tolerance in rice plants. – Food Energ Secur **2**: 96-119, 2013.

Dametto, D., Sperotto, R.A., Adamski, J.M. *et al*.: Cold tolerance in rice germinating seeds revealed by deep RNAseq analysis of contrasting *indica* genotypes. – Plant Sci **238**: 1-12, 2015.

Guo-Li W., Zhen-Fei G.: Effects of chilling stress on photosynthetic rate and chlorophyll fluorescence parameter in seedlings of two rice cultivars differing in cold tolerance. – Rice Sci **12**: 187-191, 2005.

Haldimann, P., Strasser, R.J.: Effects of anaerobiosis as probed by the polyphasic chlorophyll a fluorescence rise kinetic in pea (*Pisum sativum* L.). – Photosynth Res **62**: 67-83, 1999.

IBGE - Intituto Brasileiro de Geografia e Estatística. 2015. Available: http://www.ibge.gov.br/home/estatistica/indicadores/agropecuaria/lspa/default.shtm Accessed 10 June 2015.

Jeong S.W., Choi S.M., Lee D.S. *et al*.: Differential susceptibility of photosynthesis to light-chilling stress in rice (*Oryza sativa* L.) depends on the capacity for photochemical dissipation of light. – Mol Cell **13**: 419-428, 2002.

Murai-Hatano M., Kuwagata T., Sakurai J. *et al*: Effect of low root temperature on hydraulic conductivity of rice plants and the possible role of aquaporins. – Plant Cell Physiol **49**: 1294-1305, 2008.

Yusuf M.A., Kumar D., Rajwanshi R. *et al*: Overexpression of γ-tocopherol methyl transferase gene in transgenic Brassica juncea plants alleviates abiotic stress: physiological and chlorophyll a fluorescence measurements. – Biochim Biophys Acta **1797**: 1428-38, 2010.

Saad R.B., Fabre D., Mieulet D. *et al*.: Expression of the *Aeluropus littoralis AlSAP* gene in rice confers broad tolerance to abiotic stresses through maintenance of photosynthesis. – Plant Cell Environ **35**: 626-643, 2012.

Strasser R.J., Srivastava A., Govindjee.: Polyphasic chlorophyll a fluorescence transient in plants and cyanobacteria. – Photochem Photobiol **61**: 32-42, 1995.

Strasser B.J., Strasser R.J.: Measuring fast fluorescence transients to address environmental questions: the JIP-test. – In: Mathis P (ed.): Photosynthesis: From Light to Biosphere, Pp. 977-980. Kluwer Academic Publishers, The Netherlands 1995.

Strasser R.J., Srivastava A., Tsimilli-Michael M.: The fluorescent transient as a tool to characterise and screen photosynthesic samples. – In: Yunus M, Pathre U, Mohanty P (ed.): Probing Photosynthesis: Mechanisms, Regulation and Adaptation Pp. 445–483. Taylor and Francis, London 2000.

Strasser R.J., Srivastava A., Tsimilli-Michael M.: Analysis of the chlorophyll a fluorescence transient. – In: Papageorgiou GC, Govindjee (ed.): Advances in Photosynthesis and Respiration. vol. 19: Chlorophyll fluorescence: a Signature of photosynthesis Pp. 321-362. Kluwer Academic Publishers, The Netherlands 2004.

Tsimilli-Michael M., Strasser R.J.: In vivo assessment of plants vitality: applications in detecting and evaluating the impact of Mycorrhization on host plants. – In: Varma A. (ed.): Mycorrhiza: State of the Art, Genetics and Molecular Biology, Eco-Function, Biotechnology, Eco-Physiology, Structure and Systematics Pp. 679–703. Springer Verl., Dordrecht 2008.

Yoshida S.: Fundamentals of rice crop science. International Rice Research Institute, Los Baños, 1-63, 1981.

CONSIDERAÇÕES FINAIS

Os resultados compilados nesta Tese corroboram e expandem significativamente as informações ou conhecimentos da literatura relacionados com a tolerância ao frio em plantas de arroz.

Neste trabalho, o *screening* inicial de genótipos de arroz *indica* em relação à tolerância ao frio nas fases iniciais de desenvolvimento (germinação e vegetativa) revelou que existe uma grande variação entre os genótipos. Duas linhagens oriundas do mesmo cruzamento, porém contrastantes quanto à tolerância ao frio, foram identificadas.

A comparação dos transcriptomas dos genótipos tolerante e sensível ao frio, nas duas fases de desenvolvimento (germinação e vegetativa), possibilitou identificar genes diferencialmente expressos em cada um dos genótipos. Na fase de germinação, o genótipo tolerante parece apresentar diversos processos mais ativos do que o genótipo sensível, tais como: taxas de divisão celular e crescimento (confirmados em medições do comprimento de células epidérmicas e da quantificação de células mitóticas do coleóptilo), integridade e extensibilidade da parede celular, absorção de água e capacidade de transporte de membrana, síntese de sacarose, geração de açúcares simples, insaturação de ácidos graxos, biossíntese de cera, atividade antioxidante (confirmada pela maior atividade das enzimas CAT e APX em plântulas do genótipo tolerante) e sinalização mediada por hormônios e Ca^{+2}, levando à aclimatação e tolerância ao frio. Por outro lado, o genótipo sensível ao frio responde à baixa temperatura aumentando a síntese de proteínas de choque térmico (HSPs) e deidrinas, juntamente com uma maior taxa de degradação proteica via ubiquitinação/proteassomo e biossíntese de poliaminas.

Na fase vegetativa, vários processos são mais ativos no genótipo tolerante do que no genótipo sensível, tais como: eficiência fotossintética (confirmada através de análises de fluorescência da clorofila *a* e de trocas gasosas, descritas nos capítulos I e IV), capacidade antioxidante (confirmada pela maior atividade das enzimas SOD e CAT nas folhas das plantas do genótipo tolerante durante exposição ao frio), insaturação de ácidos graxos (confirmada pela maior concentração de ácido linoleico nas folhas das plantas do genótipo tolerante após exposição a baixa temperatura), deposição de celulose nas paredes celulares (demonstrada por meio de coloração do tecido foliar com calcoflúor) e sinalização mediada por hormônios e Ca^{+2}. Além disto, pela primeira vez a tolerância ao frio foi relacionada com a elevada expressão dos genes: *OsERD15* (NAC domain-containing transcription factor), *OsTIL-2* (Temperature-induced lipocalin-2) e *Cellulose Synthase*.

As análises do transcriptoma das duas linhagens durante duas fases de desenvolvimento (germinação e vegetativa) permitiram a identificação de genes (ou grupos de genes) candidatos, possivelmente envolvidos na tolerância a baixas temperaturas, que poderão ser úteis em futuras abordagens biotecnológicas visando a tolerância ao frio em arroz da subespécie *indica*.

Como perspectivas adicionais de trabalho nesta linha de pesquisa, destacam-se estudos relacionados aos genes *OsCESA1* e *OsCESA8.* Considerando o papel central que o complexo multiproteico da celulose sintase desempenha na planta, e a inexistência de dados de caracterização dos genes de celulose sintase em arroz, tanto a caracterização funcional destes genes quanto a avaliação do seu possível envolvimento com a tolerância ao frio são de extrema relevância. Os resultados obtidos neste trabalho sugerem que as proteínas celulose sintase desempenham papel central na tolerância ao frio. É possível que a modulação da expressão de genes capazes de alterar a composição da parece celular (como genes que codificam proteínas CESA) em plantas de arroz de genótipos sensíveis ao frio resulte em melhor desempenho destas plantas sob baixas temperaturas, possibilitando a geração de cultivares tolerantes ao frio.

Observações adicionais, realizadas durante experimentos de exposição das plantas de arroz ao frio, indicaram maior crescimento das raízes nas plantas do genótipo tolerante. Análises do transcriptoma de raízes das duas linhagens estão sendo realizadas, e poderão fornecer informações sobre outros genes candidatos possivelmente envolvidos na tolerância a baixas temperaturas em plantas de arroz.

Considerando-se que variações nos níveis de expressão dos mRNAs não são necessariamente correspondentes às variações nos níveis das proteínas que eles codificam, também estão sendo realizadas análises para detecção e caracterização de proteínas com abundância aumentada ou diminuída em resposta a baixas temperaturas nos dois genótipos avaliados. As comparações quantitativas entre os genótipos de arroz estão sendo realizadas com o uso da técnica MudPIT (*Multidimensional Protein Identification Technology*).

Portanto, esta Tese contribuiu para o avanço do conhecimento na área de respostas das plantas a estresses abióticos, produziu conhecimento com potencial aplicação na agricultura, e indicou novos caminhos a serem seguidos em investigações científicas relacionadas ao tema.

ANEXOS

Anexo 1: artigo publicado, durante o doutorado, no periódico *Food and Energy Security*.

Food and Energy Security
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REVIEW

Avoiding damage and achieving cold tolerance in rice plants

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Keywords
Germination, low temperature, *Oryza sativa*, overexpressed genes, QTLs, reproductive stage, vegetative stage.

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Abstract

Rice is a staple food for half of the human population. Unlike other cereals such as wheat and barley, rice plants are susceptible to cold stress, which often results in decreased productivity, especially in regions where the *indica* subspecies is cultivated. Low temperatures can have negative impacts on rice plants during germination, vegetative growth, and reproductive stages. Considering the expected higher frequency of extreme temperature events in the near future, cold waves could even increase the negative impacts of low temperatures in rice production. Here, we review the efforts that have been made to achieve cold tolerance in rice through breeding, the major tools used for evaluating cold tolerance in rice plants, the discovery of quantitative trait loci (QTLs) and genes related to this tolerance, and the results obtained so far by genetic transformation of rice plants with potential cold-tolerance genes. Although much progress has been achieved, joint efforts from breeders and plant biologists could speed up the production of cold-tolerant rice plants, and some possible approaches are suggested.

Introduction

Rice is one of the most important crops, being a staple food for half of the world population. Abiotic stresses directly or indirectly affect the physiological status of rice and negatively alter its overall metabolism, often with impacts on grain yield. Among these, cold temperatures can be particularly harmful due to the tropical origin of the rice species. Low temperatures comprise a major climatic problem for rice growing in 25 countries, including Korea and Japan. Yield loss due to low temperatures is a major restriction on rice cultivation not only in areas at high latitudes or high altitudes but also in tropical countries such as the Philippines and Thailand (Kaneda and Beachell 1974).

In Australia, rice farmers have suffered losses ranging from 0.5 to 2.5 t/ha in 75% of the years due to low temperature during the reproductive stage (Singh et al. 2005). Given the total area under rice cultivation in Australia, on average the rice industry suffers a loss of $23.2 million per year due to cold damage, with the existing varieties (Farrell et al. 2001).

In northern Japan, severe yield reductions have been experienced in the Tohoku region, one of the coolest rice producing areas in the world. Yield in this region is

usually higher than the Japanese average, but there are large year-to-year fluctuations, with remarkable losses in years of lower temperatures. In 1993, the average rice yield in the Tohoku region was 44% lower than normal, resulting in a net loss of 0.65 million tons of rice grain. In one particular area within this region, yield reduction was up to 80% (Shimono et al. 2007).

In the mountainous regions of Korea, rice plants can suffer from low temperatures at any stage between germination and maturity, and in years of extreme low temperatures, all rice-growing areas of that country are susceptible to cold at the reproductive stage, with grain yields dropping by up to 26% (Lee 2001). In Brazil, the largest rice producer outside Asia, the rice crop may suffer damage from cold occurrence in Rio Grande do Sul (RS), the southernmost State, where more than 60% of the Brazilian rice grain is produced. In this region, as well as in Uruguay and Argentina, *indica* rice accounts for almost the totality of cultivated rice. Low temperature can reduce up to 25% of the final yield (Bierlen et al. 1997; Clayton and Neves 2011; Lima et al. 2012).

Cold temperature can be distinguished from freezing in terms of the range of temperatures that cause the related damages (Iba 2002). Many species of tropical or subtropical origin are injured or killed by nonfreezing low temperatures, and exhibit various symptoms of chilling injury such as chlorosis, necrosis, or growth retardation. In contrast, chilling-tolerant species are able to grow at such low temperatures (Sanghera et al. 2011). In this review, we will only focus on rice chilling (cold) tolerance, which represents temperatures ranging from 0 to 15°C.

The degree of injury in rice usually depends on time of occurrence (growth stage), severity of chilling, and low temperature duration (Li et al. 1981). Low temperature has the potential to affect growth and development of rice plants during any developmental stage, from germination to grain filling (Ye et al. 2009). However, Yoshida (1981) showed that sensitivity to cold varies between stages. According to his data, rice plants have a lower threshold temperature (10–13°C) for cold damage during the early stages of development (germination and vegetative), what makes them less sensitive to cold than during the reproductive stage, which has a higher threshold temperature for damage (18–20°C).

Also, because of the resulting spikelet sterility when cold occurs in the reproductive stage, this has the highest impact in the final yield of rice. Despite these differences among stages, cool weather and cold irrigation water can damage the rice plant during any developmental stage such as germination, seedling, vegetative, reproductive, and grain maturity (Majumder et al. 1989; Andaya and Mackill 2003a; Ji et al. 2008; Xu et al. 2008).

The symptoms of cold sensitivity and damage vary according to the growth stage of the rice plant (Yoshida 1981). In the germination stage, the most common symptoms of cold temperature damage are delayed and lower percentage of germination (da Cruz and Milach 2000). At the vegetative stage, chilling damage is expressed through yellowing of the leaves, lower stature, and decreased tillering of the rice plants. When cold coincides with the reproductive stage of the rice plant, sterility of the spikelets is the most common symptom of injury, but incomplete panicle exsertion and spikelet abortion may also occur (Satake and Hayase 1970). Spikelet sterility may result from pollen abortion due to cold during microsporogenesis, when pollen grains are being formed, at the booting stage (Mackill et al. 1996). Low temperatures can also result in abnormalities at anthesis, such as cessation of anther development, nonripening of pollen, nonemergence of anthers from spikelets, partial or no anther dehiscence, pollen grains remaining in anther loculi, little or no pollen shedding, and failure of pollen to germinate after reaching stigmas (Ito et al. 1970). During the grain filling stage, chilling temperature may cause delayed and incomplete grain maturation (Ye et al. 2009).

The young microspore stage is the most susceptible stage to cold injury in rice plants (Satake and Hayase 1970). The young microspore stage occurs approximately 10 to 12 days prior to heading (Satake and Hayase 1970; Heenan 1984). The threshold temperature that induces spikelet sterility at the young microspore stage differs between cultivars and depends on factors such as the duration of low temperature exposure and growing conditions (Satake 1969). The critical air temperature that induces cold damage depends on the cultivar (Satake 1976). The duration of low temperature is also an essential element determining the extent of cold damage. Spikelet sterility increases with the increase in duration of low temperature during the young microspore stage (Hayase et al. 1969; Ito 1976, 1978; Nishiyama 1978; Heenan 1984).

Breeding for Cold Tolerance in Rice

Rice is cultivated almost all over the world, from the Latitude 50°N to 40°S and from the sea level up to 3000 m of altitude (Juliano 1993), making cold tolerance a necessary trait in many of these regions. In order to cope with low temperature sensitivity, genetic breeding is the most straightforward approach that has been used. According to Singh et al. (2005), the potential benefits from developing a variety that could withstand cold temperature 1°C below the current minimum threshold temperature for the existing varieties in Australia are US$ 79 per ha per year.

Breeding demands genetic variability. Fortunately, the rice species (*Oryza sativa* L.) has wide adaptability to

cold, and cold-tolerant ecotypes are available for breeding. The cultivated species *O. sativa* L. has two subspecies: *indica* and *japonica*. The *indica* subspecies includes cultivars better adapted to tropical environments such as India, China, and Indonesia, while *japonica* cultivars are more adapted to temperate climates such as the ones in Japan, Korea, and Java (Takahashi 1984). Studies with large number of cultivars belonging to these two subspecies showed that *japonica* genotypes have higher degree of cold tolerance at the germination stage (Lee 2001; Mertz et al. 2009) as well as at the vegetative and reproductive stages (Li et al. 1981; Mackill and Lei 1997). da Cruz and Milach (2004) also concluded that *japonica* genotypes presented higher cold tolerance at the germination stage than *indica* genotypes, although they found variability for this trait within both subspecies. This agrees with previous reports of some *indica* genotypes from high-latitude regions that may present moderate level of chilling tolerance (Jennings et al. 1979). Some *javanica* cultivars are also reported to be tolerant to cold. *Javanica* rice is considered a tropical subpopulation or an ecotype of *japonica* (Sweeney and McCouch 2007; Brar and Singh 2011; Li et al., 2012), and cold-tolerance genes from the *javanica* cultivars Silewah, Lambayque 1, and Padi Labou Alumbis were introduced into several temperate *japonica* breeding lines in Japan (Saito et al. 2001).

Transferring cold tolerance from different sources to locally adapted cultivars requires the presence of the selective agent, in this case the low temperature. However, its abiotic nature makes it unpredictable under field conditions in terms of its intensity, duration, and timing, what limits field selection for cold tolerance in rice (da Cruz and Milach 2000). A good selection method to evaluate cold tolerance in segregating populations by the use of controlled air or water temperature is, therefore, essential.

Different methodologies to screen rice genotypes for cold reaction under controlled temperature conditions at different stages of development have been described (Table 1). However, the available space to grow large plant populations under controlled temperature environments is the main limiting factor. Growth under controlled conditions leads to gain in timing and precision of the stress, but loss in the amount of populations that will be possible to test. To deal with these limitations, some rice breeding programs have implemented selection with cold water under field conditions, allowing evaluation of many different populations and thousands of plants per population (Snell et al. 2008). Several experimental stations in Japan (Nishiyama 1996; Nagano 1998) and Korea (Lee 2001) have successfully used cold water to screen rice breeding material for cold tolerance.

The trait to be evaluated to indicate the cold tolerance/sensitivity of a rice genotype depends on the developmental

Table 1. Methods and traits evaluated in different stages of plant development for cold-tolerance selection in rice.

Growth stage	Methodology of screening	Evaluated trait	Reference
Germination	10, 15, 20, and 25°C for 3 to 30 days (depending on the temperature)	Germination rate (radicle protrusion)	Bertin et al. (1996)
	17°C for 7 days	Number of germinated seeds and rate of germination	Sthapit and Witcombe (1998)
	13°C to 15°C for 7 days	Percentage of germination	Lee (2001)
	15°C for 10 days	Coleoptile length	Hou et al. (2003)
	15°C for 6 days	Germination rate	Chen et al. (2006)
Vegetative	10°C for 3, 6, and 9 days	Survival rate 10 days after the end of the cold treatment	Bertin et al. (1996)
	Cool-air treatment at 12°/10°C (day/night) for 10 days at 3-leaf stage	Growth and discoloration	Lee (2001)
	9°C for 8, 14, 16, and 18 days	Visual scale (1–9)	Andaya and Mackill (2003b); Andaya and Tai (2006)
	4°C for 6 days in the dark	Survival rate after 14 days of recovery	Koseki et al. (2010)
	6 to 10°C for 7 days	Survival percentage	Qian et al. (2000)
Reproductive	12°C at the young microspore stage for 3–5 days	% of fertility	Koike et al. (1990)
	17°C for 7 days at the anthesis stage	% of fertility	da Cruz et al. (2006b)
	Cool water (20 cm depth) at 19.4°C from the primordial stage to the completion of heading	% of fertility	Kuroki et al. (2007)
	17°C at the booting stage for 10 days	% of fertility	Suh et al. (2010); Jena et al. (2012)

stage in which the cold temperature is being imposed (Table 1). In regions where low temperature stress imposes a very poor growth and a shorter season for the rice crop, breeding for cold tolerance is sometimes addressed through indirect traits such as selection for taller plants and shorter growth duration (Bardhan Roy et al. 1982).

Besides a precise selection method, the genetic basis of cold tolerance also affects the breeding for this trait. Correlations in cold tolerance among different growth stages for rice have been reported, and it was suggested that varieties with high germination and seedling vigor under low temperature conditions are also likely to be more tolerant to low temperature exposure at the booting and flowering stages (Ye et al. 2009). However, it is reasonable to consider that tolerant genotypes, evolved under strong selection pressure, may rely on diverse gene products on different growth stages to ensure cold tolerance. Tolerance to environmental stresses often comprises redundant components, ensuring species survival even if one or two tolerance genes are lost (Moffat et al. 2012; Nishiyama et al. 2013).

Therefore, it is possible that some tolerance genes are important in all stages and others are stage specific. So far, no master genes have been clearly identified for cold tolerance in rice, although they may exist. It would be interesting to compare quantitative trait loci (QTLs) identified for different developmental stages using the same varieties and populations to investigate these possibilities. In the case of *indica* rice, genotypes that are cold tolerant at the vegetative stage and cold sensitive at the reproductive stage are very common (da Cruz and Milach 2000).

At the germination stage, rice cold tolerance seems to have a complex inheritance. da Cruz et al. (2006a) determined the inheritance and heritability of cold tolerance at the germination stage in crosses between six rice genotypes. They showed that while both additive and nonadditive gene actions were involved, the nonadditive component was relatively more important for percentage of reduction in coleoptile length and coleoptile growth. Epistatic interaction (a nonadditive effect) was also found to be important for rice germination capacity under low temperature (Chen et al. 2006). Considering that high heritability estimates have been obtained for low-temperature germinability in rice (Sthapit and Witcombe 1998; da Cruz et al. 2006a) selection for cold-tolerant genotypes is likely to be successful. However, the high importance of the nonadditive effects suggests that selection should be applied in advanced generations of breeding programs (F4 or F5), when dominance effects have been decreased due to the increase in homozygosity of the individuals.

It has been suggested that tolerance to chilling injury at the seedling stage in rice is controlled by a single dominant gene (Nagamine 1991). However, segregation analysis performed with the same F2 population showed that there was no linkage relationship between chilling injury and low-temperature chlorosis, indicating that tolerance to both stresses is controlled by different loci (Nagamine 1991).

At the vegetative stage, two major genes, *Cts1* and *Cts2*, were reported as responsible for cold tolerance, which was estimated through leaf yellowing (Kwak et al. 1984) and withering (Nagamine 1991). Although these studies suggest that major genes are involved in seedling cold tolerance, more recent studies involving QTL analysis support the idea that cold tolerance at this stage is a complex trait involving multiple genes (Andaya and Tai 2006).

It has been reported that several major genes may be involved in the cold tolerance at the reproductive stage of cultivars released in northern Japan (Moon 1984; Matsuo et al. 1995; Nakamura et al. 2000).

Despite the difficulties and limitations of selection for cold tolerance and the complex genetic basis of this trait, several cold-tolerant cultivars have already been released in different countries, proving that it is possible to improve the trait with the genetic variability available within the rice species and with the methods of selection cited. In Italy, varieties with low-temperature tolerance are available among *japonica* genotypes. Natural selection under appropriate low temperature occurring at repeated cycles resulted in the progressive and cumulative development of cold-tolerant varieties (Russo 1994). Reliable screening methods have been developed in Korea using phytotrons, growth chambers, and low water temperatures, resulting in significantly improved cold-tolerant selections. With these approaches, 57% of the rice varieties released in Korea were highly tolerant to low temperatures by the year 2000 (Lee 2001). In Japan, cold water treatment under field conditions was used to select new temperate *japonica* breeding lines developed through the use of the cultivars Silewah, Lambayque 1, and Padi Labou Alumbis as the donors of tolerance (Abe et al. 1989; Glaszmann et al. 1990; Saito et al. 2001).

Each country developed its own strategy for breeding for cold tolerance. However, it is clear that the main advancements have been obtained within the *japonica* cultivars. Therefore, the challenge still remains to develop *indica* type cultivars with adequate cold tolerance for the high-latitude regions, such as southern Brazil, where those genotypes are more productive than *japonica* ones, have grain quality well suited to the target markets, but are prone to cold damage in one or more developmental stages (Bierlen et al. 1997). An apparent simple solution could be to cross *indica* genotypes with *japonica* ones, in order to transfer genes for cold tolerance from *japonica*. However, the differences between these two rice groups

make it difficult to maintain desirable *indica* characteristics, such as the cooking quality needed for consumer acceptance.

Tools to Evaluate Cold Stress in Rice

Most of the physiological analyses to study tolerance or sensibility of rice to low temperatures have been made in two stages of development: seedling and booting. In both of them cold temperature has harmful effects on crop productivity, as in the first one the number of established plants is affected and in the booting stage pollen sterility can be induced by cold, decreasing the final number of grains. A large array of methodologies, as different cold intensities and periods of exposure, has been applied to evaluate damage and tolerance in these developmental stages (Fig. 1). Only a few of them are nondestructive.

At the seedling stage, visual characteristics, as wilting and yellowing of leaves may be related to cold stress (Su et al. 2010; Song et al. 2011; Yang et al. 2012). Tolerance to cold has been evaluated by scoring chilling injury and low-temperature chlorosis in seedlings. The degree of leaf withering was used as a criterion for scoring chilling injury (Nagamine 1991).

Cold tolerance at the seedling stage has also been evaluated by determining survival percentages, because susceptible seedlings have problems in maintaining normal metabolic rates under cold and eventually die (Morsy et al. 2007). In the past years, many studies have utilized survival data to evaluate resistance of transgenic plants to cold stress (Xiong and Yang 2003; Ito et al. 2006; Liu et al. 2007; Kawakami et al. 2008; Hu et al. 2008; Huang et al. 2009; Ma et al. 2009; Su et al. 2010; Chen et al. 2011; Li et al. 2011; Song et al. 2011; Xu et al. 2011; Zhang et al. 2011; Huang et al. 2012; Yang et al. 2012). However, an on/off trait such as survival is not ideal for use in the field, where cold tolerance is a continuous variable trait, and would also not be a useful trait for QTL selection.

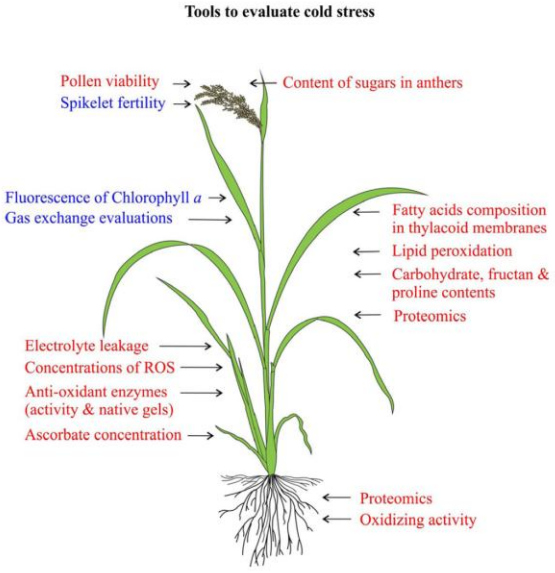

Figure 1. Tools to evaluate cold stress in rice. Methods used to access cold damage and tolerance in rice plants are shown in relation to the plant organs analyzed (roots, leaves, and panicles). Destructive methods are shown in red and nondestructive in blue. Plant survival and biomass (roots and shoots) are not represented in the figure, although often used as tolerance criteria.

Plant growth is often negatively influenced by cold stress (Sanghera et al. 2011), and quantitative analyses, as shoot and root biomass, are also used in the evaluation of contrasting genotypes (Aghaee et al. 2011) or transgenic plants (Tian et al. 2011) at any developmental stage. However, these analyses have the disadvantage of being destructive and time consuming, and are not adequate to breeding programs where a large number of lines needs to be evaluated.

One nondestructive method that allows quantifying the degree of tolerance to low temperature at the vegetative level is the determination of chlorophyll *a* fluorescence. Little changes in the structure and functioning of the photosynthetic apparatus, specifically at photosystem II (PS II), may be easily detected before irreversible structural damage becomes apparent. The technique is considered highly sensitive in the investigation of photosynthetic parameters (Lazár and Ilík 1997).

Analyses of fluorescence parameters have demonstrated structural and functional alterations in the photosynthetic apparatus of different plant genotypes or transgenic seedlings submitted to low temperature treatments (Saijo et al. 2000; Ji et al. 2003; Hirotsu et al. 2004; Lee et al. 2004a,b; Wang and Guo 2005; Kim et al. 2009; Lee et al. 2009a,b; Bonnecarrère et al. 2011; Saad et al. 2012). In most cases, the ratio F_V/F_M is used to evaluate cold sensibility or tolerance, since it indicates the maximum photochemical efficiency of PS II. A new and more responsive parameter of fluorescence, photosynthetic performance index (PI) has been used recently in evaluations of cold tolerance in rice plants (Saad et al. 2012). This parameter includes components related to capture, absorption and use of luminous energy (Stirbet and Govindjee 2011), and complements the evaluations of cold tolerance because it is closely related to the final photosynthetic activity of the plant and, therefore, to survival on stress conditions.

Quantifications of gas exchange and, in particular, of photosynthetic rate (Wang and Guo 2005), CO_2 assimilation (Saad et al. 2012), and stomatal conductance (Saad

et al. 2012) have also been used to understand the photosynthetic metabolism in different genotypes or transgenic plants of rice submitted to cold stress. The combination of gas exchange analyses and chlorophyll fluorescence determination allows the detailed understanding of the photosynthetic process (Saad et al. 2012), because cold stress impacts on luminous and carboxylation reactions of photosynthesis.

Cold stress reduces significantly the concentration of chlorophyll in susceptible rice genotypes (Dai et al. 1990; Aghaee et al. 2011). Chlorophyll content was used as a tool to evaluate the degree of cold tolerance of transgenic plants (Tian et al. 2011), to monitor plant recovery after stress (Kuk et al. 2003), and to compare chilling tolerance between distinct hybrid lines during grain filling (Wang et al. 2006).

Reactive oxygen species (ROS) are generated during cold stress in rice plants (Saruyama and Tanida 1995; Song et al. 2011; Theocharis et al. 2012; Yang et al. 2012) and are capable of causing severe damage to various cellular components such as membrane lipids, structural proteins, and enzymes. However, ROS acts as signaling molecules that modulate the expression of various genes, including those encoding antioxidant enzymes and modulators of H_2O_2 production, leading to plant stress acclimation (Theocharis et al. 2012). The antioxidant system was used as a tool to evaluate tolerance to low temperature in an investigation with transgenic rice seedlings overexpressing *OsNAC5* or suppression of *OsNAC5* expression by RNAi (Song et al. 2011). In addition, Lee et al. (2009a,b) produced genotypes of transgenic rice overexpressing *Sodc1* (encoding Cu/Zn superoxide dismutase) to obtain plants with improved tolerance to oxidative and cold stress. The stability of antioxidant enzymes and effective response of the system responsible for enzyme synthesis under stress is critical for cold tolerance. Rice plants exposed to cold during vegetative stages were evaluated in relation to the enzymes from the antioxidant defense system (Takesawa et al. 2002; Kuk et al. 2003; Morsy et al. 2007; Yang et al. 2012) and to the nonenzymatic antioxidant ascorbate (Ji et al. 2003). Activity of antioxidant enzymes has also been evaluated via native gel electrophoresis (Kuk et al. 2003; Lee et al. 2009a,b) which allows identifying specific enzyme isoforms.

Variations in the capacity to tolerate low temperatures have been related to fatty acid composition of thylakoid membranes in flag leaves during grain filling (Wang et al. 2006). Higher concentrations of fructan oligomers with different glycosidic bonds in transgenic plants were correlated to cold tolerance in rice seedlings (Kawakami et al. 2008). Moreover, proline concentration is important in the osmotic adjustment in plants and serves as an osmoprotectant in stress tolerance at low temperature. It is a compatible solute that maintains membrane integrity avoiding cellular dehydration caused by osmotic pressure (Huang et al. 2012). Proline content has been related to cold tolerance in transgenic plants (Ito et al. 2006; Zhang et al. 2011; Huang et al. 2012), as well as the contents of soluble sugars (Huang et al. 2012), which also contribute to osmotic adjustment. Furthermore, carbohydrate supply is also important as source of energy to drive reproductive development, especially considering the fact that photosynthesis is affected by cold as well (Wang and Guo 2005; Hirotsu et al. 2005).

Likewise, evaluation of damage to membrane lipids is generally a fundamental aspect of cold stress tolerance/sensibility assessment. These analyses include lipid peroxidation (Morsy et al. 2007; Song et al. 2011; Zhang et al. 2011; Yang et al. 2012) and electrolyte leakage in seedlings (Lee et al. 2004a,b; Huang et al. 2009, 2012; Zhang et al. 2011; Song et al. 2011) and in flag leaves (Ji et al. 2003), which is used to diagnose membrane damage indirectly. Total conductivity in the solutions containing plant materials is used as an indicator of electrolyte leakage in seedlings (Lee et al. 2004a,b; Huang et al. 2009). It has been pointed out that the content of unsaturated fatty acids in the plasma membrane can be related to its stability during cold temperature, preventing electrolyte leakage and, therefore, cell damage and death. Differences in the content of saturated and unsaturated fatty acids were observed among plants at the vegetative stage from tolerant and sensitive genotypes only after cold exposure (da Cruz et al. 2010). The same authors found that among the most abundant types of fatty acids, levels of linolenic acid increased in the tolerant genotypes and of palmitic acid decreased in these same genotypes, confirming an important role for the unsaturated fatty acids in maintaining membrane stability during cold stress.

In addition to the tools mentioned above, proteome analysis has provided further understanding of the molecular adaptation mechanisms developed by rice seedlings to cope with cold stress (Cui et al. 2005; Yan et al. 2006; Hashimoto and Komatsu 2007; Lee et al. 2007, 2009a,b). Stress-induced proteins are in general attributed to a wide metabolic pathway affected by stress in plants, and the list of stress proteins is far from being complete (Cui et al. 2005). Two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry were adopted to investigate the protein expression patterns of rice in response to cold stress (Lee et al. 2009a,b).

The methods described so far are mostly related to cold stress at the vegetative stage of rice plants. At the reproductive stage, mainly at anthesis, exposure of susceptible plants to cold leads to accumulation of sucrose and hexoses in anthers, starch exhaustion at the pollen grain and,

consequently, flower sterility. This happens as a function of the reduction in activity of acid invertases bound to the cellular walls of the pollen grain, responsible for sugar hydrolysis. In tolerant plants, sugar accumulation at the anthers is not observed and, consequently, there is no sterility of the pollen grain (Oliver et al. 2007). In this context, techniques of coloration for the evaluation of pollen grain viability, determination of sugars content, and invertases activity at the anthers may be used for evaluation of plant responses to cold during reproductive stage (Oliver et al. 2005). Additionally, tolerance may be evaluated based on spikelet fertility (Sato et al. 2011). Transgenic rice lines overexpressing *OsAPXa* (higher Ascorbate Peroxidase activity) were produced to obtain plants with increased ROS scavenger activity, and those plants also showed increased spikelet fertility under cold stress (Sato et al. 2011).

Therefore, analyses focused on physiological and biochemical responses to cold stress have contributed significantly with the understanding of rice pant responses to cold stress and with the identification of genotypes or transgenic rice plants tolerant to low temperature.

QTLs and Cold Tolerance in Rice

QTLs related to tolerance to low temperatures in rice have been identified by the use of restriction fragment length polymorphisms (RFLPs; Li et al. 1997; Harushima et al. 1998; Takeuchi et al. 2001) and microsatellite/simple sequence repeats (SSR) molecular markers (McCouch et al. 2002; Andaya and Mackill 2003a,b; Fujino et al. 2004; Kuroki et al. 2007; Lou et al. 2007; Suh et al. 2010). The technical efficiency and multiplex potential of SSR makes them suitable to high-throughput mapping and marker-assisted breeding. SSR markers are codominant, multiallelic, and can be efficiently used in *indica* and *japonica* germplasm (McCouch et al. 2002). Most recently, technical advances have allowed the identification of the genes (and their functions) responsible for rice QTLs, such as a combination of PCR-based screening, development of near-isogenic lines and searches for hits in EST databases (Saito et al. 2004), fine mapping based on microsatellite markers including markers identified from publicly available genomic sequences (Andaya and Tai 2006) and map-based cloning (Fujino et al. 2008).

It has been suggested that QTLs related to cold tolerance in the germination stage are independent from QTLs conferring tolerance at the vegetative and reproductive phase (Saito et al. 2001; Andaya and Mackill 2003a,b; Fujino et al. 2004), indicating that cold tolerance may be developmentally regulated and growth stage specific. Details about QTLs explaining at least 15% of the variability related to rice cold tolerance reported by different groups are shown in Table 2. These loci probably have large potential for successful application in rice genetic breeding aiming at cold tolerance in different developmental stages.

Most recently, the complete and high-quality sequence of the rice genome has provided a genome-wide SNP resource (IRGSP 2005) that leads to high-quality and reliable markers. Until the present moment, however, only few studies have been able to link cold tolerance to SNPs in rice (Koseki et al. 2010; Shirasawa et al. 2012), and only one (Kim et al. 2011) could effectively locate a SNP which resulted in amino acid substitution (I99V – Ile to Val) leading to reduced enzyme activity (glutathione transferase isoenzyme OsGSTZ2). According to the authors, this functional difference in the OsGSTZ2 isoform could explain the differential response observed between cold-tolerant and cold-sensitive rice cultivars.

QTLs related to cold tolerance at the germination stage

Low temperature stress may affect rice seed germination, avoiding development to the seedling stage and eventually leading to the heterogeneous maturation of the culture (Andaya and Mackill 2003b). A wide range of phenotypic variation of low-temperature germinability is found in rice cultivars. QTLs analyses for low-temperature germinability revealed that multiple genes control this trait. The ones with larger contribution to cold tolerance are shown in Table 2.

Three QTLs controlling low-temperature germinability using backcross inbred lines (BILs) derived from a cross between a vigorous and a weak low-temperature germinability cultivars (Italica Livorno and Hayamasari, respectively; Fujino et al. 2004; Table 2). A major QTL for low-temperature germinability on chromosome 3, *qLTG3-1*, explained 35% of the total phenotypic variation in the mapping population. High-resolution mapping placed *qLTG3-1* in a 4.8-kb region. Only one gene, Os03g0103300, was predicted to this region, and sequence analysis showed that an unknown function protein containing two known conserved domains, GRP (glycine-rich cell wall protein) and Tryp alpha amyl of the protease inhibitor/seed storage/LTP family, is encoded by this gene (Fujino et al. 2008). Using a genome-wide expression profiling analysis, Fujino and Matsuda (2010) identified 29 *qLTG3-1*-dependent genes with diverse functions. Several genes involved in defense responses were upregulated by *qLTG3-1*, indicating that *qLTG3-1* expression is required for the expression of defense response genes in low-temperature germinability in rice (Fujino and Matsuda 2010). On the other hand, Fujino et al. (2008) suggest that *qLTG3-1* may not be involved in the response to low

Table 2. QTLs related to cold tolerance in rice reported to account for at least 15% of the phenotypic variation.

Growth stage	Cross	Chromosomal location (QTL name)	Cold-tolerance variation explained (%)	Putative gene (encoded protein)	Evaluated trait	Reference
Germination	*japonica* Nipponbare × *indica* Kasalath	4 (*qLTG-4-1*)	15.0		Germination rate at 15°C	Miura et al. (2001)
	Temperate *japonica* varieties Italica	3 (*qLTG-3-1*)	35.1	Os03g0103300 (unknown function)	Germination rate at 15°C	Fujino et al. (2004)
	Livorno × Hayamasari	3 (*qLTG-3-2*)	17.4			
Vegetative	Cold-tolerant *japonica* (M-202)	4 (*qCTS4-1*)	20.8		General cold tolerance	Andaya and Mackill (2003b)
	× Cold-sensitive *indica* (IR50)	6 (*qCTS6-1*)	15.3		General cold tolerance	
		12 (*qCTS12a*)[1]	40.6		Cold-induced wilting tolerance	
		12 (*qCTS12b*)[1]	41.7		Cold-induced necrosis tolerance	
		qCTS12[1]		OsGSTZ1 (glutathione S-transferase), OsGSTZ2 (maleylacetoacetate isomerase)		Andaya and Tai (2006)
	japonica Lemont × *indica* Teqing	11 (*qSCT-11*)	29.8		Survival rate after 13 days at 10°C	Zhang et al. (2005)
	Cold-tolerant *japonica* (AAV002863) × Cold-sensitive *indica* (Zhenshan97B)	2 (*qCTS-2*)	27.4		Survival rate after 7 days at 6-10°C	Lou et al. (2007)
	japonica Asominori × *indica* IR24	1 (*qCST-1*)	24.5		Survival rate after 7 days at 6°C	Jiang et al. (2008)
	Cold-tolerant wild rice, W1943 (*Oryza rufipogon*) × Cold-sensitive *indica* Guang-lu-ai 4 (GLA4)	11 (*qCtss11*)	40.0	Os11g0615900 (NB-ARC domain), Os11g0615600	Survival rate after 6 days at 4°C	Koseki et al. (2010)
Reproductive	*japonica* 02428 × *indica* 3037	1 (*Ste1*)	32.1		Spikelet fertility	Li et al. (1997)
		1 (*Ste2*)	19.4			
		12 (*Ste3*)	16.9			
	Cold-tolerant Norin-PL8 × Cold-sensitive Kirara397	4 (*Ctb-1*)	-	Ctb1 (F-box protein)	Anther length	Saito et al. (2010)
	Cold-tolerant *japonica* Koshihikari	7 (*qCT-7*)	22.1		Spikelet fertility	Takeuchi et al. (2001)
	× Cold-sensitive *japonica* Akihikari	1 (*qCL-1*)	31.1		Culm length (cm)	
		3 (*qHD-3-2*)	15.5		Heading date (day)	
		6 (*qHD-6*)	50.5			
	japonica M-202 × *indica* IR50	2 (*qCTB2a*)	16.8		Spikelet fertility	Andaya and Mackill (2003a)
		3 (*qCTB3*)	16.5			
	japonica varieties Kunmingxiaobaigu × Towada	7 (*qRCT7*)	20.6		Spikelet fertility	Dai et al. (2004)
	Cold-tolerant Hokkai-PL9 × Cold-sensitive Hokkai287	8 (*qCTB8*)	26.6	Monodehydroascorbate reductase (MDAR)	Seed fertility	Kuroki et al. (2007)

Table 2. Continued

Growth stage	Cross	Chromosomal location (QTL name)	Cold-tolerance variation explained (%)	Putative gene (encoded protein)	Evaluated trait	Reference
	Cold-tolerant *japonica* Kunmingxiaobaigu (KMXBG) × Cold-sensitive *japonica* Towada	10 (*qCTB-10-2*)	15.0		Spikelet fertility	Xu et al. (2008)
	Cold-tolerant Lijiangheigu × Cold-sensitive Reiziq	10 (*qLTSPKST10.1*)	20.5		Spikelet fertility	Ye et al. (2010)
	Cold-tolerant ZL1929-4 × Cold-sensitive *japonica* Towada	7 (*qCTB7*)	21.0	Os07g0576100 and Os07g0576500 (indole-3-acetic acid-amido synthetases), Os07g0575800 and Os07g0577300 (glucan endo-1, 3-beta-glucosidase), Os07g0577400 (ubiquitin-conjugating enzyme E2)	13 cold tolerance-related traits	Zhou et al. (2010)
	japonica TR22183 × *indica* Dasanbyeo	2 (*QTL 2.1*)	16.7		Spikelet fertility	Jiang et al. (2011)
		8 (*QTL 8.1*)	24.8			
		10 (*QTL 10.1*)	22.9			
	Cold-tolerant Ukei 840 × Hitomebore	3 (*qLTB3*)	24.4	Os03g0790700 (putative aldehyde oxidase)	Seed fertility	Shirasawa et al. (2012)

[1]QTLs *qCTS12a* and *qCTS12b* were shown to be the same and named *qCTS12* by Andaya and Tai (2006).

temperature, but rather in seed germination itself, under different kinds of stress.

QTLs related to cold tolerance at the vegetative stage

Cold tolerance at the seedling stage is an important trait affecting stable rice production. Different groups have located chromosome regions responsible for cold tolerance during the vegetative stage of rice growth (Table 2).

Recombinant inbred lines (RILs) derived from a cross between a cold-tolerant temperate *japonica* cultivar (M-202) and a cold-sensitive *indica* cultivar (IR50) were evaluated using microsatellite markers (Andaya and Mackill 2003b). Fifteen QTLs were identified, mostly with small effects. The QTL identified on chromosome 4, designated as *qCTS4-1*, accounted for about 21% of the phenotypic variation for general cold tolerance. In this same category, a QTL on chromosome 6, *qCTS6-1*, explained 15% of the variation. A major QTL was identified on chromosome 12, named *qCTS12a*, which accounted for 41% of the phenotypic variation in tolerance to cold-induced wilting tolerance. Another QTL on chromosome 12, *qCTS12b*, explained 42% of the cold-induced necrosis tolerance (Table 2). A further study by Andaya and Tai (2006) raised the possibility that these two QTLs on chromosome 12 are in fact the same, naming them as *qCTS12*. Although the injuries from wilting and necrosis appeared to be distinct, there was strong correlation between the two traits. Moreover, both QTLs mapped to the same position on chromosome 12. Fine mapping of this locus was performed by saturating the short arm of chromosome 12 with microsatellite markers. Ten open reading frames were identified in this region, and two of them (*OsGSTZ1* and *OsGSTZ2* – two zeta class glutathione *S*-transferases) were considered the best candidates to correspond to *qCTS12* (Andaya and Tai 2006; Table 2).

Recently, Kim et al. (2011) suggested that cold sensitivity in rice is strongly correlated with a naturally occurring Ile99Val mutation in the multifunctional glutathione transferase isoenzyme GSTZ2.

A mapping population of F2 plants derived from a cold-tolerant wild rice, W1943 (*Oryza rufipogon*), and a sensitive *indica* cultivar, Guang-lu-ai 4, was used to identify QTLs associated with cold tolerance at the seedling stage. The work was based on phenotypic evaluation and development of SNP markers. Three QTLs were detected on chromosomes 3, 10, and 11. A major locus, *qCtss11*, was located on the long arm of chromosome 11, explaining about 40% of the phenotypic variation (Koseki et al. 2010; Table 2). Additional markers were developed to enable fine mapping of the *qCtss11* region, where six putative open reading frames were identified. Two of those candidate genes (Os11g0615600 and Os11g0615900) were considered the best candidates for *qCtss11*. The protein encoded by the Os11g0615900 gene was characterized as containing a NB-ARC domain (nucleotide-binding adaptor shared by APAF-1, R protein and CED-4). A comparison of the genetic location of *qCtss11* and *qCTS11-2* (identified by Andaya and Mackill 2003b) suggests that these loci are likely coincident and therefore possibly allelic.

QTLs related to cold tolerance at the reproductive stage

Rice cold tolerance at the booting stage is a quantitative trait controlled by multiple genes. Identification of QTLs is based on phenotypical evaluation of distinct genotypes, an approach that is not prone to allow the simultaneous identification of multiple genes. To be able to combine a large number of genes in one genotype, it has been considered that the most effective approach is to identify single genes, based on phenotype analyses, and then combine the genes by marker-assisted selection (Shirasawa et al. 2012). Although the genetic mechanisms responsible for low temperature tolerance during the booting stage are not well known, some QTLs have been successfully identified (Table 2).

Saito et al. (2001) detected two closely linked QTLs (*Ctb-1* and *Ctb-2*) for cold tolerance and suggested their association with anther length. Later, Saito et al. (2004) reported the physical mapping of *Ctb1* and confirmed the association of *Ctb1* with anther length. According to the authors, seven open reading frames (ORFs) were found within the 56-kb region where *Ctb1* was located: two receptor-like protein kinases, three ubiquitin-proteasome pathway-associated proteins (two of which encoded F-box proteins), a protein with an OTU domain and an unknown protein. Recently, Saito et al. (2010) indicated that an F-box protein gene confers the cold-tolerance trait and that cold tolerance is associated with larger anther length. Moreover, the F-box protein interacts with a subunit of the E3 ubiquitin ligase, Skp1, suggesting that an ubiquitin-proteasome pathway is involved in cold tolerance at the booting stage (Saito et al. 2010). The amount of pollen available for fertilization is directly related to anther length, and cold affects pollen grain maturation, reducing fertility as a consequence. Cold-tolerant varieties hold larger anthers and, consequently, they produce a larger number of pollen grains than susceptible varieties. Therefore, a strong correlation was suggested to exist between cold-tolerance QTLs and anther length QTLs, being the pollen amount an important component of the tolerance mechanism (Saito et al. 2001).

Takeuchi et al. (2001) had constructed a linkage map with RFLP and RAPD molecular markers for detection of QTLs controlling cold tolerance, and a total of eight QTLs were found. Among them, the ones with higher contributions were associated with general cold tolerance (*qCT-7*, on chromosome 7), and cold tolerance related to culm length (*qCL-1*, on chromosome 1) and heading date (*qHD-3-2*, on chromosome 3 and *qHD-6*, on chromosome 6), explaining 22.1%, 31.1%, 15.5%, and 50.5% of the respective phenotypic variation (Table 2).

A QTL for booting stage cold tolerance was detected on chromosome 8 (*qCTB8*) through the analysis of F2, F3, and F7 populations, using SSR markers. This QTL explains 26.6% of the phenotypic variance (Kuroki et al. 2007; Table 2). About 30 open reading frames were identified at the *qCTB8* region. One of them encodes monodehydroascorbate reductase (MDAR), which was shown to be upregulated by cold treatment in rice anthers during the young microspore stage.

A QTL for cold tolerance at the booting stage was also reported on chromosome 7, named *qCTB7*. The QTL explained 9 and 21% of the phenotypic variances in the F2 and F3 generations, respectively. Twelve putative cold-tolerance genes from this QTL region were identified by fine mapping and candidate gene cloning (Zhou et al. 2010; Table 2). On the basis of genetical and physical mapping, the authors suggested that two other QTLs previously identified in the same location (*qRCT7* – Dai et al. 2004; and *qCT-7* – Takeuchi et al. 2001) may correspond to the same locus as *qCTB7*. Although identified in diverse genetic backgrounds and environments, the three QTLs explained similar percentages of the phenotypic variance, ranging from 20.6% to 22.1%.

A single QTL for booting stage cold tolerance was reported on the long arm of chromosome 3. This QTL was named *qLTB3* and explained 24.4% of the phenotypic variance (Shirasawa et al. 2012; Table 2). Seven SNP markers were identified in five genes within the *qLTB3* region, all of them causing amino acid substitutions. One of those SNPs (in the Os03g0790700 gene) caused a mutation in a conserved amino acid and was considered the strongest candidate for conferring cold tolerance. The Os03g0790700 gene encodes a protein similar to the *Arabidopsis* AAO2 aldehyde oxidase, which is believed to function in ABA biosynthesis (Koiwai et al. 2004; Seo et al. 2004).

Gene Discovery and Transgenic Cold-Tolerant Rice Plants

Conventional breeding methods limit cold tolerance improvement by the available genetic diversity within existing germplasm collections, by the complexity of stress tolerance traits and lack of efficient selection criteria. It is important, therefore, to look for alternative strategies to develop cold stress-tolerant crops (Sanghera et al. 2011). Gene expression analysis has contributed to increase our knowledge about the physiological mechanisms related to abiotic stresses in plants, including low temperature tolerance (Gao et al. 2008). Several rice genes have been isolated and characterized as responsive to chilling stress. Most appear to be involved in cold stress tolerance and encode proteins which act as enzymes required for biosynthesis of osmoprotectants (MaNeil et al. 1999), signaling components (Saijo et al. 2000; Wen et al. 2002; Xiong and Yang 2003; Chen et al. 2011; Xie et al. 2012), chaperones (Lee et al. 2005; Mittal et al. 2009), and transcription factors (Huang et al. 2005; Ohnishi et al. 2005; Nakashima et al. 2007; Chaikam and Karlson 2008; Huang et al. 2008; Kim et al. 2009; Ye et al. 2009; Hossain et al. 2010; Tao et al. 2011), especially from the CBF/DREB1 family (C-repeat binding factor/dehydration-responsive element binding; Chen et al. 2003; Dubouzet et al. 2003; Lee et al. 2004a,b; Ito et al. 2006; Wang et al. 2008; Zhang et al. 2009). Screening of T-DNA-tagged rice plants submitted to cold stress has also been used to identify cold stress-responsive genes. Using this approach coupled to inverse PCR, Lee et al. (2004a,b) and Koh et al. (2007) were able to detect and characterize at the molecular level the following genes: *OsRLK1* (a putative leucine rich repeat-type receptor-like protein kinase), *OsDMKT1* (a putative demethylmenaquinone methyltransferase), and *OsGSK1* (glycogen synthase kinase3-like 1). *OsGSK1*, an ortholog of the *Arabidopsis brassinosteroid insensitive 2* (BIN2), promoted cold tolerance in rice plants when knocked out, suggesting that *OsGSK1* might function as a negative regulator of brassinosteroid (BR)-signaling (Koh et al. 2007). Analyses of these responsive genes have shown that numerous physiological and molecular changes occur during cold acclimation and that several metabolic pathways are affected by low temperature stress, indicating that cold tolerance is more complex than perceived and a better understanding of the basic mechanisms is still needed.

Transgenic technology looks promising as a tool to improve cold tolerance in rice plants by introduction or disruption of specific DNA sequences. Advances in recombinant DNA technology and the use of efficient gene transfer protocols have resulted in efficient transformation and generation of transgenic lines (Sanghera et al. 2011).

Overexpression of transcription factors

Several low-temperature stress-inducible genes have been overexpressed and produced transgenic rice plants with

stress-tolerant phenotypes (Table 3). Most of these genes (13 of 24) encode transcription factors responsible for transcriptional regulation of different stress-inducible genes. Some of these regulatory pathways are not cold specific and are also involved in drought and high-salinity stress responses (Seki et al. 2003; Sanghera et al. 2011).

The CBF/DREB1 genes represent one of the most significant discoveries in the field of low temperature adaptation and signal transduction (Sanghera et al. 2011). Transgenic rice plants overexpressing *OsDREB1* or *AtDREB1* genes showed improved tolerance to low temperature, drought, and high-salt stresses, and elevated contents of osmoprotectants such as free proline and various soluble sugars. However, the transgenic plants show growth retardation under normal growth conditions (Ito et al. 2006). Overexpression of *OsDREB1F* gene also led to transgenic rice plants with enhanced stress tolerance, but no growth retardation effect was found under normal growth conditions (Wang et al. 2008). Another rice *DREB1* gene (*OsDREB1D*) was overexpressed in *Arabidopsis* plants, resulting in transgenic plants in which the degree of cold tolerance was correlated with the level of *OsDREB1D* expression (Zhang et al. 2009). *OsDREB1D* and *OsDREB1A* genes may be redundant in function (Zhang et al. 2009), since the level of cold tolerance that can be achieved by independent overexpression of each one of them is very similar, along with the fact that both overexpressed genes resulted in constitutive expression of *COR15A*, *RD29A*, and *KIN1*, three genes involved in plant cold tolerance (Dubouzet et al. 2003). Rice plants overexpressing the *OsDREB1D* gene have not been generated so far, but would probably have the same tolerant phenotype of rice plants overexpressing other *OsDREB1* genes. Recently, Xu et al. (2011) overexpressed a maize CBF gene (*ZmCBF3*) in rice plants, and the resulting transgenics showed growth retardation only at the seedling stage, with no yield penalty under field conditions. As expected, transgenic plants were cold tolerant (Xu et al. 2011).

Transcription factors belonging to several other families have been overexpressed aiming to generate cold-tolerant rice plants. Overexpression of *OsCOIN* (*O. sativa* cold inducible), a transcription factor belonging to a family of bZIP zinc finger proteins, enhanced tolerance to cold, drought, and salt treatment in transgenic rice plants, along with higher proline levels after cold treatment, compared to WT plants (Liu et al. 2007). Overexpression of *OsZFP245*, a C_2H_2-type zinc finger protein, also led to rice plants with enhanced proline levels and high tolerance to cold and drought stresses. Transgenic plants also showed higher superoxide dismutase (SOD) and peroxidase (POD) activities than WT plants under low temperature conditions, suggesting a better modulation of oxidative stress responses (Huang et al. 2009). Recently, overexpression of *OsZFP182*, a TFIIIA-type zinc finger protein, resulted in cold tolerance and accumulation of compatible osmolytes, such as free proline and soluble sugars. The authors also suggested that OsZFP182 functions as the upstream regulator of *OsDREB1A* and *OsDREB1B*, under both normal and drought conditions (Huang et al. 2012). Another zinc finger protein (A20/AN1-type) from the halophytic grass, *Aeluropus littoralis* (*AlSAP* – Stress-Associated Protein), was previously characterized as stress associated (Saad et al. 2010). Rice plants overexpressing this gene show enhanced tolerance to various stress conditions (Saad et al. 2012). All WT plants died and did not produce seeds under stress conditions, while transgenic plants yielded 60% of the seed set under normal conditions. According to the authors, *AlSAP* overexpression probably generates stress tolerance in rice plants through maintenance of the photosynthetic apparatus integrity (Saad et al. 2012).

Two groups overexpressed plant genes belonging to the NAC (NAM, ATAF, and CUC) family of transcription factors in rice. More than 50% of the transgenic rice plants overexpressing the *SNAC2* gene remained vigorous when almost all WT plants died after severe cold stress (Hu et al. 2008), probably because of the higher cell membrane stability of transgenic plants. Song et al. (2011) overexpressed the *OsNAC5* gene and also found enhanced stress tolerance. Accumulation of proline and soluble sugars was positively correlated with *OsNAC5* expression levels and transgenic plants showed reduced accumulation of malondialdehyde (MDA) and H_2O_2, suggesting that overexpression of *OsNAC5* renders plants less susceptible to oxidative damage.

MYB transcription factors play central roles in plant responses to abiotic stresses. Transgenic rice plants overexpressing *OsMYB3R-2* exhibited higher transcript levels of several G2/M phase-specific genes, including cyclin genes, than WT plants in response to cold treatment (Ma et al. 2009). Together with increased levels of cellular free proline, the cold resistance mechanism in *OsMYB3R-2*-overexpressing rice plants must be mediated by cell cycle regulation. On the other hand, transgenic rice constitutively expressing the *OsMYBS3* gene tolerated 4°C for at least 1 week and the degree of cold tolerance correlated with the *OsMYBS3* expression level. Interestingly, *OsMYBS3* overexpression repressed the well-known DREB1/CBF-dependent cold signaling pathway in rice, which mediates the fast cold shock response. The authors showed that *OsMYBS3* responds slowly to cold stress, which suggests that this gene acts in long-term cold adaptation in rice (Su et al. 2010). In this way, it seems that distinct pathways act sequentially and complementarily for adapting to short- and long-term cold stress in rice. It would be interesting to test whether these two systems

Table 3. Available reports on generation of cold-tolerant rice plants by genetic transformation.

Overexpressed gene(s)/ promoter	Gene(s) function	Cold treatment/ Plant age or stage	Characteristics of transgenic plants	Deleterious effects of transgenic plants	Reference
OsDREB1 and *AtDREB1* genes/*CaMV 35S* and maize *Ubiquitin*	Transcription factors	2°C for 4 days/ 17 days old	Higher survival rates (up to 90%) than the WT plants under low temperature, drought, and high salt (only 16% of the WT plants survived)/Elevated contents of osmoprotectants such as free proline and various soluble sugars	Growth retardation under normal growth conditions/Dwarf phenotype even at the reproduction stage in some transgenic lines	Ito et al. (2006)
OsDREB1F/CaMV 35S	Transcription factor	10°C for 7 days/ 16 days old	Enhanced tolerance to low temperature, drought, and high salt[1]	–	Wang et al. (2008)
ZmCBF3/maize *Ubiquitin*	Transcription factor	4°C for 1 day/ 21 days old	Transgenic lines showed a remarkable increase in survival rates (ranging from 67% to 93%) when compared with WT plants (32%) under cold treatment/Transgenic lines also showed increased tolerance to drought and salt stresses/Reduced contents of malondialdehyde and relative conductivity under stress conditions	Growth retardation at the seedling stage under normal growth conditions/The yield of transgenic rice under field conditions was not affected	Xu et al. (2011)
OsCOIN/maize *Ubiquitin*	Transcription factor	4°C for up to 3.5 days/14 days old	Enhanced cold tolerance (up to 76% survival, compared to 52% survival in WT plants), salt, and drought treatment/Higher (3.2-fold) content of cellular proline after cold treatment, compared to WT plants	—	Liu et al. (2007)
OsZFP245/CaMV 35S	Transcription factor	4°C for 4 days/ four-leaf stage	High tolerance to cold and drought stresses (~80% survival, compared to 20% survival in WT plants)/ Increased free proline levels and elevated expression of rice pyrroline-5-carboxylatesynthetase and proline transporter genes under stress conditions/Enhanced activities of reactive oxygen species-scavenging enzymes under stress conditions and increased tolerance of rice seedlings to oxidative stress	Transgenic plants did not exhibit growth retardation, but showed growth sensitivity against exogenous abscisic acid	Huang et al. (2009)
OsZFP182/CaMV 35S	Transcription factor	4°C for 4 days/ four-leaf stage	Survival rates of transgenic lines (~80%) were higher than those of WT plants (~17%) under cold treatment, and also under salt and drought treatments/Accumulation of compatible osmolytes, such as free proline and soluble sugars	–	Huang et al. (2012)
AeluropuslittoralisAlSAP/ CaMV 35S	Transcription factor	4°C for 1 day followed by 12°C for 3 days/six-leaf stage	Enhanced cold tolerance (100% survival after cold treatment), drought, and salt stresses/Cold treatment imposed a much smaller reduction in leaf gas exchange rates in transgenic plants/Photosynthetic rates were reestablished to the prestress level following the 7-day recovery period in transgenic plants	–	Saad et al. (2012)

Table 3. Continued

Overexpressed gene(s)/ promoter	Gene(s) function	Cold treatment/ Plant age or stage	Characteristics of transgenic plants	Deleterious effects of transgenic plants	Reference
SNAC2/maize *Ubiquitin*	Transcription factor	4°C for 5 days/ four-leaf stage	Significantly enhanced cold tolerance (after 7 days of recovery, more than 50% of the transgenic plants remained vigorous while almost all WT plants died) as well as to salinity and dehydration stresses/ Transgenic plants had significantly lower cell membrane penetrability than WT plants	–	Hu et al. (2008)
OsNAC5/maize *Ubiquitin*	Transcription factor	4°C for 6 days/ 14 days old	Enhanced tolerance to cold (~70% survival, compared to 50% survival in WT plants), drought, and salt stresses/Accumulation of proline and soluble sugars was positively correlated with *OsNAC5* expression levels/Reduced accumulation of malondialdehyde and H_2O_2 (less susceptible to oxidative damage)/ Transgenic plants exhibited lower relative electrolyte leakage than WT plants	–	Song et al. (2011)
OsMYB3R-2/maize *Ubiquitin*	Transcription factor	2°C for up to 3.5 days/three-leaf stage	Enhanced tolerance to 72 and 84 h of cold stress (~50% and 20% survival in transgenic plants, while all WT plants died)/Increased free proline levels (300 μg/g fresh weight compared with 188 μg/g fresh weight in the wild-type plants)/Under cold conditions, the mitotic index in the overexpressing lines was markedly higher than that of the WT	Growth retardation under normal growth conditions up to the heading stage/Shorter roots in the overexpression transgenic lines	Ma et al. (2009)
OsMYBS3/maize *Ubiquitin*	Transcription factor	4°C for 7 days/ 10 days old	Enhanced cold tolerance (up to 86% survival, compared to 10% survival in WT plants)/Repression of the well-known DREB1/CBF-dependent cold signaling pathway in rice	Under greenhouse growth conditions, transgenic plants were 20% shorter, had 30% lower tiller numbers, and headed 1 week later than WT plants	Su et al. (2010)
OsMYB2/maize *Ubiquitin*	Transcription factor	2°C for 3 days/ 14 days old	Enhanced tolerance to cold (~80% survival, compared to 20% survival in WT plants), dehydration, and salt stresses	–	Yang et al. (2012)
LeTERF2/*CaMV35S*	Transcription factor	6°C for 3 days/ 8 days old	Enhanced cold tolerance[1]/Increased accumulation of osmotic substances (proline and soluble sugars) and chlorophyll/Reduced reactive oxygen species (ROS) and malondialdehyde (MDA) content under cold stress/Decreased electrolyte leakage under cold stress	–	Tian et al. (2011)
OsCDPK7/*CaMV 35S*	Ca^{2+}-dependent protein kinase	4°C for 1 day/ 10 days old	Enhanced cold tolerance[1], salt, and drought/The extent of cold tolerance correlated well with the level of *OsCDPK7* expression/Chlorophyll fluorescence recovered to nearly normal levels in transgenic plants 48 hours after cold treatment/Transgenic plants enhanced induction of specific stress-responsive genes	–	Saijo et al. (2000)

Table 3. Continued

Overexpressed gene(s)/ promoter	Gene(s) function	Cold treatment/ Plant age or stage	Characteristics of transgenic plants	Deleterious effects of transgenic plants	Reference
			in response to salinity and drought, but not to cold		
OsMAPK5/*CaMV 35S*	Mitogen-activated protein kinase (MAPK)	4°C for 3 days/ 14 days old	*OsMAPK5* overexpressing plants showed increased tolerance to cold[1], drought, and salt stresses/ Suppression of *OsMAPK5* expression resulted in the constitutive expression of pathogenesis-related (*PR*) genes and significantly enhanced resistance to fungal (*Magnaporthe grisea*) and bacterial (*Burkholderia glumae*) pathogens	Starting from the late vegetative stage (~2 months after germination), irregular brownish stripes developed on mature leaves of suppressed lines. Nevertheless, each suppressed line proceeded to the reproductive stage and had normal seed setting	Xiong and Yang (2003)
Triticum aestivum TaWFT1 and *TaWFT2*/ *CaMV 35S*	Carbohydrate metabolism	5°C for 11 days/ 10 days old	Enhanced cold tolerance (up to almost 90% survival, compared to fewer than 10% survival in WT plants)/ Higher accumulation of fructans in transgenic plants than in WT plants	–	Kawakami et al. (2008)
OsAsr1/maize *Ubiquitin*	Unknown	4°C for 4 days/ 10 days old	Enhanced cold tolerance (~68% of the transgenic plants showed wilting after 6 days of cold treatment; 96% of the WT plants had wilted)/Photosynthetic efficiency about twofold higher than in WT plants after 1 day of cold treatment	–	Kim et al. (2009)
OsPRP3/maize *Ubiquitin*	Cell wall protein	4°C for up to 4 weeks/42 days old	Higher cold tolerance than WT plants[1]/Increased proline content (more than fourfold within a week of cold treatment compared to the free proline accumulation in WT plants)/Mesophyll cells of the transgenic plants maintained their cell structure and retained the cell wall integrity	–	Gothandam et al. (2010)
OsRAN2/maize *Ubiquitin*	Cell division	4°C for 3 days/ 14 days old	Enhanced cold tolerance (up to 80% survival, compared to 14% survival in WT plants)/Transgenic plants maintained cell division, decreased proportion of cells with intranuclear tubulin, and formation of a normal nuclear envelope under the cold condition	–	Chen et al. (2011)
OsTPS1/*CaMV 35S*	Carbohydrate metabolism	4°C for 5 days/ 14 days old	Enhanced tolerance to cold (up to 90% survival, compared to 4% survival in WT plants), drought, and salt/Increased trehalose and proline levels	Slight dwarfing and shorter leaf length under normal conditions (not sufficient to impact the normal growth of the transgenic lines)	Li et al. (2011)
OsOVP1/maize *Ubiquitin*	H^+-transport protein	4°C for up to 10 days/15 days old	Enhanced cold tolerance (up to 55% survival, compared to 20% survival in WT plants)/Increased integrity of cell membrane/Decreased MDA content/ Higher accumulation of proline than WT plants	–	Zhang et al. (2011)
OsAPXa/ *E0082*[2]	Antioxidant enzyme	12°C for 6 days/ Booting stage	Spikelet fertility was significantly higher in transgenic lines than in WT plants after cold treatment[1]/H_2O_2 levels and MDA content were significantly lower than	–	Sato et al. (2011)

Table 3. Continued

Overexpressed gene(s)/ promoter	Gene(s) function	Cold treatment/ Plant age or stage	Characteristics of transgenic plants	Deleterious effects of transgenic plants	Reference
			in WT plants/APX activity showed negative correlations with levels of H_2O_2 and MDA content		
OsGSTl maize *Ubiquitin*	Cellular detoxification	Germination at 13°C/ Growth after germination at 15°C for 13 days	Number of days required for 50% germination was nine for transgenic plants and 16 for WT plants/Root and shoot growth was higher in transgenic plants than in WT plants	–	Takesawa et al. (2002)
Triticum aestivum TaABA8′OH1/OsG6B[3]	ABA catabolism	12°C for 5 days/ Reproductive stage	Sterility percentage was reduced in transgenic plants (30–63% compared with WT plants)/Spikelet ABA accumulation was reduced in transgenic cold-stressed plants (58–76% of the WT plants)	–	Ji et al. (2011)

[1]Survival rate not calculated on the cited reference.
[2]*E0082* promoter drives expression of a downstream gene in rice green tissues and spikelets.
[3]*OsG6B* promoter is tapetum specific.

could be maximized and further enhance the cold tolerance in rice, by overexpression of both *OsDREB1* and *OsMYBS3*. Recently, Yang et al. (2012) generated a transgenic rice overexpressing a R2R3-type MYB gene, *OsMYB2*, and found increased tolerance to various stresses, adding one more transcription factor to the list of genes with potential usefulness for improving stress tolerance in rice.

One member of the AP2/ERF superfamily has been previously identified in tomato (*TERF2/LeERF2*), and its overexpression in tomato and tobacco plants leads to enhanced tolerance to freezing temperatures (Zhang and Huang 2010). In rice, its overexpression presents a similar effect, with enhanced tolerance to cold without affecting growth or agronomic traits. Physiological responses of transgenic plants include increased accumulation of osmotic substances (proline and soluble sugars), along with reduced reactive oxygen species (ROS), MDA, and electrolyte leakage levels (Tian et al. 2011).

Overexpression of other genes

Several other genes (not transcription factors, 11 of 24 in Table 3) with diverse cellular functions have been used to acquire cold tolerance in rice plants, evidencing that low temperature adaptation is a complex trait, which can be achieved by several mechanisms. Saijo et al. (2000) suggested that *OsCDPK7* (Ca^{2+}-dependent protein kinase) promotes cold and salt/drought tolerance through different pathways, since induction of some stress-related genes only occurs in response to salinity and drought, but not to cold in transgenic rice plants overexpressing this gene. *OsMAPK5*, which encodes a mitogen-activated protein kinase, can inversely modulate broad-spectrum disease resistance, through negative regulation of pathogenesis-related genes, and abiotic stress tolerance, through positive regulation of cold, drought, and salt tolerance (Xiong and Yang 2003). Overexpression of two fructan synthesizing-enzymes of wheat (*TaWFT1* and *TaWFT2*) generates transgenic rice plants which accumulate large amounts of fructans, probably activating a signal cascade that induces expression of multiple genes, such as cold-responsive genes, increasing tolerance (Kawakami et al. 2008). One member of the plant-specific *Asr* gene family (*OsAsr1*) plays an important role during low temperature stress, and cold-tolerant overexpressing rice plants have photosynthetic efficiency about twofold higher than those of WT plants after cold treatment (Kim et al. 2009). Also, the cold-tolerant phenotype in rice has already been achieved by overexpression of a cell wall protein (OsPRP3), which maintains the structure of mesophyll cells and retains cell wall integrity under low temperature (Gothandam et al. 2010).

Maintenance of cell division under cold stress is also an efficient approach to generate transgenic cold-tolerant rice plants, as previously seen by Chen et al. (2011), through the overexpression of the *OsRAN2* gene, which is essential for mitosis and can promote intranuclear tubulin export at the end of mitosis. Accumulation of trehalose, a nonreducing disaccharide sugar, was proved to be an excellent manner to acquire cold tolerance in rice (Li et al. 2011), obtained by overexpressing the trehalose-6-phosphate synthase gene (*OsTPS1*). A H^+-pump (*OsOVP1*) was also overexpressed, leading to transgenic rice plants with higher accumulation of proline and also increased integrity of cell membrane, which resulted in cold tolerance (Zhang et al. 2011).

Only three reports have analyzed cold tolerance with temperatures higher than 10°C. Sato et al. (2011) enhanced chilling tolerance (12°C) of rice plants at the booting stage by overexpressing an ascorbate peroxidase gene (*OsAPXa*). The authors suggested that ectopic expression of this gene enhances H_2O_2-scavenging capacity and protects rice spikelets from lipid peroxidation, increasing spikelet fertility under chilling stress. In order to develop a rice cultivar that would be suitable for direct-seeding cultivation in cooler temperate regions, Takesawa et al. (2002) analyzed germination and growth after germination under chilling stress (13 and 15°C, respectively) of WT and glutathione S-transferase (*OsGST*) overexpressing rice plants. Transgenic plants showed faster germination and root penetration, which increase the chance of seedling establishment, and had higher root and shoot growth, probably reducing oxidative stress caused by the chilling treatment (Takesawa et al. 2002). Recently, Ji et al. (2011) found that overexpression of the wheat *TaABA8′OH1* gene can lead to reduced spikelet ABA levels without disruption of the anther sink strength, improving cold stress (12°C) tolerance at the reproductive stage, which is evidenced by lower sterility percentage in transgenic plants.

It is important to highlight that, in most of the cited reports, researches kept in mind that a transgenic cold-tolerant rice plant must perform at least as well as WT plants under control conditions. Five of the 24 transgenic approaches shown in Table 3 generated plants with some kind of growth retardation under unstressed conditions, which would undermine the release of these transgenic lines in noncold regions. Instead of using constitutive promoters (as *CaMV 35S* or maize *Ubiquitin*, which overexpress the transgene in all cells, throughout the life cycle of the plant), an alternative approach would be to use tissue- and/or stage-specific promoters to control transgene expression, or even cold-driven promoters. Another important issue to be considered is that each new transgenic plant should be field validated to prove its efficacy as a possible new cold-tolerant cultivar. Also, 20 of the 24 transgenic strategies employed a very low temperature (about 4°C or even less) to evaluate cold tolerance, which is, in practice, not common in regions where the rice crop is cultivated. The use of extremely low temperatures, and also of constitutive promoters, results in limited usefulness of the transgenic experimental results. They prove the complexity of the cold temperature tolerance trait in rice, as a large number of transcription factors and genes can be manipulated to enhance cold stress response, but their practical utility has yet to be shown.

Future Directions

It is clear that much progress has been achieved in the understanding of cold tolerance in rice plants. However, decreased productivity caused by low temperatures remains as a problem, especially in places where *indica* rice is cultivated. Considering that the frequency of extreme temperature events is expected to increase as part of the general global warming phenomena (IPCC 2007), damage caused by cold temperatures may increase in some areas in Africa and Latin America (Marengo and Camargo 2008).

New cold-tolerant cultivars will need to be designed according to the growth stage when plants will be exposed to cold in a particular region, and a combined strategy that considers breeding and genetic engineering as tools to be used hand-in-hand could lead to successful projects. The use of specific promoters and pyramiding of genes should also be encouraged.

Most of the search for genes potentially able to confer cold tolerance in rice plants has been based on QTL or gene expression analysis. Future efforts in this direction will certainly be more elucidative if high-throughput techniques (such as microarray analysis, large scale sequencing, and proteomics) are more intensely used. Next-generation sequence technology allows the sequencing of several related genomes to evaluate genetic diversity within and between germplasm pools. Such genomic tool can greatly improve the precision of genetic variations identification (such as SNPs and QTLs), which in turn can efficiently determine the functional relevance of candidate genes for complex stress tolerance traits through genetic association (Parida et al. 2012) and also predict phenotypes from genotypes. This is the basis of genomics-assisted breeding (Varshney et al. 2009). According to Kumar et al. (2012), the connection of three interlaced research areas is needed to effectively accelerate the development of improved cultivars with enhanced stress tolerance: next-generation sequencing, its associated bioinformatics challenges, and the applications of SNPs in

genetic studies. The use of available mutant populations of rice plants (by T-DNA insertion or by activation of the Tos17 transposon) should also be intensified. Screening of T-DNA mutants has provided a few candidate genes involved in cold tolerance or susceptibility, but mutants from these populations would also be excellent tools to confirm the role of candidate genes revealed by other techniques, in a reverse genetics approach. The same could also be done with gene silencing using RNA interference, which was rarely used for cold tolerance in rice. Another useful tool to be explored is the *A. thaliana* collection of independent lines overexpressing rice genes (Rice FOX – Full-length Over-eXpressor Arabidopsis lines) (http://ricefox.psc.riken.jp/; Sakurai et al. 2011). These lines could be used in a general screening for cold tolerance (forward genetics) or to confirm the role of candidate genes (reverse genetics approach).

Practical progress in the field would probably be achieved faster if there was more integration between breeders and plant biologists. Although some genes have been identified based on previous QTL discovery, there is a lot to be done in the search for genes within QTL regions that are effectively shown to be important for cold tolerance in rice. The genes chosen so far for overexpression do not seem to have been picked from papers describing cold tolerance QTLs, but rather based on gene expression data. The only coincidence between genes related to QTLs and overexpressed genes reported in our Tables 2 and 3 relates to glutathione S-transferases. However, the transgenic plants were generated four years before a major QTL was related to a GST gene. It would be very productive if groups interested in rice cold tolerance could join efforts to identify all genes responsible for the QTLs shown in Table 3 and to test the relevance of those genes by both overexpression (in rice or testing the corresponding FOX lines) and silencing (in insertion mutants or by RNAi).

Integration between breeders and plant biologists would also have positive outcomes if a set of genes with the most promising results based on experimental data were sequenced in two groups of rice genotypes, with contrasting cold tolerance. This could allow the identification of important mutations to be used as markers in breeding programs.

Hopefully, the scientific community will be able to deliver answers and products to ensure food security to the future generations. Cold-tolerant rice plants may be part of these important achievements.

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Conflict of Interest

None declared.

References

Abe, N., S. Kotaka, K. Toriyama, and M. Kobayashi. 1989. Development of the "rice Norin-PL8" with high tolerance to cool temperature at the booting stage. Res. Bull. Hokkaido Natl. Agric. Exp. Stat. 152:9–17.

Aghaee, A., F. Moradi, H. Zare-Maivan, F. Zarinkamar, H. Pour Irandoost, and P. Sharifi. 2011. Physiological responses of two rice (*Oryza sativa* L.) genotypes to chilling stress at seedling stage. Afr. J. Biotechnol. 10:7617–7621.

Andaya, V. C., and D. J. Mackill. 2003a. QTLs conferring cold tolerance at the booting stage of rice using recombinant inbred lines from a *japonica* × *indica* cross. Theor. Appl. Genet. 106:1084–1090.

Andaya, V. C., and D. J. Mackill. 2003b. Mapping of QTLs associated with cold tolerance during the vegetative stage in rice. J. Exp. Bot. 54:2579–2585.

Andaya, V. C., and T. H. Tai. 2006. Fine mapping of the *qCTS12* locus, a major QTL for seedling cold tolerance in rice. Theor. Appl. Genet. 113:467–475.

Bardhan Roy, S. K., G. F. Pateña, and B. S. Vergara. 1982. Feasibility of selection for traits associated with cold tolerance in rice under rapid generation advance method. Euphytica 31:25–31.

Bertin, P., J. M. Kinet, and J. Bouharmont. 1996. Evaluation of chilling sensitivity in different rice varieties. Relationship between screening procedures applied during germination and vegetative growth. Euphytica 89:201–210.

Bierlen, R., E. J. Wailes, and G. L. Cramer. 1997. The Mercosur rice economy. Ark. Exp. Stat. Bull. 954:1–58.

Bonnecarrère, V., O. Borsanib, P. Díazb, F. Capdeviellea, P. Blancoc, and J. Monza. 2011. Response to photoxidative stress induced by cold in japonica rice is genotype dependent. Plant Sci. 180:726–732.

Brar, D. S., and K. Singh. 2011. Oryza. Pp. 321–366 *in* C. Kole, ed. Wild crop relatives: genomic breeding and resources: cereals. Springer-Verlag, Berlin, Heidelberg.

Chaikam, V., and D. Karlson. 2008. Functional characterization of two cold shock domain proteins from *Oryza sativa*. Plant Cell Environ. 31:995–1006.

Chen, J. Q., Y. Dong, Y. J. Wang, Q. Liu, J. S. Zhang, and S. Y. Chen. 2003. An AP2/EREBP-type transcription-factor gene from rice is cold-inducible and encodes a nuclear-localized protein. Theor. Appl. Genet. 107:972–979.

Chen, L., Q. Lou, Z. Sun, Y. Xing, X. Yu, and L. Luo. 2006. QTL mapping of low temperature on germination rate of rice. Rice Sci. 13:93–98.

Chen, N. A., Y. Xu, X. Wang, C. Du, J. Du, M. Yuan, et al. 2011. *OsRAN2*, essential for mitosis, enhances cold tolerance in rice by promoting export of intranuclear tubulin and

maintaining cell division under cold stress. Plant Cell Environ. 34:52–64.

Clayton, S., and P. C. Neves. 2011. Country snapshot: Brazil. Rice Today 10:16–17.

da Cruz, R. P., and S. C. K. Milach. 2000. Breeding for cold tolerance in irrigated rice. Ciência Rural 30:909–917.

da Cruz, R. P., and S. C. K. Milach. 2004. Cold tolerance at the germination stage of rice: methods of evaluation and characterization of genotypes. Sci. Agric. 61:1–8.

da Cruz, R. P., S. C. K. Milach, and L. C. Federizzi. 2006a. Inheritance of rice cold tolerance at the germination stage. Genet. Mol. Biol. 29:314–320.

da Cruz, R. P., S. C. K. Milach, and L. C. Federizzi. 2006b. Rice cold tolerance at the reproductive stage in a controlled environment. Sci. Agric. 63:255–261.

da Cruz, R. P., J. I. Golombieski, M. T. Bazana, C. Cabreira, T. F. Silveira, and L. P. da Silva. 2010. Alterations in fatty acid composition due to cold exposure at the vegetative stage in rice. Braz. J. Plant Physiol. 22:199–207.

Cui, S., F. Huang, J. Wang, X. Ma, X. Cheng, and J. Liu. 2005. A proteomic analysis of cold stress responses in rice seedlings. Proteomics 5:3162–3172.

Dai, K., B. S. Vergara, and R. M. Visperas. 1990. Amelioration of cold injury in rice (*Oryza sativa* L.): improving root oxidizing activity by plant growth regulators. Philipp. J. Crop Sci. 15:49–54.

Dai, L., X. Lin, C. Ye, K. Ise, K. Saito, A. Kato, et al. 2004. Identification of quantitative trait loci controlling cold tolerance at the reproductive stage in Yunnan landrace of rice, Kunmingxiaobaigu. Breed. Sci. 54:253–258.

Dubouzet, J. G., Y. Sakuma, Y. Ito, M. Kasuga, E. G. Dubouzet, S. Miura, et al. 2003. *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought, high-salt- and cold-responsive gene expression. Plant J. 33:751–763.

Farrell, T. C., R. L. Williams, and S. Fukai. 2001. The cost of low temperature to the NSW rice industry. Proc. 10th Aust. Agron. Conf. 1:1300–1430.

Fujino, K., and Y. Matsuda. 2010. Genome-wide analysis of genes targeted by *qLTG3-1* controlling low-temperature germinability in rice. Plant Mol. Biol. 72:137–152.

Fujino, K., H. Sekiguchi, T. Sato, H. Kiuchi, Y. Nonoue, Y. Takeuchi, et al. 2004. Mapping of quantitative trait loci controlling low-temperature germinability in rice (*Oryza sativa* L.). Theor. Appl. Genet. 108:794–799.

Fujino, K., H. Sekigushi, Y. Matsuda, K. Sugimoto, K. Ono, and 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.

Gao, J. P., D. Y. Chao, and H. X. Lin. 2008. Toward understanding molecular mechanisms of abiotic stress responses in rice. Rice 1:36–51.

Glaszmann, J. C., R. N. Kaw, and G. S. Khush. 1990. Genetic divergence among cold tolerant rices (*Oryza sativa* L.). Euphytica 45:95–104.

Gothandam, K. M., E. Nalini, S. Karthikeyan, and J. S. Shin. 2010. OsPRP3, a flower specific proline-rich protein of rice, determines extracellular matrix structure of floral organs and its overexpression confers cold-tolerance. Plant Mol. Biol. 72:125–135.

Harushima, Y., M. Yano, A. Shomura, M. Sato, T. Shimano, Y. Kuboki, et al. 1998. A high-density rice genetic linkage map with 2,275 markers using a single F_2 population. Genetics 148:479–494.

Hashimoto, M., and S. Komatsu. 2007. Proteomic analysis of rice seedlings during cold stress. Proteomics 7:1293–1302.

Hayase, H., T. Satake, I. Nishiyama, and N. Ito. 1969. Male sterility caused by cooling treatment at the meiotic stage in rice plants. II. The most sensitive stage to cooling and the fertilizing ability of pistils. Proc. Crop Sci. Soc. Jpn. 38:706–711.

Heenan, D. P. 1984. Low temperature induced floret sterility in the rice cultivars Calrose and Inga as influenced by nitrogen supply. Aust. J. Exp. Agric. Anim. Husb. 24:255–259.

Hirotsu, N., A. Makino, A. Ushio, and T. Mae. 2004. Changes in the thermal dissipation and the electron flow in the water–water cycle in rice grown under conditions of physiologically low temperature. Plant Cell Physiol. 45:635–644.

Hirotsu, N., A. Makino, S. Yokota, and T. Mae. 2005. The photosynthetic properties of rice leaves treated with low temperature and high irradiance. Plant Cell Physiol. 46:1377–1383.

Hossain, M. A., J. I. Cho, M. Han, C. H. Ahn, J. S. Jeon, G. An, et al. 2010. The ABRE-binding bZIP transcription factor OsABF2 is a positive regulator of abiotic stress and ABA signaling in rice. J. Plant Physiol. 167:1512–1520.

Hou, M. Y., L. Jiang, C. M. Wang, and J. M. Wan. 2003. Detection and analysis of QTLs for low temperature germinability in rice (*Oryza sativa* L.) Rice Genet. Newslett. 20:52–55.

Hu, H., J. You, Y. Fang, X. Zhu, Z. Qi, and L. Xiong. 2008. Characterization of transcription factor gene *SNAC2* conferring cold and salt tolerance in rice. Plant Mol. Biol. 67:169–181.

Huang, I., J. F. Wang, Q. H. Wang, and H. S. Zhang. 2005. Identification of a rice zinc finger protein whose expression is transiently induced by drought, cold but not by salinity and abscisic acid. DNA Seq. 16:130–136.

Huang, J., M. M. Wang, Y. Jiang, Y. M. Bao, X. Huang, H. Sun, et al. 2008. Expression analysis of rice A20/AN1-type zinc finger genes and characterization of *ZFP177* that contributes to temperature stress tolerance. Gene 420:135–144.

Huang, J., S. J. Sun, D. Q. Xu, X. Yang, Y. M. Bao, Z. F. Wang, et al. 2009. Increased tolerance of rice to cold, drought and oxidative stresses mediated by the overexpression of a gene that encodes the zinc finger protein ZFP245. Biochem. Biophys. Res. Commun. 389:556–561.

Huang, J., S. Sun, D. Xu, H. Lan, H. Sun, Z. Wang, et al. 2012. A TFIIIA-type zinc finger protein confers multiple abiotic stress tolerances in transgenic rice (*Oryza sativa* L.). Plant Mol. Biol. 80:337–350.

Iba, K. 2002. Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. Annu. Rev. Plant Biol. 53:225–245.

Intergovernmental Panel on Climate Change (IPCC). 2007. Summary for policymakers. Pp. 1–18 *in* S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, M. Tignor and H.L. Miller, eds. Climate change 2007: the physical science basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, Cambridge University Press, Cambridge and New York, NY.

IRGSP. 2005. The map-based sequence of the rice genome. Nature436:793–800.

Ito, N. 1976. Male sterility caused by cooling treatment at the young microspore stage in rice plants. XV. Effect of moderate cooling before or after the critical stage on the sterility induced by cooling at the critical stage. Proc. Crop Sci. Soc. Jpn. 45:558–562.

Ito, N. 1978. Male sterility caused by cooling treatment at the young microspore stage in rice plants. XVI. Changes in carbohydrates, nitrogenous and phosphorus compounds in rice anthers after cooling treatment. Jpn. J. Crop Sci. 47:318–323.

Ito, N., H. Hayase, T. Satake, and I. Nishiyama. 1970. Male sterility caused by cooling treatment at the meiotic stage in rice plants. III. Male abnormalities at anthesis. Proc. Crop Sci. Soc. Jpn. 39:60–64.

Ito, Y., K. Katsura, K. Maruyama, T. Taji, M. Kobayashi, M. Seki, et al. 2006. Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant Cell Physiol. 47:141–153.

Jena, K. K., S. M. Kim, J. P. Suh, C. I. Yang, and Y. G. Kim. 2012. Identification of cold-tolerant breeding lines by quantitative trait loci associated with cold tolerance in rice. Crop Sci. 52:517–523.

Jennings, P. R., W. R. Coffman, and H. E. Kauffman. 1979. Pp. 83–186 *in* Rice improvement. International Rice Research Institute, Los Baños.

Ji, B. H., Y. Y. Cao, H. S. Xie, S. Q. Zhu, Q. Ma, and D. M. Jiao. 2003. Changes in violaxanthin deepoxidase activity and unsaturation of thylakoid membrane lipids in *indica* and *japonica* rice under chilling condition and strong light. Acta Bot. Sin. 45:1063–1070.

Ji, S. L., L. Jiang, Y. H. Wang, S. J. Liu, X. Liu, H. Q. Zhai, et al. 2008. QTL and epistasis for low temperature germinability in rice. Acta Agronom. Sin. 34:551–556.

Ji, X., B. Dong, B. Shiran, M. J. Talbot, J. E. Edlington, T. Hughes, et al. 2011. Control of abscisic acid catabolism and abscisic acid homeostasis is important for reproductive stage stress tolerance in cereals. Plant Physiol. 156:647–662.

Jiang, L., M. Xun, J. Wang, and J. Wan. 2008. QTL analysis of cold tolerance at seedling stage in rice (*Oryza sativa* L.) using recombination inbred lines. J. Cereal Sci. 48:173–179.

Jiang, W., Y. M. Jin, J. Lee, K. I. Lee, R. Piao, L. Han, et al. 2011. Quantitative Trait Loci for cold tolerance of rice recombinant inbred lines in low temperature environments. Mol. Cells 32:579–587.

Juliano, B. O. 1993. Pp. 1–15 *in* Rice in human nutrition. International Rice Research Institute and Food and Agriculture Organization of the United Nations, Los Baños.

Kaneda, C., and H. M. Beachell. 1974. Breeding rice for cold tolerance. Saturday Seminar Paper 9. Philippines International Rice Research Institute, Los Baños.

Kawakami, A., Y. Sato, and M. Yoshida. 2008. Genetic engineering of rice capable of synthesizing fructans and enhancing chilling tolerance. J. Exp. Bot. 59:793–802.

Kim, S. J., S. C. Lee, S. K. Hong, K. An, G. An, and S. R. Kim. 2009. Ectopic expression of a cold-responsive *OsAsr1* cDNA gives enhanced cold tolerance in transgenic rice plants. Mol. Cells 27:449–458.

Kim, S. I., V. C. Andaya, and T. H. Tai. 2011. Cold sensitivity in rice (*Oryza sativa* L.) is strongly correlated with a naturally occurring I99V mutation in the multifunctional glutathione transferase isoenzyme GSTZ2. Biochem. J. 435:373–380.

Koh, S., S. C. Lee, M. K. Kim, J. H. Koh, S. Lee, G. An, et al. 2007. T-DNA tagged knockout mutation of rice *OsGSK1*, an orthologue of *Arabidopsis BIN2*, with enhanced tolerance to various abiotic stresses. Plant Mol. Biol. 65:453–466.

Koike, S., K. Takeda, and T. Satake. 1990. Comparison of cold tolerance between isogenic pairs differing in grain size. Rice Genet. Newslett. 7:133–134.

Koiwai, H., K. Nakaminami, M. Seo, W. Mitsuhashi, T. Toyomasu, and T. Koshiba. 2004. Tissue-specific localization of an abscisic acid biosynthetic enzyme, AAO3, in *Arabidopsis*. Plant Physiol. 134:1697–1707.

Koseki, M., N. Kitazawa, S. Yonebayashi, Y. Maehara, Z. X. Wang, and Y. Minobe. 2010. Identification and fine mapping of a major quantitative trait locus originating from wild rice, controlling cold tolerance at the seedling stage. Mol. Genet. Genomics 284:45–54.

Kuk, Y. I., J. S. Shin, N. R. Burgos, T. E. Hwang, O. Han, B. H. Cho, et al. 2003. Antioxidative enzymes offer protection from chilling damage in rice plants. Crop Sci. 43:2109–2117.

Kumar, S., T. W. Banks, and S. Cloutier. 2012. SNP discovery through next-generation sequencing and its applications. Int. J. Plant Genomics 2012:Article ID 831460.

Kuroki, M., K. Saito, S. Matsuba, N. Yokogami, H. Shimizu, I. Ando, et al. 2007. A quantitative trait locus for cold tolerance at the booting stage on rice chromosome 8. Theor. Appl. Genet. 115:593–600.

Kwak, T. S., B. S. Vergara, J. S. Nanda, and W. R. Coffman. 1984. Inheritance of seedling cold tolerance in rice. Sabrao J. 16:83–86.

Lazár, D., and P. Ilík. 1997. High-temperature induced chlorophyll fluorescence changes in barley leaves: comparison of the critical temperatures determined from fluorescence induction and from fluorescence temperature curve. Plant Sci. 124:159–164.

Lee, M. H. 2001. Low temperature tolerance in rice: the Korean experience. Pp. 109–117 *in* S. Fukai and J. Basnayake, eds. Increased lowland rice production in the Mekong Region. Proceedings of an international workshop, Vientiane, Laos, 30 October to 2 November 2000. Australian Center for International Agricultural Research, Canberra, Australia.

Lee, S. C., J. Y. Kim, S. H. Kim, S. J. Kim, K. Lee, S. K. Han, et al. 2004a. Trapping and characterization of cold-responsive genes from T-DNA tagging lines in rice. Plant Sci. 166:69–79.

Lee, S. C., K. W. Huh, K. An, G. An, and S. R. Kim. 2004b. Ectopic expression of a cold-inducible transcription factor, CBF1/DREB1b, in transgenic rice (*Oryza sativa* L.). Mol. Cells 18:107–114.

Lee, S. C., M. Y. Lee, S. J. Kim, S. H. Jun, G. An, and S. R. Kim. 2005. Characterization of an abiotic stress-inducible dehydrin gene, *OsDhn1*, in rice (*Oryza sativa* L.). Mol. Cells 19:212–218.

Lee, D. G., N. Ahsan, S. H. Lee, K. Y. Kang, J. J. Lee, and B.-H. Lee. 2007. An approach to identify cold-induced low-abundant proteins in rice leaf. C. R. Biol. 330:215–225.

Lee, D. G., N. Ahsan, S. H. Lee, J. J. Lee, J. D. Bahk, K. Y. Kang, et al. 2009a. Chilling stress-induced proteomic changes in rice roots. J. Plant Physiol. 166:1–11.

Lee, S. C., S. Y. Kwon, and S. R. Kim. 2009b. Ectopic expression of a cold-responsive Cu/Zn superoxide dismutase gene, *SodCc1*, in transgenic rice (*Oryza sativa* L.). J. Plant Biol. 52:154–160.

Li, T. G., R. M. Visperas, and B. S. Vergara. 1981. Correlation of cold tolerance at different growth stages in rice. Acta Bot. Sin. 23:203–207.

Li, H. B., J. Wang, A. M. Liu, K. D. Liu, Q. Zhang, and J. S. Zou. 1997. Genetic basis of low-temperature-sensitive sterility in *indica-japonica* hybrids of rice as determined by RFLP analysis. Theor. Appl. Genet. 95:1092–1097.

Li, H. W., B. S. Zang, X. W. Deng, and X. P. Wang. 2011. Overexpression of the trehalose-6-phosphate synthase gene *OsTPS1* enhances abiotic stress tolerance in rice. Planta 234:1007–1018.

Li, W. J., B. Zhang, G. W. Huang, G. P. Kang, M. Z. Liang, and L. B. Chen. 2012. Chloroplast DNA polymorphism and evolutional relationships between Asian cultivated rice (*Oryza sativa*) and its wild relatives (*O. rufipogon*). Genet. Mol. Res. 11:4418–4431.

Lima, M. G. S., N. F. Lopes, P. D. Zimmer, G. E. Meneghello, C. R. Mendes, and L. Amarante. 2012. Enzyme expression in *indica* and *japonica* rice cultivars under saline stress. Acta Sci. Biol. Sci. 34:473–481.

Liu, K., L. Wang, Y. Xu, N. Chen, Q. Ma, F. Li, et al. 2007. Overexpression of *OsCOIN*, a putative cold inducible zinc finger protein, increased tolerance to chilling, salt and drought, and enhanced proline level in rice. Planta 226:1007–1016.

Lou, Q., L. Chen, Z. Sun, Y. Xing, J. Li, X. Xu, et al. 2007. A major QTL associated with cold tolerance at seedling stage in rice (*Oryza sativa* L.). Euphytica 158:87–94.

Ma, Q., X. Dai, Y. Xu, J. Guo, Y. Liu, N. Chen, et al. 2009. Enhanced tolerance to chilling stress in *OsMYB3R-2* transgenic rice is mediated by alteration in cell cycle and ectopic expression of stress genes. Plant Physiol. 150:244–256.

Mackill, D. J., and X. Lei. 1997. Genetic variation for traits related to temperate adaptation of rice cultivars. Crop Sci. 37:1340–1346.

Mackill, D. J., W. R. Coffman, and D. P. Garrity. 1996. P. 242 *in* Rainfed lowland rice improvement. International Rice Research Institute, Manila.

Majumder, M. K., D. V. Seshu, and V. V. Shenoy. 1989. Implications of fatty acids and seed dormancy in a new screening procedure for cold tolerance in rice. Crop Sci. 29:1298–1304.

MaNeil, S. D., M. L. Nuccio, and A. D. Hanson. 1999. Betaines and related osmoprotectants. Targets for metabolic engineering of stress resistance. Plant Physiol. 120:945–949.

Marengo, J. A., and C. Camargo. 2008. Surface air temperature trends in Southern Brazil for 1960-2002. Int. J. Climatol. 28:893–904.

Matsuo, T., K. Kumazawa, R. Ishii, K. Ishihara, and H. Hirata. 1995. Science of the rice plant 2: Physiology. Food and Agriculture Policy Research Center, Tokyo, Japan.

McCouch, S. R., L. Teytelman, Y. Xu, K. B. Lobos, K. Clare, M. Walton, et al. 2002. Development and mapping of 2,240 new SSR markers for rice (*Oryza sativa* L.). DNA Res. 9:199–207.

Mertz, L. M., F. A. Henning, R. C. Soares, R. F. Baldiga, F. B. Peske, and D. M. Moraes. 2009. Physiological changes in rice seeds exposed to cold in the germination phase. Rev. Bras. Sement. 31:254–262.

Mittal, D., S. Chakrabarti, A. Sarkar, A. Singh, and A. Grover. 2009. Heat shock factor gene family in rice: genomic organization and transcript expression profiling in response to high temperature, low temperature and oxidative stresses. Plant Physiol. Biochem. 47:785–795.

Miura, K., S. Y. Lin, M. Yano, and T. Nagamine. 2001. Mapping quantitative trait loci controlling low temperature germinability in rice (*Oryza sativa* L.). Breed. Sci. 51:293–299.

Moffat, C. S., R. A. Ingle, D. L. Wathugala, N. J. Saunders, H. Knight, and M. R. Knight. 2012. ERF5 and ERF6 play redundant roles as positive regulators of JA/Et-mediated defense against *Botrytis cinerea* in *Arabidopsis*. PLoS ONE 7:1–11.

Moon, H. P. 1984. Inheritance of low-temperature induced-sterility and its relationship to agronomic characters in rice (*Oryza sativa*). Diss. Abst. Int. 44: 2065.

Morsy, M. R., L. Jouve, J. F. Hausman, L. Hoffmann, and J. D. Stewart. 2007. Alteration of oxidative and carbohydrate metabolism under abiotic stress in two rice (*Oryza sativa* L.) genotypes contrasting chilling tolerance. J. Plant Physiol. 164:157–167.

Nagamine, T. 1991. Genetic control of tolerance to chilling injury at seedling in rice, *Oryza sativa* L. Jpn. J. Breed. 41:35–40.

Nagano, K. 1998. Development of new breeding techniques for cold tolerance and breeding of new rice cultivars with highly cold tolerance, Hitomebore and Jyoudeki. Pp. 143–148 *in* Proceedings of International Workshop on Breeding and Biotechnology for Environmental Stress in Rice, Sapporo, Japan.

Nakamura, T., M. Chiba, S. Koike, I. Nishiyama, T. Satake, and M. Shibata. 2000. Number of pollen grains in rice cultivars with different cool-weather resistance at the young microspore stage. Plant Prod. Sci. 3:299–305.

Nakashima, K., L. S. P. Tran, D. V. Nguyen, M. Fujita, K. Maruyama, D. Todaka, et al. 2007. Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. Plant J. 51:617–630.

Nishiyama, I. 1978. Male sterility caused by cooling treatment at the young microspore stage in rice plants. XVIII. Some enzyme activities in anthers during and after the cooling. Jpn. J. Crop Sci. 47:551–556.

Nishiyama, I. 1996. Strategies for the research to overcome cool weather damage in rice plants. Pp. 246–251 *in* Proceedings of the 2nd Asian Crop Science Conference, Fukui, Japan.

Nishiyama, R., Y. Watanabe, M. A. Leyva-Gonzalez, C. Van Ha, Y. Fujita, M. Tanaka, et al. 2013. Arabidopsis AHP2, AHP3, and AHP5 histidine phosphotransfer proteins function as redundant negative regulators of drought stress response. Proc. Natl. Acad. Sci. USA 110:4840–4845.

Ohnishi, T., S. Sugahara, T. Yamada, K. Kikuchi, Y. Yoshiba, H. Y. Hirano, et al. 2005. *OsNAC6*, a member of the NAC gene family, is induced by various stresses in rice. Genes Genet. Syst. 80:135–139.

Oliver, S. N., J. T. V. Dongen, S. C. Alfred, E. A. Mamun, X. Zhao, H. S. Saini, et al. 2005. Cold-induced repression of the rice anther-specific cell wall invertase gene *OSINV4* is correlated with sucrose accumulation and pollen sterility. Plant Cell Environ. 28:1534–1551.

Oliver, S. N., X. Zhao, E. S. Dennis, and R. Dolferus. 2007. The molecular basis of cold-induced pollen sterility in rice. Pp. 205–207 *in* Z. Xu, J. Li, Y. Xue and W. Yang, eds. Biotechnology and sustainable agriculture 2006 and beyond. Springer, the Netherlands.

Parida, S. K., M. Mukerji, A. K. Singh, N. K. Singh, and T. Mohaptra. 2012. SNPs in stress-responsive rice genes: validation, genotyping, functional relevance and population structure. BMC Genomics 13:426.

Qian, Q., D. Zeng, P. He, X. Zheng, Y. Chen, and L. Zhu. 2000. QTL analysis of the rice seedling cold tolerance in a double haploid population derived from anther culture of a hybrid between *indica* and *japonica* rice. Chin. Sci. Bull. 45:448–453.

Russo, S. 1994. Breeding and genetical research in Italian rice. Cah. Options Méditerr. 8:43–47.

Saad, B. R., N. Zouari, B. W. Ramdhan, J. Azaza, D. Meynard, E. Guiderdoni, et al. 2010. Improved drought and salt stress tolerance in transgenic tobacco overexpressing a novel A20/AN1 zinc-finger '*AlSAP*' gene isolated from the halophyte grass *Aeluropus littoralis*. Plant Mol. Biol. 72:171–190.

Saad, B. R., D. Fabre, D. Mieulet, D. Meynard, M. Dingkuhn, A. Al-Doss, et al. 2012. Expression of the *Aeluropus littoralis AlSAP* gene in rice confers broad tolerance to abiotic stresses through maintenance of photosynthesis. Plant Cell Environ. 35:626–643.

Saijo, Y., S. Hata, J. Kyozuka, K. Shimamoto, and K. Izui. 2000. Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J. 23:319–327.

Saito, K., K. Miura, K. Nagano, Y. Hayano-Saito, H. Araki, and A. Kato. 2001. Identification of two closely linked quantitative trait loci for cold tolerance on chromosome 4 of rice and their association with anther length. Theor. Appl. Genet. 103:862–868.

Saito, K., Y. H. Saito, W. M. Funatsuki, Y. Sato, and A. Kato. 2004. Physical mapping and putative candidate gene identification of a quantitative trait locus *Ctb1* for cold tolerance at the booting stage of rice. Theor. Appl. Genet. 109:515–522.

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

Sakurai, T., Y. Kondou, K. Akiyama, A. Kurotani, M. Higuchi, T. Ichikawa, et al. 2011. RiceFOX: a database of Arabidopsis mutant lines overexpressing rice full-length cDNA that contains a wide range of trait information to facilitate analysis of gene function. Plant Cell Physiol. 52:265–273.

Sanghera, G. S., S. H. Wani, W. Hussain, and N. B. Singh. 2011. Engineering cold stress tolerance in crop plants. Curr. Genomics 12:30–43.

Saruyama, H., and M. Tanida. 1995. Effect of chilling on activated oxygen-scavenging enzymes in low temperature-sensitive and -tolerant cultivars of rice (*Oryza sativa* L.). Plant Sci. 109:105–113.

Satake, T. 1969. Research on cold injury of paddy rice plants in Japan. Jpn. Agric. Res. Q. 4:5–10.

Satake, T. 1976. Sterile-type cool injury in paddy rice plants. Pp. 281–300 *in* International Rice Research Institute. Climate and rice. Los Baños, Philippines.

Satake, T., and H. Hayase. 1970. Male sterility caused by cooling treatment at the young microspore stage in rice plants. V. Estimation of pollen developmental stage and the most sensitive stage to coolness. Proc. Crop Sci. Soc. Jpn. 39:468–473.

Sato, Y., Y. Masuta, K. Saito, S. Murayama, and K. Ozawa. 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.

Seki, M., A. KameiK. Yamaguchi-Shinozaki, and K. Shinozaki. 2003. Molecular responses to drought, salinity and frost: common and different paths for plant protection. Curr. Opin. Plant Biol. 14:194–199.

Seo, M., H. Aoki, H. Koiwai, Y. Kamiya, E. Nambara, and T. Koshiba. 2004. Comparative studies on the *Arabidopsis* aldehyde oxidase (AAO) gene family revealed a major role of AAO3 in ABA biosynthesis in seeds. Plant Cell Physiol. 45:1694–1703.

Shimono, H., M. Okada, E. Kanda, and I. Arakawa. 2007. Low temperature-induced sterility in rice: evidence for the effects of temperature before panicle initiation. Field Crops Res. 101:221–231.

Shirasawa, S., T. Endo, K. Nakagomi, M. Yamaguchi, and T. Nishio. 2012. Delimitation of a QTL region controlling cold tolerance at booting stage of a cultivar, 'Lijiangxintuanheigu', in rice, *Oryza sativa* L. Theor. Appl. Genet. 124:937–946.

Singh, R. P., J. P. Brennan, T. Farrell, R. Williams, R. Reinke, L. Lewin, et al. 2005. Economic analysis of breeding for improved cold tolerance in rice in Australia. Aust. Agribus. Rev. 13:1–9.

Snell, P., D. Johnston, and R. Ford. 2008. Cold tolerant rice varieties – a matter of need for Australia. IREC Farmers' Newslett. 177:4–5.

Song, S. Y., Y. Chen, J. Chen, X. Y. Dai, and W. H. Zhang. 2011. Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress. Planta 234:331–345.

Sthapit, B. R., and J. R. Witcombe. 1998. Inheritance of tolerance to chilling stress in rice during germination and plumule greening. Crop Sci. 38:660–665.

Stirbet, A., and Govindjee. 2011. On the relation between the Kautsky effect (chlorophyll a fluorescence induction) and photosystem II: basics and applications of the OJIP fluorescence transient. J. Photochem. Photobiol., B 104:236–257.

Su, C. F., Y. C. Wang, T. H. Hsieh, C. A. Lu, T. H. Tseng, and S. M. Yu. 2010. A novel MYBS3-dependent pathway confers cold tolerance in rice. Plant Physiol. 153:145–158.

Suh, J. P., J. U. Jeung, J. I. Lee, Y. H. Choi, J. D. Yea, P. S. Virk, et al. 2010. Identification and analysis of QTLs controlling cold tolerance at the reproductive stage and validation of effective QTLs in cold-tolerant genotypes of rice (*Oryza sativa* L.). Theor. Appl. Genet. 120:985–995.

Sweeney, M., and S. McCouch. 2007. The complex history of the domestication of rice. Ann. Bot. 100:951–957.

Takahashi, N. 1984. Differentiation of ecotypes in *Oryza sativa* L. Pp. 31–67 *in* N. Takahashi and S. Tsunoda, eds. Biology of rice. Japan Science Society, Tokyo, Japan.

Takesawa, T., M. Ito, H. Kanzaki, N. Kameya, and I. Nakamura. 2002. Over-expression of *glutathione S-transferase* in transgenic rice enhances germination and growth at low temperature. Mol. Breeding 9:93–101.

Takeuchi, Y., H. Hayasaka, B. Chiba, I. Tanaka, T. Shimano, M. Yamagishi, et al. 2001. Mapping quantitative trait loci controlling cool-temperature tolerance at booting stage in temperate *japonica* rice. Breed. Sci. 51:191–197.

Tao, Z., Y. Kou, H. Liu, X. Li, J. Xiao, and S. Wang. 2011. *OsWRKY45* alleles play different roles in abscisic acid signaling and salt stress tolerance but similar roles in drought and cold tolerance in rice. J. Exp. Bot. 62:4863–4874.

Theocharis, A., C. Clément, and E. A. Barka. 2012. Physiological and molecular changes in plants at low temperatures. Planta 235:1091–1105.

Tian, Y., H. Zhang, X. Pan, X. Chen, Z. Zhang, X. Lu, et al. 2011. Overexpression of ethylene response factor *TERF2* confers cold tolerance in rice seedlings. Transgenic Res. 20:857–866.

Varshney, R. K., S. N. Nayak, G. D. May, and S. A. Jackson. 2009. Next-generation sequencing technologies and their implications for crop genetics and breeding. Trends Biotechnol. 27:522–530.

Wang, G., and Z. Guo. 2005. Effects of chilling stress on photosynthetic rate and chlorophyll fluorescence parameter in seedlings of two rice cultivars differing cold tolerance. Rice Sci. 12:187–191.

Wang, J., C. Zhang, G. Chen, P. Wang, D. Shi, and C. Lu. 2006. Responses of photosynthetic functions to low temperature in flag leaves of rice genotypes at the milky stage. Rice Sci. 13:113–119.

Wang, Q., Y. Guan, Y. Wu, H. Chen, F. Chen, and 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.

Wen, J. Q., K. Oono, and R. Imai. 2002. Two novel mitogen-activated protein signaling components, OsMEK1

and OsMAP1, are involved in a moderate low-temperature signaling pathway in rice. Plant Physiol. 129:1880–1891.

Xie, G., H. Kato, and R. Imai. 2012. Biochemical identification of the OsMKK6-OsMPK3 signaling pathway for chilling stress tolerance in rice. Biochem. J. 443:95–102.

Xiong, L., and Y. Yang. 2003. Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. Plant Cell 15:745–759.

Xu, L. M., L. Zhou, Y. W. Zeng, F. M. Wang, H. L. Zhang, S. Q. Shen, 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.

Xu, M., L. Li, Y. Fan, J. Wan, and L. Wang. 2011. *ZmCBF3* overexpression improves tolerance to abiotic stress in transgenic rice (*Oryza sativa*) without yield penalty. Plant Cell Rep. 30:1949–1957.

Yan, S. P., Q. Y. Zhang, Z. C. Tang, W. A. Su, and W. N. Sun. 2006. Comparative proteomic analysis provides new insights into chilling stress responses in rice. Mol. Cell. Proteomics 5:484–496.

Yang, A., X. Dai, and W. H. Zhang. 2012. A R2R3-type MYB gene, *OsMYB2*, is involved in salt, cold, and dehydration tolerance in rice. J. Exp. Bot. 63:2541–2556.

Ye, H., H. Du, N. Tang, X. Li, and L. Xiong. 2009. Identification and expression profiling analysis of TIFY family genes involved in stress and phytohormone responses in rice. Plant Mol. Biol. 71:291–305.

Ye, C., S. Fukai, I. D. Godwin, H. Koh, R. Reinke, Y. Zhou, et al. 2010. A QTL controlling low temperature induced spikelet sterility at booting stage in rice. Euphytica 176:291–301.

Yoshida, S. 1981. Pp. 1–63 *in* Fundamentals of rice crop science. International Rice Research Institute, Los Baños.

Zhang, Z., and 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. H., L. Su, W. Li, W. Chen, and Y. G. Zhu. 2005. A major QTL conferring cold tolerance at the early seedling stage using recombinant inbred lines of rice (*Oryza sativa* L.). Plant Sci. 168:527–534.

Zhang, Y., C. Chen, X. F. Jin, A. S. Xiong, R. H. Peng, Y. H. Hong, et al. 2009. Expression of a rice *DREB1* gene, *OsDREB1D*, enhances cold and high-salt tolerance in transgenic *Arabidopsis*. BMB Reports 42:486–492.

Zhang, J., J. Li, X. Wang, and J. Chen. 2011. *OVP1*, a vacuolar H^+-translocating inorganic pyrophosphatase (V-PPase), overexpression improved rice cold tolerance. Plant Physiol. Biochem. 49:33–38.

Zhou, Y., Y. Zeng, W. Zheng, B. Tang, S. Yang, H. Zhang, et al. 2010. Fine mapping a QTL *qCTB7* for cold tolerance at the booting stage on rice chromosome 7 using a near-isogenic line. Theor. Appl. Genet. 121:895–905.

Anexo 2: artigo não relacionado ao tema da Tese, submetido para publicação no periódico *Journal of Plant Nutrition and Soil Science*.

Suppression of iron toxicity in rice plants by alternative irrigation management

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Keywords: *Oryza sativa*, soil solution, suppression of irrigation, iron toxicity, BR-IRGA 409, IRGA 425

Abstract

Iron toxicity is a major nutritional disorder in rice plants, especially in flooded areas. The use of alternative crop management practices, such as interruptions in irrigation, may mitigate negative impacts of iron toxicity in rice. This study aimed to evaluate the impact of alternative water management on agronomical and physiological parameters in rice plants from two cultivars, resistant and sensitive to iron toxicity, grown in a field location with iron toxicity history. Rice (*Oryza sativa* L. ssp. *indica*) cultivars BR-IRGA 409 (sensitive) and IRGA 425 (resistant to iron toxicity) were tested. Irrigation management comprised three treatments: continuous irrigation (CI), one cycle of water suppression between stages V_6 and V_8 (1S) and two cycles of water suppression, between stages V_6 and V_8 and between V_8 and V_{10} (2S). Evaluations included the ionic composition of soil

solution and leaf tissues, grain yield, antioxidant responses and gene expression. Grain yield was higher in IRGA 425 plants than in BR-IRGA 409 under CI and 1S treatments. However, the 2S treatment resulted in increased grain yield in BR-IRGA 409. With two cycles of irrigation suppression, the MDA concentration in the sensitive cultivar decreased to levels equivalent to the ones found in the resistant cultivar. Resistance to iron toxicity in IRGA 425 plants seem be related to limited Fe translocation to shoots, increased tolerance to oxidative stress in leaves and higher expression of Ferritin genes. Plants from the BR-IRGA 409 cultivar (sensitive to Fe toxicity) improved growth, development and yield under suppression of irrigation, probably due to lower levels of oxidative stress.

Anexo 3: artigo não relacionado ao tema da Tese, submetido para publicação no periódico *Journal of Experimental Botany*.

Physiological and molecular alterations promoted by *Schizotetranychus oryzae* mite infestation in rice leaves

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Abstract

Infestation of phytophagous mite *Schizotetranychus oryzae* in rice leaves causes critical rice yield losses. To better understand the rice-*S. oryzae* interaction, we employed a proteomic approach to identify differentially expressed proteins in healthy and infested rice leaves. Multidimensional Protein Identification Technology (MudPIT) analysis led to the identification of 19 unique proteins in control and 872 in infested-leaves, respectively. We detected 1,574 proteins that were expressed in both conditions. Of these, 32 were statistically more abundant in control leaves. *S. oryzae* infestation caused decreased abundance of proteins related to photosynthesis (mostly photosystem II-related, confirmed by chlorophyll *a* fluorescence experiments), carbon assimilation and energy production, chloroplast reactive oxygen species detoxification, defense mechanism (protease inhibitors), fatty acid biosynthesis and gibberellin levels (reduced growth). On the other hand, infestation caused increased abundance of proteins involved in jasmonate biosynthesis, protein modification and degradation, amino acid synthesis, gene expression at the translation level, protein partitioning to different organelles, lipid metabolism (β-oxidation of fatty acids), actin cytoskeleton remodeling and molecular chaperones

synthesis. Our results also suggest that *S. oryzae* infestation promotes cell wall remodeling and interferes with ethylene biosynthesis in rice leaves. Proteomic data were positively correlated with enzymatic assays and RT-qPCR results of selected proteins/genes. Our findings describe the protein expression patterns of rice leaves infested by *S. oryzae*, and suggest that the acceptor side of PSII is probably the major damaged target in the photosynthetic apparatus of rice plants infested by phytophagous mites. These data will be useful in future biotechnological approaches aiming to induce phytophagous mite resistance in rice.

Keywords

MudPIT; photosynthesis; phytophagous mite; rice infestation; *Schizotetranychus oryzae*; shotgun proteomics.

Anexo 4: Atividades (não curriculares) realizadas durante o curso de Doutorado.

Resumos expandidos publicados em anais de congressos:

ADAMSKI, J. M.; TERRA, T.F.; CARGNELUTTI, D.; SPEROTTO, R.A.; CRUZ, R.P.; ROSA, L.M.G.; FETT, J.P. Alteration of photosynthetic and oxidative metabolism under low temperature stress in two indica rice genotypes contrasting in cold tolerance. In: VIII Congresso Brasileiro de Arroz Irrigado, 2013, Santa Maria. p. 237-240.

SILVA, C.S.F.; PEIXOTO, C.R.M.; CASTILHOS, H.; **ADAMSKI, J.M.**; JERENKOW, J.A.; MOURA, N.F. Composição fitoquímica do falso guaraná (Bunchosia glandulifera). In: III Simpósio Internacional de Plantas Medicinais e Nutracêuticas (3ISMNP)/III Conferência do Instituto Nacional de Ciência e Tecnologia de Frutos Tropicais, 2012, Aracaju.

Resumos publicados em anais de congressos:

DAMETTO, A.; **ADAMSKI, J.M.**; TERRA, T.F.; CARGNELUTTI, D.; SPEROTTO, R.A.; RICACHENEVSKY, F.; FETT, J.P. Deep RNAseq analysis in two contrasting indica rice genotypes reveals cold tolerance mechanisms during seed germination. In: 60 Congresso Brasileiro de Genética, 2014, Guarujá, 2014.

BLASI, E.A.R.; BUFFON, G.; SILVA, R.Z.; STEIN, C.; **ADAMSKI, J.M.**; SILVA, W.O.B.; SPEROTTO, R.A. Physiological and molecular alterations in leaves of rice plants infested by *Schizotetranychus oryzae* (Acari: Tetranychidae). In: XV Congreso Latino Americano, XXX Reunión Argentina de Fisiologia Vegetal, 2014, Mar del Plata.

ADAMSKI, J.M.; SPEROTTO, R.A.; TERRA, T.F.; SOROKA, V.; FETT, J.P. Identification of genes associated with cold tolerance in indica rice genotypes during the vegetative stage. In: IV Simpósio Brasileiro de Genética Molecular de Plantas, 2013, Bento Gonçalves.

SPEROTTO, R.A.; **ADAMSKI, J.M.**; SOROKA, V.; CARGNELUTTI, D.; CRUZ, R.P.; FETT, J.P . Deep RNA sequencing reveals cold tolerance mechanisms of indica rice plants during early vegetative stage. In: IV Simpósio Brasileiro de Genética Molecular de Plantas, 2013, Bento Gonçalves, 2013.

ADAMSKI, J.M.; TERRA, T.F.; SPEROTTO, R.A.; FETT, J.P. Participação do gene *WCOR413* na resposta ao frio em linhagens de arroz (*Oriza sativa* L.) tolerante e sensível a baixa temperatura. In: XIV Congresso Brasileiro de Fisiologia Vegetal, 2013, Poços de Caldas.

ADAMSKI, J.M.; TERRA, T.F.; SOROKA, V.; ROSA, L.M.G.; FETT, J.P. Fluorescência da clorofila em genótipos contrastantes de arroz da subespécie indica em relação à tolerância ao frio. In: 63 Congresso Nacional de Botânica, 2012, Joinville.

TERRA, T.F.; **ADAMSKI, J.M.**; SOROKA, V.; CARGNELUTTI, D.; FETT, J P. Mecanismo antioxidante em genótipos contrastantes de arroz da subespécie indica em relação à tolerância ao frio. In: 63 Congresso Nacional de Botânica, 2012, Joinville.

CARVALHO, J.B.; **ADAMSKI, J.M.**; CARGNELUTTI, D.; CARMONA, F.C. ; FETT, J.P. Mecanismo antioxidante em resposta ao excesso de ferro em arroz sob condições de manejo de irrigação com intermitência. In: 63 Congresso Nacional de Botânica, 2012, Joinville.

Formação complementar

Curso Teórico-Prático de Citometria de Fluxo em Plantas. (Carga horária: 10h). Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

Fluorescência da clorofila. (Carga horária: 20h). Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

The soil, the leaf and the ecosystem. (Carga horária: 45h). Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

Co-orientação de alunos de iniciação científica:

1 - Suellen Godoy da Silva (acadêmica do curso de Agronomia – UFRGS).

2 - Amanda Rodrigues (acadêmica do curso de Biotecnologia – UFRGS).

3- Fernanda Otesbelgue (acadêmica do curso de Biotecnologia – UFRGS).

4 - Vinícius Duarte Soroka (acadêmico do curso de Biotecnologia – UFRGS).