(i) Title: Rice calcium-dependent protein kinase OsCPK17 targets plasma membrane intrinsic protein and sucrose phosphate synthase and is required for a proper cold stress response

Running title: OsCPK17 role in cold stress signaling

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ABSTRACT

Calcium-dependent protein kinases (CDPKs) are involved in plant tolerance mechanisms to abiotic stresses. Although CDPKs are recognized as key messengers in signal transduction, the specific role of most members of this family remains unknown. Here we test the hypothesis that OsCPK17 plays a role in rice cold stress response by analyzing *OsCPK17* knockout, silencing, and overexpressing rice lines under low temperature. Altered *OsCPK17* gene expression compromises cold tolerance performance, without affecting the expression of key cold stress-inducible genes. A comparative phosphoproteomic approach led to the identification of six potential *in vivo* OsCPK17 targets, which are associated with sugar and nitrogen metabolism, and with osmotic regulation. To test direct interaction, *in vitro* kinase assays were performed, showing that the sucrose phosphate synthase OsSPS4, and the aquaporin OsPIP2;1/OsPIP2;6 are phosphorylated by OsCPK17 in a calcium-dependent manner. Altogether, our data indicates that OsCPK17 is required for a proper cold stress response in rice, likely affecting the activity of membrane channels and sugar metabolism.

Rice production is severely affected by different abiotic stresses, including cold. Cold perception is mediated by calcium signals that activate kinases to elicit the adequate cellular response. In this work, we show the involvement of the rice calcium-dependent protein kinase 17 (OsCPK17) in such a process. We show that altered *OsCPK17* gene expression in transgenic lines affects cold tolerance performance,

and that OsCPK17 targets proteins are associated with osmotic regulation, and sugar and nitrogen metabolism.

Keyword index: CDPK, abiotic stress, cold stress, signal transduction, phosphoproteomics, phosphorylation

INTRODUCTION

Rice is essential to feed the human population, and is a key component in food security programs. Over half of the human population uses rice as a staple food. Worldwide rice production is, however, severely constrained by different abiotic stresses. In particular, low temperatures (atmospheric or irrigation waters) limit rice yield in several countries, including some of the world top rice producers such as China, India, Thailand, the Philippines, Brazil, Japan, and Korea (Cruz et al. 2013). Although the rice plant is more sensitive to cold stress at the booting stage, its growth and development can be negatively affected by cold stress at any developmental stage, resulting in lower productivity (Andaya and Mackill 2003, Li et al. 1981; Satake and Hayase 1970, Ye et al. 2009a).

Plants respond and adapt to abiotic stress via a diversity of molecular and physiological mechanisms. Calcium (Ca^{2+}) signaling plays a vital role in a plant's perception and response to stress stimuli. When the organism senses a stressful condition, induced transient elevations of cytosolic Ca^{2+} concentration are decoded by different calcium binding proteins. Among these proteins, the calcium-dependent protein kinases (CDPKs) are essential sensor-transducer molecules that were shown

to be involved in the regulation of plant response to several environmental stresses (reviewed in Boudsocq and Sheen 2013, Schulz et al. 2013).

CDPKs are typically composed of three domains: 1) an N-terminal domain, 2) a serine/threonine kinase domain, and 3) a CDPK activation domain (CAD), with usually four functional EF-hands (Liese and Romeis 2013, Schulz et al. 2013). Upon Ca^{2+} binding, the protein undergoes a conformational alteration and exposes its active site (Liese and Romeis 2013, Schulz et al. 2013, Wernimont et al. 2010). The CDPK is then prone to autophosphorylation and/or to promote phosphorylation of its downstream targets, which include transcription factors, ion channels, or metabolic enzymes (Boudsocq and Sheen 2013).

CDPKs are found across the plant kingdom, as well as in some protozoans (Harmon et al. 2001). In plants, large multigene families of CDPKs have been identified, e.g. 31 members in rice (Ray et al. 2007, Ye et al. 2009b), and 34 in *Arabidopsis* (Uno et al. 2009). Moreover, CDPKs isoforms seem to be involved in different signal transduction pathways (Boudsocq and Sheen 2013, Harmon et al. 2001). Supporting this idea, they were found in various subcellular locations (Dammann et al. 2003, Harper et al. 2004), and while some CDPKs are ubiquitously expressed, others are only expressed upon particular stimuli or in specific tissues (Asano et al. 2012, Boudsocq and Sheen 2013, Campo et al. 2014, Sebastià et al. 2004, Zhang et al. 2014, Zhang and Chollet 1997). In rice, several CDPKs were shown to be involved in different aspects of the abiotic stress response. Namely, *OsCPK13* increases the plant tolerance to salinity, cold and drought in transgenic rice plants (Saijo et al. 2000) while *OsCPK4* is involved in salt and drought stress responses (Campo et al. 2014). *OsCPK21* positively regulates ABA and salt

signaling pathways (Asano et al. 2011). *OsCPK7* is involved in the sensing of sugar starvation and plays a major role in drought tolerance (Ho et al. 2013), while *OsCPK23* functions as a sucrose synthase kinase (Asano et al. 2002). Additionally, *OsCPK18* was associated with biotic stress response (Campos-Soriano et al. 2011, Xie et al. 2014), and *OsCPK19* and *OsCPK25/26* with aspects of rice development (Morello et al. 2000, Frattini et al. 1999, Zhang et al. 2014).

Although it has been observed that several CDPK genes are transcriptionally modulated by cold stress (Kong et al. 2013, Li et al. 2008, Ray et al. 2007, Wan et al. 2007), only a few were functionally characterized under low temperature. In rice and *Arabidopsis*, to our knowledge, only *OsCPK7*, *OsCPK13*, and *AtCPK1*, have been analyzed and implicated in cold stress response (Abbasi et al. 2004, Bohmer and Romeis 2007, Komatsu et al. 2007, Saijo et al. 2000). In addition, *PeCPK10*, in *Populus euphratica* (Chen et al. 2013), and *VaCPK20*, in *Vitis amurensis* (Dubrovina et al. 2015), were identified as positive regulators of cold stress tolerance, whereas *ZmCPK1*, in *Zea mays*, has been shown to act as a negative regulator of cold stress signaling (Weckwerth et al. 2015). Evidence indicates that CDPKs certainly participate in plant cold stress tolerance. However, the mechanisms of action remain mostly elusive (Boudsocq and Sheen 2013). In particular, *OsCPK17* (LOC_Os07g06740) contains multiple stress-responsive cis-elements and has been shown to be transcriptionally-regulated under biotic and abiotic stresses (Wan et al. 2007). Moreover, *OsCPK17* has been identified as co-localizing with four QTLs related to ion homeostasis and associated with salinity response (Negrão et al. 2011). In previous studies, when comparing nearly 400 rice accessions by EcoTILLING, we could identify five allelic variants of *OsCPK17*, although no

correlation between these coding region variations and salt-associated phenotypes could be found (Negrao et al. 2013).

Here we provide evidence supporting the role of OsCPK17 in rice cold stress response. By combining the characterization of rice transgenic lines with phosphoproteomics and *in vitro* biochemical analysis, we demonstrate that OsCPK17 is an active calcium-dependent protein kinase that activates a rice cold stress response mechanism. The network regulated by OsCPK17 involves the phosphorylation of aquaporin channels and a mild attenuation of primary metabolism.

MATERIALS AND METHODS

Screening loss of function line

The T-DNA insertion line for *OsCPK17* (4A-50582) was obtained from Postech (www.postech.ac.kr/life/pfg/risd, developed by Gynheung An). The wild-type (WT) background of this line is the accession Dongjin (*O. sativa ssp. japonica* cv. Dongjin). The insertion line seeds were germinated, self-crossed and screened by PCR analysis for homozygosity. Genotyping primers are listed in Table S1 (OsCPK17_gen_Fw/Rv and T_DNA_LeftB). The T-DNA insertion position in *OsCPK17* was determined by sequencing. As a second T-DNA insertion was confirmed in locus Os01g04470, self-crossed progeny was selected to exclude this additional T-DNA insertion. The expression of *OsCPK17* in the *oscpk17* line was analyzed by semi-quantitative RT-PCR (RT-PCR), as described below, with primers OsCPK17-RT-Fw and OsCPK17-RT-Rv (Table S1).

Transformation and screening of transgenic plants

To develop rice transgenic plant lines overexpressing *OsCPK17*, the coding region of *OsCPK17* was amplified from WT cDNA samples, using primers *OsCPK17_OE_Fw/Rv* (Table S1). The fragment was initially inserted into pJET 1.2/blunt cloning vector (Thermo Scientific) and subsequently digested with *Bam*HI and *Bsp*120I. The digested fragment was then ligated (T4 ligase, Thermo Scientific) to pE3C vector (Dubin et al. 2008), previously digested with *Bam*HI and *Not*I, that adds a six c-MYC tag at the protein C-terminal. The *OsCPK17*-MYC sequences were verified by sequencing and then transferred to pH7WG3, a vector modified from the pH7WG2 (University of Ghent, Belgium), under the control of the maize ubiquitin promoter (*Ubi1*) (Christensen and Quail 1996, Lourenço et al. 2011) (Figure 1C).

To generate *OsCPK17* RNA interference (RNAi) silencing lines, a fragment of the last exon plus 3'UTR of *OsCPK17* was isolated by PCR (primers *iRNAattB1_Fw/Rv*, in Table S1) and cloned in pDONR221 (Gateway system, InvitrogenTM). The sequence was confirmed by sequencing and transferred to pANDA vector (Miki and Shimamoto 2004).

Both constructs pH7WG3:*OsCPK17*-MYC and pANDA:*OsCPK17* were verified by restriction digestion and sequencing and introduced independently into *Agrobacterium tumefaciens*, strain EHA105. *Agrobacterium*-mediated transformation of rice was performed as described in Hiei and Komari (2008), using calluses induced from mature seeds of Dongjin accession. Transgenic plants were selected by hygromycin resistance and PCR genotyping. The T₃ or T₄ seeds of the transgenic lines were used for this work.

Growth conditions and stress treatment

To characterize the behaviour of the transgenic lines under control growth conditions, seeds were heat-treated at 50°C, for 5 days, to break dormancy, and germinated at 28°C, in the dark for 2 days. Uniformly germinated seedlings were transferred to soil pots with 5 plants per pot (4 pots per genotype), and grown in the greenhouse. Several growth parameters were evaluated: germination rate, heading date, tiller number, plant height, panicle length (principal panicle), grain weight per plant, number of grains per plant, and seed fertility (measured as the percentage of filled spikelets in the principal panicle).

A cold acclimation assay was set as described in Almeida et al. (2016). Briefly, after breaking dormancy and germinating the seeds in water-soaked filter paper, the uniformly germinated seedlings were transferred to seedling floats on trays with Yoshida solution (Yoshida 1976). Plants were placed in a growth chamber with 50-70% relative humidity, at 28°C, with a 12h photoperiod, under cool-white lights (200 $\mu\text{mol}/\text{m}^2/\text{s}$). Three-leaf stage seedlings were then submitted to a pre-treatment of 24h at 10°C (acclimation), followed by a chilling period of 18 days (5°C/4°C, day/night). The irradiance was reduced from 200 $\mu\text{mol}/\text{m}^2/\text{s}$ to 100 $\mu\text{mol}/\text{m}^2/\text{s}$.

For biochemical and molecular analyses, shoot samples (from a minimum of 5 plants per time point and per plant line) were collected at 0h, 30 min, 3h, 24h and 72h after incubation at low temperature. The samples were flash frozen in liquid nitrogen and stored at -80°C until analysis. All samples were reduced to a fine powder in liquid nitrogen.

Electrolyte leakage

The percentage of electrolyte leakage was measured before (datapoint 0) and after 4, 7, 10 and 18 days of cold stress imposition, as described in Almeida et al. 2016.

The second fully expanded leaves (six replicates from 3-5 random plants per line and timepoint) were used for the measurements. The electrical conductivity of each sample (S1) was measured in a GLP31 conductometer (Crison, Spain), and the relative degree of electrolyte leakage was calculated using the formula: $EL (\%) = S1/S2 \times 100$.

Gene expression analysis

Total RNA was extracted from shoots using Zymo Research Direct-zol RNA miniprep, following manufacturer's instructions, and was then treated with TURBO DNA-free kit (Ambion, Life Technologies) to eliminate any possible DNA contamination. The first-strand cDNA was synthesized from 2 µg total RNA with an anchored-oligo (dT)₁₈ primer, according to the instructions from Transcriptor High Fidelity cDNA Synthesis Kit (Roche).

For gene expression analysis by semi-quantitative RT-PCR (RT-PCR), the gene-specific primers listed in Table S1 were used. For the PCR amplification, 2 µL of 5-fold diluted cDNA was used in a 20 µL reaction mixture, with a first cycle of 5 min at 95°C, followed by 23-35 cycles of 30 s at 95°C, 40 s at 58-61°C, 1 min at 72°C, and a final extension of 5 min at 72°C. After electrophoresis on a 1% agarose gel and staining with EtBr, the band intensities were quantified using ImageJ (<http://imagej.nih.gov/ij/>). The relative transcript level of each gene was normalized

for the amplification of the rice *Eukaryotic Elongation Factor 1- α* (*eELF1- α* ; Os03g08060) and expressed protein (*EP*; Os05g08980) (Maksup et al. 2013). Results were obtained from at least three technical replicates.

The quantitative real-time PCR (qPCR) analysis was performed using the LightCycler 480 system and the SYBR Green I Master mix (both from Roche, Basel, Switzerland). The qPCR reactions were carried out with the primers listed in Table S1. The PCR running conditions were: one cycle of 5 min at 95°C and 45 cycles of amplification of 10 s at 95°C, 10 s at 58–60°C and 10 s at 72°C. The CT values were calculated using three technical replicates and gene expression was assessed relative to the internal controls rice *Eukaryotic Elongation Factor 1- α* (*eELF1- α* ; Os03g08060) and expressed protein (*EP*; Os05g08980) (Maksup et al. 2013) transcripts.

Western blot

Total protein extracts were obtained by homogenizing frozen powdered shoots in 5 volumes (w/v) of Laemmli Buffer 5x (with 13% β -mercaptoethanol). After incubation at 99°C for 5 min, samples were centrifuged for 20 min at 12,000 $\times$ g, the supernatant collected to a new tube and further centrifuged (5 min) to ensure cell debris removal. The protein extracts were quantified using Pierce 660 nm Protein Assay Kit (Thermo Scientific), following manufacturer's instructions. For immunoblots, 30 μ g of protein extracts were resolved on a 10% SDS-PAGE gel and later transferred to PVDF membranes (Perkin Elmer). To obtain a loading control, membranes were stained with Ponceau S solution (Sigma-Aldrich) or Coomassie blue R350 (Sigma-Aldrich). For immunodetection of OsCPK17-MYC in

overexpressing lines, the membrane was probed overnight, at 4°C, with α -MYC (ABM), followed by 1h at RT with α -mouse horseradish peroxidase-conjugated antibody.

Sample preparation before nanoflow LC-MS/MS analysis

To a powdered sample of 150 mg, 1 mL of lysis buffer [7 M Urea (PlusOne, GE), 2 M Thiourea (PlusOne, GE), 30 mM Tris (PlusOne, GE), 4% (w/v) CHAPS (PlusOne, GE) supplemented with 1 μ M Pepstatin (Roche), Benzodase Mix (Merck), cOmplete EDTA-free protease inhibitor cocktail (Roche) and PhosStop phosphatase inhibitor cocktail (Roche)] was added. After incubation on ice for 10 min, each sample was centrifuged at 12,000 $\times g$ for 25 min at 4°C and the resulting supernatant was transferred to a new microtube. Samples were further centrifuged, four more times at 12,000 $\times g$ for 15 min each, until all debris was removed and a clear supernatant was obtained. Samples were freeze-dried and stored at -80°C until further analysis.

Protein extracts were processed and analyzed by LC-MS/MS at the MS & Proteomics Resource of the WM Keck Foundation Biotechnology Resource Laboratory services at Yale, USA (see Supplemental Experimental Procedures for details on sample preparation and MS analysis).

Carbohydrate quantification

Soluble sugars were extracted from approximately 50 mg powdered frozen shoots, as described in Ramalho et al. 2013. Briefly, samples were homogenized in cold MilliQ water (in a proportion of 1 mL per 75 mg of tissue) and placed on ice for 20 min, for extraction. Samples were then centrifuged at 12,000 x *g* for 5 min, at 4°C and the collected supernatant was boiled for 3 min for protein denaturation, cooled on ice for 6 min, centrifuged again at 12,000 x *g* for 5 min, at 4°C and filtered (0.45 µm, nylon). Sugar determination was performed by injection of 20 µL filtered extract aliquots into an HPLC system, equipped with a refractive index detector (Model 2414, Waters). Sugars were separated with a Sugar-Pak I column (300 x 6.5 mm, Waters) at 90°C, with H₂O as the eluent (containing 50 mg EDTA-Ca L⁻¹ H₂O) at a flow rate of 0.5 mL min⁻¹. Standard curves were used to quantify each sugar.

Recombinant OsCPK17 production

OsCPK17 coding sequence was amplified with primers OsCPK17_BamHI_Fw and OsCPK17_XhoI_Rv (Table S1). These primers carry recognition sites for *Bam*HI and *Xho*I, respectively, to clone in pET28 vector. The construct was confirmed by sequencing before transformation into *Escherichia coli* Rosetta pLysS cells (Invitrogen) for protein expression. Cells were grown in LB-medium until OD₆₀₀ reached 0.5. Induction of protein production was done with 200 mM isopropyl-d-1-thiogalactopyranoside (IPTG) for 4 h at 30°C. Cells were harvested by centrifugation at 6,400 x *g*, 4°C for 10 min. Cells were then resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM imidazole, 50 mg/L of lysozyme, 5 µg/mL

DNase, 10 mM MgCl_2) and frozen at -20°C . Before purification, 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to the cell extract. Protein extracts were loaded onto a HisTrap chelating column (Amersham Biosciences) and proteins eluted in 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 250 mM imidazole. The purified protein was analyzed by SDS-PAGE and by mass spectrometry on TripleTOF 6600 at the UniMS – Mass Spectrometry Unit, ITQB/IBET, Oeiras, Portugal, to confirm its correspondence to OsCPK17.

***In vitro* kinase assay**

OsCPK17 kinase activity was determined in 30 μL of phosphorylation buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl_2 and 1 mM CaCl_2 or 1 mM EGTA, for presence or absence of calcium, respectively), containing 0.125 μg of recombinant OsCPK17 and 100 μM of synthetic peptide (see Table S2) or 3 μg of histone III-S (Sigma-Aldrich), as substrates. The reactions were initiated by adding 100 μM ATP spiked with 2.5 μCi [γ ^{32}P]-ATP incubated for 30 min at 30°C and stopped by spotting an aliquot of 20 μL of each reaction on P81 phosphocellulose paper (1.5x1.5 cm, Millipore). The phosphocellulose papers were immediately immersed in 250 mL ice-cold 75 mM phosphoric acid, washed 3x for 10 min and allowed to air dry. Radiolabeled substrates were measured by scintillation counting. As a negative control, OsCPK17 was denatured for 20 min at 75°C before the phosphorylation assay.

Accession numbers

Sequence data from this article can be found in the Rice Database under the following accession numbers: OsFd-GOGAT (Os07g46460), OsPIP2;1 (Os07g26690), OsPIP2;6 (Os04g16450), OsSPS4 (Os08g20660), OsSPS5 (Os11g12810), OsNR1 (Os08g36480), OsPDHE1-A (Os02g50620), *eELF1- α* (Os03g08060), *EP* (Os05g08980), OsMYB4 (Os04g43680), OsDREB1b (Os09g35010), OsNAC6 (Os01g66120), OsP5CS1 (Os05g38150), OsLip9 (Os02g44870), OsUbc (Os02g42314).

RESULTS

Altered expression of OsCPK17 affects plant growth and seed production

CDPKs are described as involved in signaling pathways regulating not only plant growth and development but also plant response to biotic and abiotic stresses (Boudsocq and Sheen 2013, Schulz et al. 2013). To investigate the biological function of *OsCPK17*, we used a T-DNA insertion line (4A-50582, from Postech) with ‘Dongjin’ genetic background. We obtained the progeny seeds from primary transgenic plants and selected the *oscpk17* homozygous plants from the segregating population using a PCR-based strategy. We localized, by sequencing, the T-DNA insertion in the first intron of *OsCPK17* (908 bp from ATG, 127 bp from intron start) (Figure 1A). We then assessed the *OsCPK17* transcript levels by semi-quantitative RT-PCR (RT-PCR) and by real-time PCR (qPCR) in the WT and the *oscpk17* line, respectively. We could not detect *OsCPK17* transcripts in the homozygous *oscpk17* plants (Figure 1B), thus, confirming the gene knockout in this line.

We developed *OsCPK17* overexpressing and silencing lines (Figure 1C-D) to further characterize the role of *OsCPK17* in rice plants. Over 25 independent T_0 *OsCPK17* RNAi lines were obtained, with a transformation success rate of over 2.25%. We then selected the lines with the lowest *OsCPK17* gene expression, RNAi2, and RNAi29, with approx. 15% and 20% of the WT *OsCPK17* expression, respectively (Figure 1B). We observed a very low transformation success (around 0.025%) for *OsCPK17* overexpression lines, with only two independent lines obtained, OX1 and OX2. The T_0 overexpressing plants displayed growth retardation and a severely low fertility rate, but the following generations showed an attenuation of this phenotype, in particular of growth retardation. This milder phenotype can be correlated to a reduction in *OsCPK17*-MYC levels throughout the generations (Figure 1E). We used T_3 or T_4 seeds of all the transgenic lines in further studies. In T_4 plants, the overexpression level of *OsCPK17* in OX1 and OX2 seedlings (compared to WT), was estimated by RT-PCR to be at least 2- or 3-fold, respectively (Figure 1B).

Next, we evaluated the effects of *OsCPK17* altered expression on plant growth and development under normal growth conditions. The germination rate of the lines was not affected, with all transgenic plants showing a 99% germination rate. Moreover, the transgenic plants appeared to have WT-like developmental stages, including similar flowering time, and also similar photosynthetic capacity (Figure S1). The most significant differences observed were related to plant height and spikelet fertility (Figure 1F-H and Figure S2). Overexpressing lines were significantly shorter than WT (Figure 1F). While the number of spikelets per panicle was similar, spikelet fertility was affected in all transgenic lines (Figure 1G and 1H). The severity of the phenotype could be correlated to the levels of *OsCPK17* transcripts with silencing

lines showing an intermediate phenotype to *oscpk17*. Overexpressing lines were an exception to this tendency, suggesting that the control of the expression of OsCPK17 is more important than its levels, and thus that the ectopic expression of OsCPK17 has an adverse impact on plant performance.

OsCPK17 is an active kinase

To confirm the kinase activity of OsCPK17 and to assess its calcium dependence, we produced recombinant OsCPK17 in *Escherichia coli* and performed *in vitro* kinase assays in the presence or absence of calcium, using histone III-S as a substrate. OsCPK17 has kinase activity that is calcium-dependent, as clearly shown in Figure 2.

Controlled regulation of OsCPK17 levels is critical for proper rice responses to cold stress

To clarify the putative function of *OsCPK17* in cold stress response, we monitored the performance of WT, *oscpk17*, OX1, OX2, RNAi2 and RNAi29 under low temperature conditions at seedling stage. At this stage, rice is particularly sensitive to cold stress, which can have dramatic consequences for seedling development and survival. In a preliminary assay, we found that the application of an acclimation period considerably enhances the survival rate after cold stress, as compared to the direct imposition of chilling temperatures (cold shock) (Figure S2). This result agrees with previous studies (Pan et al. 2013). Thus, to induce a cold acclimation response, we exposed seedlings to temperatures of 5°C/4°C (day/night) during 18 days (chilling

period), after a pre-treatment period of one day at 10°C (acclimation period) (Figure 3A). After 18 days of cold treatment, leaves of WT plants revealed considerably less visual cold injury symptoms than all other lines (Figure 3B). OX1, OX2, and *oscpk17* lines showed high leaf chlorosis and rolling, with the overexpressing lines also showing a high degree of wilting. RNAi lines displayed intermediate phenotypes between WT and *oscpk17* plants.

Low temperatures can affect cell membranes rapidly, triggering an increased membrane rigidity and leading to increased electrolyte leakage (EL) (Campos et al. 2003, Cruz et al. 2013, Theocharis et al. 2012). As an indirect estimation of membrane injury promoted by cold, we evaluated shoot EL at five time points, namely: immediately before transferring the seedlings to cold (0), and after 4, 7, 10 and 18 days of cold exposure. The obtained data is consistent with the cold injury phenotypes described above. As shown in Figure 3C, WT plants maintained a fairly low level of EL throughout the cold incubation period while *oscpk17* plants (knockouts) could only avoid the leakage of electrolytes in the first seven days at low temperature. After this period, *oscpk17* plants presented an increasingly higher degree of membrane injury symptoms. RNAi silencing lines revealed intermediate EL profiles between WT and *oscpk17*. Also, OX1 and OX2 showed the highest EL, from the very beginning of the cold treatment.

These results suggest that both knockout and overexpressing plants have a lower tolerance to cold stress, as compared to WT and RNAi lines, indicating the need for a precise regulation of *OsCPK17* gene expression for some cold stress adaptation in rice.

The *OsCPK17*-mediated rice response to cold stress does not affect the transcription of well-recognized cold stress markers

Several abiotic stresses regulate CDPKs at the transcriptional level. To check whether *OsCPK17* is also transcriptionally regulated by cold stress, we monitored *OsCPK17* transcript profile in shoots, by qPCR, during the first 72h of cold treatment. As can be seen in Figure 4A, the *OsCPK17* expression in WT seedlings is significantly downregulated by cold, with an approximate reduction of 85% after 72h stress. *OsCPK17* in OX1 plants was not affected by cold stress, suggesting that the protein levels are not affected by this treatment (Figure 4B).

To investigate the role of *OsCPK17* in the cold stress-signaling pathway, we examined the expression pattern of several key cold stress-inducible genes in the shoots of WT and *oscpk17* seedlings under the imposed cold stress conditions. We examined the profile of three transcription factors that are important regulons of cold response, namely: i) *OsDREB1B* (Dubouzet et al. 2003), which codes for the Drought Response Element Binding 1B; ii) *OsMyb4*, which encodes a Myb transcription factor (Pandolfi et al. 1997) and; iii) *OsNAC6* (Kikuchi et al. 2000), which encodes a NAC-type transcription factor. Moreover, we also examined the expression of downstream stress-inducible genes: *OsP5CS1*, which encodes a Δ^1 -pyrroline-5-carboxylate synthetase, involved in proline biosynthesis (Igarashi et al. 1997) and the dehydrin *OsLIP9*, which encodes a low-temperature-inducible protein (Aguan et al. 1991). We found similar cold-induced transcript profiles for all genes in WT and *oscpk17* seedlings (Figure 4C). From these results, it seems that the role of *OsCPK17* does not involve the transcriptional modulation of the most commonly recognized cold stress response mechanisms.

Phosphoproteomic analysis reveals putative OsCPK17 substrates

Despite the fact that *OsCPK17* expression is downregulated under cold stress, we anticipate the putative requirement of *OsCPK17* in the regulation of the initial phases of the cold acclimation process. To further investigate potential *in vivo* target proteins of *OsCPK17*, we performed a quantitative phosphoproteomic analysis by high-resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS), in whole protein extracts of WT and *oscpk17* seedlings, which enabled the comparison of global changes in phosphorylation. We analyzed seedling samples collected just before (0h, control conditions) and after incubation at 10°C for 30 min (30 min, cold acclimation conditions). This analysis allowed the identification of 58 unique phosphopeptides in 47 phosphoproteins, from a total of 2245 unique peptides identified (belonging to 487 proteins), using a false discovery rate threshold of 1%. Assuming that direct or indirect *OsCPK17* targets will increase their phosphorylation levels in WT plants when comparing with *oscpk17*, we then assessed the phosphorylation patterns of the two lines, upon cold stress imposition. We identified six peptides that showed unaltered phosphorylation levels in the *oscpk17* after cold stress while presenting increased levels in WT (Figure 5). Hence, these phosphopeptides are putative targets of *OsCPK17* in the initial process of cold stress acclimation. The details on the identified phosphopeptides and corresponding phosphoproteins are listed in Table 1.

We found a peptide common to the plasma membrane intrinsic protein 2-1 (*OsPIP2;1*) and *OsPIP2;6* that revealed a much higher phosphorylation level in WT cold-treated seedlings in residue S285 or S275, respectively (Figure 5 and S4.C-D). Phosphorylation in these C-terminus *OsPIP2;1*/*OsPIP2;6* residues was also reported in other studies (Chang et al. 2012, Nakagami et al. 2010, Whiteman et al. 2008,

Yao et al. 2014). OsPIP2;1/OsPIP2;6 are aquaporins and are likely playing a role as channel proteins, mediating the passive transport of water and small neutral solutes across cell membranes, similar to most members of this family (reviewed in Chevalier and Chaumont 2015, Maurel et al. 2015).

Increased cold-induced phosphorylation in WT was also observed for the chloroplastic ferredoxin-dependent glutamate synthase (OsFd-GOGAT), which is involved in leaf glutamate biosynthesis (Hayakawa et al. 2003, Suzuki and Knaff 2005). Glutamate and products from glutamate metabolism such as arginine, proline and γ -aminobutyric acid (GABA) play key roles in abiotic stress tolerance (Forde and Lea 2007). In our study, we found that OsFd-GOGAT is differentially phosphorylated in the residue S1300. To the best of our knowledge, this is the first report to describe the phosphorylation of OsFd-GOGAT.

Another protein with increased cold-induced phosphorylation in WT is the pyruvate dehydrogenase E1 component subunit alpha-1 (OsPDHE1-A) (Figure 5), which is the first component enzyme of the pyruvate dehydrogenase complex (mtPDC), localized in the mitochondria. mtPDC catalyzes the conversion of pyruvate into acetyl-CoA, and the reaction catalyzed by E1 is considered to be the rate-limiting step (Reid et al. 1977). The precise residue differentially phosphorylated in our analysis remains ambiguous: it could be either S293 or S295. Interestingly, the phosphorylation of S293 in rice OsPDHE1-A, also identified in other studies (Cheng et al. 2014, Han et al. 2014), corresponds to the mammalian phosphorylation site 1, which correlates with major inactivation of the complex (reviewed in Patel and Korotchikina 2001, Tovar-Mendez et al. 2003).

Nitrate reductase [NADH]1, OsNR1, presented higher phosphorylation in T533 in the WT seedlings after low temperature incubation (Figure 5). Nitrate reductase is a key enzyme involved in the first step of plant nitrate assimilation, synthesizing nitrate from nitrite (Campbell 1999). Phosphorylations in T531 and nearby residue S532 of OsNR1 were previously described, whereas S532 was revealed to be a conserved phosphorylated site in orthologous Arabidopsis AtNR1 and AtNR2 (Nakagami et al. 2010). Several studies have shown regulation of NR activity by phosphorylation of a serine residue localized in hinge 1: Arabidopsis (S534), spinach (S543) or tobacco (S521) (Figure S4.A-B) (Bachmann et al. 1996, Douglas et al. 1995, Lillo et al. 2003, Su et al. 1996). The phosphorylation of these residues seems to create a binding site for 14-3-3 proteins resulting in the inhibition of the NR activity. Additionally, NR mediates one of the possible pathways for nitric oxide (NO) production (Cantrel et al. 2011, Gupta et al. 2011), which plays an important role in the regulation of cold stress response (Cantrel et al. 2011, Zhao et al. 2009). The formation of NO, through NR, seems to be negatively affected by phosphorylation in the aforementioned 14-3-3 protein binding conserved site (Lea et al. 2004).

Interestingly, two members of the sucrose-phosphate synthase family, OsSPS4 and OsSPS5 (also named OsSPS8 and OsSPS11, respectively) were differentially phosphorylated upon cold stress in WT and *oscpk17* (Figure 5). SPSs are essential enzymes involved in sucrose synthesis. We observed a higher cold-induced phosphorylation level in residue S170 of OsSPS4 in WT as compared to *oscpk17* plants. This phosphorylation in OsSPS4 has been identified in other studies (Nakagami et al. 2010, Yao et al. 2014). We also identified a cold-regulated phosphosite, S105 in OsSPS5, which has not been previously described. However, S105 lays close to the phosphosite identified in OsSPS4 (i.e. S170) when protein

primary sequences of OsSPS4 and OsSPS5 are aligned (Figure S4.E-F). Also, we identified another phosphosite (S48) in OsSPS5, although with similar abundance in WT as compared to *oscpk17* lines. Because differences in phosphopeptide abundances could be related to changes in the protein amount, we quantified, whenever possible, the protein abundance for each of the putative targets identified. For this purpose, we calculated the sum of individual quantifications of all peptides identified for the target protein in each sample. OsFd-GOGAT and OsPDHE1-A revealed no significant differences in protein levels in all samples analyzed (Figure 6A). As for OsPIP2;1/ OsPIP2;6, the relatively small protein increase observed in the WT by cold treatment (approximately 1.3 and 2.1 fold for OsPIP2;1 and for OsPIP2;6, respectively) is not sufficient to explain the differences found in the corresponding phosphorylation patterns (28.6 fold increase, see Figure 5 and Figure 6A). On the other hand, for OsNR1, OsSPS4, and OsSPS5, total protein quantification was not possible because only phosphopeptides were detected. Thus, for these putative targets, we evaluated their gene expression by RT-PCR in WT and *oscpk17* seedlings throughout the cold stress period, and observed no significant transcriptional changes that could lead, per se, to distinct protein levels between the lines after 30 min of cold treatment (Figure 6B). The transient upregulation of *OsN/A1* 30 min after cold stress was similar in WT and *oscpk17* plants (Figure 6B), unlike the phosphorylation profiles of OsNR1 in both lines in the same period (Figure 5). Hence, there is no indication that the distinct phosphorylation patterns of OsNR1, OsSPS4 and OsSPS5 are able to be attributed to differences in protein amount, although we cannot rule out such hypothesis.

Next, we investigated whether the proteins identified as potential targets for OsCPK17 could be directly phosphorylated by this kinase. To address this question, we used an *in vitro* phosphorylation assay with purified recombinant OsCPK17, and synthetic peptides specifically designed for each of the putative target proteins as substrates (Table S2). While no *in vitro* phosphorylation of OsFd-GOGAT, OsNR1, OsPDHE1-A, and OsSPS5 was detected, OsSPS4 and OsPIP2;1/OsPIP2;6 peptides were revealed to be significantly phosphorylated *in vitro* by OsCPK17 (Figure 6C). This result suggests that the aquaporin OsPIP2;1/OsPIP2;6 and the sucrose-phosphate synthase OsSPS4 are direct substrates of OsCPK17, being phosphorylated by this enzyme upon incubation under cold stress conditions. As for the remaining putative targets, the modulation of their phosphorylation status by OsCPK17 is probably not due to a direct interaction, but instead, may result from the intermediary action of other proteins in a phosphorylation cascade regulated by OsCPK17. To investigate which of the two potential phosphorylation sites in the OsPIP2;1/OsPIP2;6 peptide (i.e. S285 or S288) is the target of OsCPK17, we designed additional peptides carrying mutations for each or both serine to alanine residues. Our *in vitro* kinase assays unequivocally showed that S285 is the sole target for OsCPK17 within our peptide (Figure 6D).

Considering the fundamental role that sucrose metabolism plays in plant adaptation to cold stress (Morsy et al. 2007, Ruan 2014, Theocharis et al. 2012), the regulation of sucrose biosynthesis by SPSs is undoubtedly an important issue. Moreover, in the phosphoproteomic analysis, we found that OsCPK17 might lead, directly or indirectly, to the phosphorylation of two SPSs at the beginning of cold stress imposition. Therefore, we sought to investigate the OsCPK17 effect on sucrose biosynthesis further by determining sucrose content in shoots of WT,

oscpk17, and OX1 plants, under cold conditions. No relevant differences were observed in shoot sucrose content during the first 3h of cold treatment. However, with longer cold incubation periods (24h and 72h) WT seedlings seem to accumulate higher amounts of sucrose than OX1, whereas *oscpk17* show an intermediate value (Figure 7).

DISCUSSION

Low temperature significantly affects rice productivity worldwide. Along with the threat of global warming, climate change is also associated with frequent and unpredictable temperature fluctuations, thus intensifying environmental stresses and imposing major constraints to crop production. A better understanding of the rice molecular mechanisms of low temperature perception and translation may help develop plants with increased cold tolerance, which is particularly damaging to seedling development and reproductive stages.

In this work, we have characterized OsCPK17 and demonstrate that it has the properties of a typical CDPK and that the recombinant OsCPK17 shows kinase activity, which is calcium dependent, similarly to most CDPKs from the same subgroup (Boudsocq et al. 2012). Our study shows that OsCPK17 can transmit the information encrypted in a stimulus-specific calcium-signaling signature under cold stress in rice.

In a cold acclimation experiment, we show that the absence or silencing of *OsCPK17* leads to lower tolerance towards cold. This result suggests that *OsCPK17* is involved in the cold stress signaling response in rice. *OsCPK17* seems to have fairly constitutive expression throughout the rice life cycle, in all analyzed tissues, according to the Genevestigator database (Hruz et al. 2008) although presenting a downregulation under cold stress (Figure S5). Wan et al. 2007 observed a considerable downregulation of *OsCPK17* after 12h under cold stress. We also show that the expression of *OsCPK17* in the WT is downregulated under cold-stress, which suggests that the *OsCPK17* role must take place during the early stages of the cold-sensing process. Our transcript analyses indicate that in non-stressed WT plants, *OsCPK17* protein is already present in the cell. Thus, the protein is most probably in an inactive form and ready to be activated upon a Ca^{2+} stimulus. Upon cold stress, which results in a specific calcium signature (Martí et al. 2013), *OsCPK17* is possibly activated by calcium binding and transduces the signal by phosphorylating its downstream targets. After transmitting the signaling information, *OsCPK17* appears to be shut down through the downregulation of its gene expression. Thus, the higher cold stress sensitivity observed in *oscpk17* could be related to the lack of the initial *OsCPK17*-mediated signaling transmission, whereas for the OX lines it could be related to the absence of this *OsCPK17* downregulation mechanism or the effects of the ectopic expression of *OsCPK17*.

Additionally, we show that *OsCPK17* is not involved in the upregulation of the key cold stress-inducible genes analyzed. This observation is consistent with other reports, for example, *OsCPK13* contributes to cold tolerance without affecting transcription modulation of stress-responsive genes (Saijo et al. 2000). Also, *AtCPK1* regulates the phosphoproteome in cold conditions, although it does not affect CBF

regulation (Bohmer and Romeis 2007). The same case occurs under salinity with *oscpk12* and OsCPK12 OX plants showing expression levels of stress-inducible genes comparable to those of WT plants (Asano et al. 2012). Thus, we hypothesized that the cold-sensitive phenotype of *oscpk17* results from an alteration in the post-translation modifications of other proteins involved in cold stress response.

● Our phosphoproteomic analysis identified six proteins as candidate targets of OsCPK17 (direct or indirect), in rice cold stress response. These putative target proteins are involved in nitrogen metabolism (OsFd-GOGAT and OsNR1), sugar metabolism (OsSPS4, OsSPS5, and OsPDHE1) or water permeability across the plasma membrane (hydraulic conductivity) (OsPIP2;1/OsPIP2;6). Moreover, we provide evidence of *in vitro* phosphorylation of OsSPS4 and OsPIP2;1/OsPIP2;6 peptides by OsCPK17, which supports the idea that these peptides are OsCPK17 direct targets.

Phosphorylations of PIPs are important for their subcellular localization or gating (reviewed in Chevalier and Chaumont 2015, Maurel et al. 2015). The distinctly phosphorylated peptide ALG_pSFR revealed in our study, locates at the C-terminal tail of either OsPIP2;1 or OsPIP2;6 (Figure S4.C-D). Phosphorylation of the residues S274 and S115 of OsPIP2;1 is suggested to open the water channel and modulate water transport (Tornroth-Horsefield et al. 2006, Johansson et al. 1998, Johansson et al. 1996). Moreover, in transgenic Arabidopsis, using phosphomimetic and phosphorylation-deficient variants of AtPIP2;1, the phosphorylation at Ser280 and Ser283 (corresponding to S285 and S288 of OsPIP2;1, respectively) is crucial to enhance leaf hydraulic conductivity in Arabidopsis veins, in the darkness (Prado et al. 2013). Hence, we can infer that the cold-induced phosphorylation of

OsPIP2;1/OsPIP2;6 by OsCPK17, may result in increased water permeability across the plasma membrane, at least at the onset of the cold period (Figure 8).

Furthermore, low temperature reduces root hydraulic conductivity and causes water deficit in leaves (leaf dehydration) due to an imbalance between root water uptake and transpiration rate (reviewed in Aroca et al. 2012). Cold-tolerant plants can recover from the drastic reduction of root hydraulic conductance after cold stress imposition, and by closing their stomata faster, they prevent water loss by transpiration when root hydraulic conductivity is compromised (Bloom et al. 2004, Gazquez et al. 2015, Wilkinson et al. 2001). Aquaporins seem to play a role in the maintenance of this chilling tolerance associated with root hydraulic conductivity (Aroca et al. 2001). The phosphorylation at S121 of AtPIP2;1 (corresponding to S115 of SoPIP2;1) has been suggested to play a role in ABA-induced stomatal closure by increasing water permeability of the guard cells (Grondin et al. 2015). One could hypothesize that OsCPK17-induced phosphorylation of OsPIP2;1/OsPIP2;6 increases water conductivity in the guard cells, promoting a fast stomatal closure under cold stress and increasing tolerance. Also, the presence of aquaporins in the veins and their regulation by phosphorylation, shown previously for Arabidopsis, can determine the amount of water that reaches the mesophyll (Prado et al. 2013). Both effects could be contributing to our *oscpk17* seedlings phenotype under cold. *oscpk17* showed a higher degree of leaf dehydration, compared to the WT, particularly obvious after 18 days under low temperature. Although higher leaf dehydration in the *oscpk17* could be due to a lower activity of OsPIP2;1/OsPIP2;6 aquaporins, one cannot exclude additional aspects such as the lower capacity of osmotic adjustment or a decline in membrane integrity. On the other hand, the OX low cold stress tolerance could be due to an off-place increase in aquaporin

transport and resulting water imbalance. Additionally, the PIP2 class of aquaporins (Bots et al. 2005), and in particular OsPIP2;1 in rice (Hayashi et al. 2015), are implicated in critical dehydration processes during reproductive stages. This involvement is interesting as it could eventually be related to the decreased fertility rate observed in both *oscpk17* and OX, compared to WT plants.

● Sucrose is both a nutrient and a signaling molecule playing fundamental roles in plant development, productivity and stress response, namely by acting as an osmotic protectant under cold conditions (Theocharis et al. 2012). Sucrose is synthesized in the cytosol by the sequential action of two enzymes: SPS and Sucrose-phosphate phosphatase (SPP) (Figure 8). SPSs mediate the rate-limiting step of sucrose synthesis and are encoded by a small gene family with five genes described in rice (Castleden et al. 2004). We observed a cold-induced phosphorylation of OsSPS4 at S170, only in WT plants (Figure 5 and Figure S4.E-F), which supports the results of other studies such as the increased phosphorylation at the same site in cold-acclimated *Arabidopsis* (S159 of AtSPSA2) (Wolschin et al. 2005). In spinach, it was demonstrated that phosphorylation at this site (conserved in plant SPSs) (Castleden et al. 2004, Taneja and Das 2014), was responsible for the dark inactivation of SPS (Toroser et al. 1999). Hence, OsSPS4 phosphorylation, possibly mediated by OsCPK17, could lead to a reduction in OsSPS4 activity in the early stages of cold stress.

Increased sucrose accumulation under cold conditions has been correlated with increased SPS activity (Chen et al. 2012, Deiting et al. 1998). We observed the expected increase in sucrose levels during cold response and this accumulation was somehow impaired in *oscpk17* and OX plants, which may explain their overall increased sensitivity to stress. However, the global sucrose levels at 30 min of stress do not correlate with our assumption of a phosphorylation-induced decrease in SPS activity. Since only OsSPS4 and OsSPS5 phosphorylation was affected in our study and SPSs have distinct tissue distribution, putative alterations in these SPSs activities could be diluted (Okamura et al. 2011, Sun et al. 2011, Volkert et al. 2014). So, local adjustments on sucrose content may not appear under our experimental conditions. Additionally, in both *oscpk17* and OX plants mannitol accumulation appears to be affected, being higher than in WT (Figure S6). This may reflect a preferential use of fructose-6-P (F6P) for the biosynthesis of mannitol instead of sucrose in the transgenic lines, since F6P is a common substrate for sucrose and mannitol pathways (Hare et al. 1998). Overall, these results suggest that OsCPK17 affects plant sugar metabolism (Figure 8).

Interestingly, two of OsCPK17 targets, NR and SPSs share some similarities in the regulation of their activity (Campbell 1999, Huber et al. 1992, Huber et al. 1989, Jones et al. 1998, Toroser et al. 1998), and are both negatively regulated by sucrose non-fermenting-1 (SNF1) related kinase (SnRK1) (Sugden et al. 1999). In spinach, SPS and NR are inactivated by SnRK1 kinases via phosphorylation of S158 and S543, respectively (Figure S4A and S4E). Because these regulation sites in spinach correspond to the cold-induced phosphorylated sites identified in our study (or the nearby residue, in the case of OsNR1), we may hypothesize that OsCPK17 inactivates OsSPS4, OsSPS5, and OsNR1, via SnRK1 activity modulation. We could

not detect the rice SnRK1 kinase in our phosphoproteomic analysis, so we cannot verify if SnRK1 is differently phosphorylated in WT vs. *oscpk17* in response to stress. We tested, by *in vitro* phosphorylation, the ability of OsCPK17 to modify the SnRK1 conserved phosphorylatable activation residue (Crozet et al. 2014, Shen et al. 2009), contained in the synthetic peptide RGHFLKTSCGS. We found that this peptide is not a direct target of OsCPK17 (Figure S7). Nevertheless, indirect OsCPK17-dependent effects on SnRK1 activity cannot be excluded. If this is the case, the penalties in plant growth and development observed in OsCPK17 OX plants could be due to a misregulation of SnRK1 by the constitutive *OsCPK17* overexpression. A similar effect was seen in *Arabidopsis* SnRKs overexpressing lines that displayed delays in seed germination, vegetative growth and flowering time (Baena-Gonzalez et al. 2007, Tsai and Gazzarrini 2012).

In sum, under cold stress, the activated OsCPK17 phosphorylates its targets, leading to cell osmotic regulation, by activating the aquaporin OsPIP2;1/OsPIP2;6. Simultaneously, OsCPK17 may modulate general metabolism, by decreasing the activity of several components of sugar and nitrogen metabolisms: OsSPS4, OsSPS5, OsNR1 and OsPDHE1-A (Figure 8). This negative effect on metabolism might allow the plants to redirect their efforts into important cold response mechanisms thus leading to increased cold tolerance.

Altogether our work suggests new possibilities for the dissection of cold tolerance mechanisms in rice.

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REFERENCES

- Abbasi F., Onodera H., Toki S., Tanaka H. & Komatsu S. (2004) OsCDPK13, a calcium-dependent protein kinase gene from rice, is induced by cold and gibberellin in rice leaf sheath. *Plant Molecular Biology*, **55**, 541-552.
- Aguan K., Sugawara K., Suzuki N. & Kusano T. (1991) Isolation of Genes for Low-Temperature-Induced Proteins in Rice by a Simple Subtractive Method. *Plant and Cell Physiology*, **32**, 1285-1289.
- Almeida D.M., Almadanim M.C., Lourenco T., Abreu I.A., Saibo N.J. & Oliveira M.M. (2016) Screening for Abiotic Stress Tolerance in Rice: Salt, Cold, and Drought. *Methods in Molecular Biology*, **1398**, 155-182.
- Andaya V.C. & Mackill D.J. (2003) Mapping of QTLs associated with cold tolerance during the vegetative stage in rice. *Journal of Experimental Botany*, **54**, 2579-2585.
- Aroca R., Porcel R. & Ruiz-Lozano J.M. (2012) Regulation of root water uptake under abiotic stress conditions. *Journal of Experimental Botany*, **63**, 43-57.
- Aroca R., Tognoni F., Irigoyen J.J., Sanchez-Diaz M. & Pardossi A. (2001) Different root low temperature response of two maize genotypes differing in chilling sensitivity. *Plant Physiology and Biochemistry*, **39**, 1067-1073.
- Asano T., Hakata M., Nakamura H., Aoki N., Komatsu S., Ichikawa H., ..., Ohsugi R. (2011) Functional characterisation of OsCPK21, a calcium-dependent protein kinase that confers salt tolerance in rice. *Plant Molecular Biology*, **75**, 179-191.

- Asano T., Hayashi N., Kobayashi M., Aoki N., Miyao A., Mitsuhashi I., ..., Ohsugi R. (2012) A rice calcium-dependent protein kinase OsCPK12 oppositely modulates salt-stress tolerance and blast disease resistance. *The Plant Journal*, **69**, 26-36.
- Asano T., Kunieda N., Omura Y., Ibe H., Kawasaki T., Takano M., ..., Shimada H. (2002) Rice SPK, a calmodulin-like domain protein kinase, is required for storage product accumulation during seed development: phosphorylation of sucrose synthase is a possible factor. *The Plant Cell*, **14**, 619-628.
- Bachmann M., Shiraishi N., Campbell W.H., Yoo B.C., Harmon A.C. & Huber S.C. (1996) Identification of Ser-543 as the major regulatory phosphorylation site in spinach leaf nitrate reductase. *The Plant Cell*, **8**, 505-517.
- Baena-Gonzalez E., Rolland F., Thevelein J.M. & Sheen J. (2007) A central integrator of transcription networks in plant stress and energy signalling. *Nature*, **448**, 938-942.
- Bloom A.J., Zwieniecki M.A., Passioura J.B., Randall L.B., Holbrook N.M. & St. Clair D.A. (2004) Water relations under root chilling in a sensitive and tolerant tomato species. *Plant, Cell & Environment*, **27**, 971-979.
- Bohmer M. & Romeis T. (2007) A chemical-genetic approach to elucidate protein kinase function in planta. *Plant Molecular Biology*, **65**, 817-827.
- Bots M., Vergeldt F., Wolters-Arts M., Weterings K., van As H. & Mariani C. (2005) Aquaporins of the PIP2 Class Are Required for Efficient Anther Dehiscence in Tobacco. *Plant Physiology* **137**, 1049-1056.

- Boudsocq M., Droillard M.J., Regad L. & Lauriere C. (2012) Characterization of Arabidopsis calcium-dependent protein kinases: activated or not by calcium? *Biochemical Journal*, **447**, 291-299.
- Boudsocq M. & Sheen J. (2013) CDPKs in immune and stress signaling. *Trends in Plant Science*, **18**, 30-40.
- Campbell W.H. (1999) Nitrate reductase structure, function and regulation: Bridging the Gap between Biochemistry and Physiology. *Annual Review of Plant Physiology and Plant Molecular Biology*, **50**, 277-303.
- Campo S., Baldrich P., Messeguer J., Lalanne E., Coca M. & San Segundo B. (2014) Overexpression of a Calcium-Dependent Protein Kinase Confers Salt and Drought Tolerance in Rice by Preventing Membrane Lipid Peroxidation. *Plant Physiology*, **165**, 688-704.
- Campos P.S., Quartin V., Ramalho J.C. & Nunes M.A. (2003) Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of Coffea sp. plants. *Journal of Plant Physiology*, **160**, 283-292.
- Campos-Soriano L., Gomez-Ariza J., Bonfante P. & San Segundo B. (2011) A rice calcium-dependent protein kinase is expressed in cortical root cells during the presymbiotic phase of the arbuscular mycorrhizal symbiosis. *BMC Plant Biology* **11**, 90.
- Cantrel C., Vazquez T., Puyaubert J., Reze N., Lesch M., Kaiser W.M., ..., Baudouin E. (2011) Nitric oxide participates in cold-responsive phosphosphingolipid formation and gene expression in Arabidopsis thaliana. *New Phytologist*, **189**, 415-427.

- Castleden C.K., Aoki N., Gillespie V.J., MacRae E.A., Quick W.P., Buchner P., ..., Lunn J.E. (2004) Evolution and function of the sucrose-phosphate synthase gene families in wheat and other grasses. *Plant Physiology*, **135**, 1753-1764.
- Chang I.F., Hsu J.L., Hsu P.H., Sheng W.A., Lai S.J., Lee C., ..., Chen C.C. (2012) Comparative phosphoproteomic analysis of microsomal fractions of *Arabidopsis thaliana* and *Oryza sativa* subjected to high salinity. *Plant Science*, **185-186**, 131-142.
- Chen J., Xue B., Xia X. & Yin W. (2013) A novel calcium-dependent protein kinase gene from *Populus euphratica*, confers both drought and cold stress tolerance. *Biochemical and Biophysical Research Communications*, **441**, 630-636.
- Chen T., Pei H., Zhang Y. & Qian Q. (2012) Seasonal changes in non-structural carbohydrates and sucrose metabolism enzymes in two *Sabina* species. *Acta Physiologiae Plantarum*, **34**, 173-180.
- Cheng H., Deng W., Wang Y., Ren J., Liu Z. & Xue Y. (2014) dbPPT: a comprehensive database of protein phosphorylation in plants. *Database (Oxford)*, **2014**, bau121.
- Chevalier A.S. & Chaumont F. (2015) Trafficking of plant plasma membrane aquaporins: multiple regulation levels and complex sorting signals. *Plant and Cell Physiology*, **56**, 819-829.
- Christensen A.H. & Quail P.H. (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research*, **5**, 213-218.

- Crozet P., Margalha L., Confraria A., Rodrigues A., Martinho C., Adamo M., ..., Baena-Gonzalez E. (2014) Mechanisms of regulation of SNF1/AMPK/SnRK1 protein kinases. *Frontiers in Plant Science*, **5**.
- Cruz R.P.d., Sperotto R.A., Cargnelutti D., Adamski J.M., de FreitasTerra T. & Fett J.P. (2013) Avoiding damage and achieving cold tolerance in rice plants. *Food and Energy Security*, **2**, 96-119.
- Dammann C., Ichida A., Hong B., Romanowsky S.M., Hrabak E.M., Harmon A.C., ..., Harper J.F. (2003) Subcellular targeting of nine calcium-dependent protein kinase isoforms from Arabidopsis. *Plant Physiology*, **132**, 1840-1848.
- Deiting U., Zrenner R. & Stitt M. (1998) Similar temperature requirement for sugar accumulation and for the induction of new forms of sucrose phosphate synthase and amylase in cold-stored potato tubers. *Plant, Cell & Environment*, **21**, 127-138.
- Douglas P., Morrice N. & MacKintosh C. (1995) Identification of a regulatory phosphorylation site in the hinge 1 region of nitrate reductase from spinach (*Spinacea oleracea*) leaves. *FEBS Letters*, **377**, 113-117.
- Dubin M.J., Bowler C. & Benvenuto G. (2008) A modified Gateway cloning strategy for overexpressing tagged proteins in plants. *Plant Methods*, **4**, 3.
- Dubouzet J.G., Sakuma Y., Ito Y., Kasuga M., Dubouzet E.G., Miura S., ..., 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.

- Dubrovina A.S., Kiselev K.V., Khristenko V.S. & Aleynova O.A. (2015) VaCPK20, a calcium-dependent protein kinase gene of wild grapevine *Vitis amurensis* Rupr., mediates cold and drought stress tolerance. *Journal of Plant Physiology*, **185**, 1-12.
- Forde B.G. & Lea P.J. (2007) Glutamate in plants: metabolism, regulation, and signalling. *Journal of Experimental Botany*, **58**, 2339-2358.
- Fratini M., Morello L. & Breviario D. (1999) Rice calcium-dependent protein kinase isoforms OsCDPK2 and OsCDPK11 show different responses to light and different expression patterns during seed development. *Plant Molecular Biology* **41**, 753-764.
- Gazquez A., Maiale S.J., Rachoski M.M., Vidal A., Ruiz O.A., Menéndez A.B. & Rodríguez A.A. (2015) Physiological Response of Multiple Contrasting Rice (*Oryza sativa* L.) Cultivars to Suboptimal Temperatures. *Journal of Agronomy and Crop Science*, **201**, 117-127.
- Grondin A., Rodrigues O., Verdoucq L., Merlot S., Leonhardt N. & Maurel C. (2015) Aquaporins Contribute to ABA-Triggered Stomatal Closure through OST1-Mediated Phosphorylation. *The Plant Cell*, **27**, 1945-1954.
- Gupta K.J., Fernie A.R., Kaiser W.M. & van Dongen J.T. (2011) On the origins of nitric oxide. *Trends in Plant Science*, **16**, 160-168.
- Han C., Yang P.F., Sakata K. & Komatsu S. (2014) Quantitative Proteomics Reveals the Role of Protein Phosphorylation in Rice Embryos during Early Stages of Germination. *Journal of Proteome Research*, **13**, 1766-1782.

- Hare P.D., Cress W.A. & Van Staden J. (1998) Dissecting the roles of osmolyte accumulation during stress. *Plant Cell and Environment*, **21**, 535-553.
- Harmon A.C., Gribskov M., Gubrium E. & Harper J.F. (2001) The CDPK superfamily of protein kinases. *New Phytologist*, **151**, 175-183.
- Harper J.F., Breton G. & Harmon A. (2004) Decoding Ca(2+) signals through plant protein kinases. *Annual Review of Plant Biology*, **55**, 263-288.
- Hayakawa T., Sakai T., Ishiyama K., Hirose N., Nakajima H., Takezawa M., ..., Yamaya T. (2003) Organization and Structure of Ferredoxin-Dependent Glutamate Synthase Gene and Intracellular Localization of the Enzyme Protein in Rice Plants. *Plant Biotechnology*, **20**, 43-55.
- Hayashi H., Ishikawa-Sakurai J., Murai-Hatano M., Ahamed A. & Uemura M. (2015) Aquaporins in developing rice grains. *Bioscience, Biotechnology, and Biochemistry*, **79**, 1422-1429.
- Hiei Y. & Komari T. (2008) Agrobacterium-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nature Protocols*, **3**, 824-834.
- Ho S.L., Huang L.F., Lu C.A., He S.L., Wang C.C., Yu S.P., ..., Yu S.M. (2013) Sugar starvation- and GA-inducible calcium-dependent protein kinase 1 feedback regulates GA biosynthesis and activates a 14-3-3 protein to confer drought tolerance in rice seedlings. *Plant Molecular Biology*, **81**, 347-361.
- Hruz T., Laule O., Szabo G., Wessendorp F., Bleuler S., Oertle L., ..., Zimmermann P. (2008) Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. *Advances in Bioinformatics*, **2008**, 420747.

Huber J.L., Huber S.C., Campbell W.H. & Redinbaugh M.G. (1992) Reversible light/dark modulation of spinach leaf nitrate reductase activity involves protein phosphorylation. *Archives of Biochemistry and Biophysics*, **296**, 58-65.

Huber J.L., Huber S.C. & Nielsen T.H. (1989) Protein phosphorylation as a mechanism for regulation of spinach leaf sucrose-phosphate synthase activity. *Archives of Biochemistry and Biophysics*, **270**, 681-690.

Igarashi Y., Yoshida Y., Sanada Y., Yamaguchi-Shinozaki K., Wada K. & Shinozaki K. (1997) Characterization of the gene for delta1-pyrroline-5-carboxylate synthetase and correlation between the expression of the gene and salt tolerance in *Oryza sativa* L. *Plant Molecular Biology*, **33**, 857-865.

Johansson I., Karlsson M., Shukla V.K., Chrispeels M.J., Larsson C. & Kjellbom P. (1998) Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation. *The Plant Cell*, **10**, 451-459.

Johansson I., Larsson C., Ek B. & Kjellbom P. (1996) The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca^{2+} and apoplastic water potential. *The Plant Cell*, **8**, 1181-1191.

Jones T.L., Tucker D.E. & Ort D.R. (1998) Chilling delays circadian pattern of sucrose phosphate synthase and nitrate reductase activity in tomato. *Plant Physiology*, **118**, 149-158.

Kikuchi K., Ueguchi-Tanaka M., Yoshida K.T., Nagato Y., Matsusaka M. & Hirano H.Y. (2000) Molecular analysis of the NAC gene family in rice. *Molecular and General Genetics*, **262**, 1047-1051.

- Komatsu S., Yang G., Khan M., Onodera H., Toki S. & Yamaguchi M. (2007) Over-expression of calcium-dependent protein kinase 13 and calreticulin interacting protein 1 confers cold tolerance on rice plants. *Molecular Genetics and Genomics*, **277**, 713-723.
- Kong X., Lv W., Jiang S., Zhang D., Cai G., Pan J. & Li D. (2013) Genome-wide identification and expression analysis of calcium-dependent protein kinase in maize. *BMC Genomics*, **14**, 433.
- Lea U.S., Ten Hoopen F., Provan F., Kaiser W.M., Meyer C. & Lillo C. (2004) Mutation of the regulatory phosphorylation site of tobacco nitrate reductase results in high nitrite excretion and NO emission from leaf and root tissue. *Planta*, **219**, 59-65.
- Li A.L., Zhu Y.F., Tan X.M., Wang X., Wei B., Guo H.Z., ..., Mao L. (2008) Evolutionary and functional study of the CDPK gene family in wheat (*Triticum aestivum* L.). *Plant Molecular Biology*, **66**, 429-443.
- Li T.G., Visperas R.M. & Vergara B.S. (1981) Correlation of cold tolerance at different growth stages in rice. *Acta Botanica Sinica*, **23**, 203–207.
- Liese A. & Romeis T. (2013) Biochemical regulation of in vivo function of plant calcium-dependent protein kinases (CDPK). *Biochimica et Biophysica Acta*, **1833**, 1582-1589.
- Lillo C., Lea U.S., Leydecker M.T. & Meyer C. (2003) Mutation of the regulatory phosphorylation site of tobacco nitrate reductase results in constitutive activation of the enzyme in vivo and nitrite accumulation. *The Plant Journal*, **35**, 566-573.

- Lourenço T., Saibo N., Batista R., Pinto Ricardo C. & Oliveira M.M. (2011) Inducible and constitutive expression of HvCBF4 in rice leads to differential gene expression and drought tolerance. *Biologia Plantarum*, **55**, 653-663.
- Maksup S., Supaibulwatana K. & Selvaraj G. (2013) High-quality reference genes for quantifying the transcriptional responses of *Oryza sativa* L. (ssp. indica and japonica) to abiotic stress conditions. *Chinese Science Bulletin*, **58**, 1919-1930.
- Marti M.C., Stancombe M.A. & Webb A.A. (2013) Cell- and stimulus type-specific intracellular free Ca²⁺ signals in *Arabidopsis*. *Plant Physiology*, **163**, 625-634.
- Maurel C., Boursiac Y., Luu D.T., Santoni V., Shahzad Z. & Verdoucq L. (2015) Aquaporins in Plants. *Physiological Reviews*, **95**, 1321-1358.
- Miki D. & Shimamoto K. (2004) Simple RNAi vectors for stable and transient suppression of gene function in rice. *Plant and Cell Physiology*, **45**, 490-495.
- Morello L., Frattini M., Giani S., Christou P. & Breviario D. (2000) Overexpression of the calcium-dependent protein kinase OsCDPK2 in transgenic rice is repressed by light in leaves and disrupts seed development. *Transgenic Research* **9**, 453-462.
- Morsy M.R., Jouve L., Hausman J.F., Hoffmann L. & Stewart J.M. (2007) Alteration of oxidative and carbohydrate metabolism under abiotic stress in two rice (*Oryza sativa* L.) genotypes contrasting in chilling tolerance. *Journal of Plant Physiology*, **164**, 157-167.
- Nakagami H., Sugiyama N., Mochida K., Daudi A., Yoshida Y., Toyoda T., ..., Shirasu K. (2010) Large-Scale Comparative Phosphoproteomics Identifies Conserved Phosphorylation Sites in Plants. *Plant Physiology*, **153**, 1161-1174.

- Negrão S., Almadanim M.C., Pires I.S., Abreu I.A., Maroco J., Courtois B., ..., Oliveira M.M. (2013) New allelic variants found in key rice salt-tolerance genes: an association study. *Plant Biotechnology Journal*, **11**, 87-100.
- Negrão S., Courtois B., Ahmadi N., Abreu I., Saibo N. & Oliveira M.M. (2011) Recent Updates on Salinity Stress in Rice: From Physiological to Molecular Responses. *Critical Reviews in Plant Sciences*, **30**, 329-377.
- Okamura M., Aoki N., Hirose T., Yonekura M., Ohto C. & Ohsugi R. (2011) Tissue specificity and diurnal change in gene expression of the sucrose phosphate synthase gene family in rice. *Plant Science*, **181**, 159-166.
- Pan X.-W., Li Y.-c., Li X.-x., Liu W.-q., Ming J., Lu T.-t., ..., Sheng X.-n. (2013) Differential Regulatory Mechanisms of CBF Regulon Between Nipponbare (Japonica) and 93-11 (Indica) During Cold Acclimation. *Rice Science*, **20**, 165-172.
- Pandolfi D., Solinas G., Valle G. & Coraggio I. (1997) Cloning of a cDNA encoding a novel myb gene (acc. No. Y11414) highly expressed in cold stressed rice coleoptiles (PGR 97-079). *Plant Physiology*, **114**.
- Patel M.S. & Korotchkina L.G. (2001) Regulation of mammalian pyruvate dehydrogenase complex by phosphorylation: complexity of multiple phosphorylation sites and kinases. *Experimental & Molecular Medicine*, **33**, 191-197.
- Prado K., Boursiac Y., Tournaire-Roux C., Monneuse J.M., Postaire O., Da Ines O., ..., Maurel C. (2013) Regulation of Arabidopsis leaf hydraulics involves light-

dependent phosphorylation of aquaporins in veins. *The Plant Cell*, **25**, 1029-1039.

Ramvalho J.C., Rodrigues A.P., Semedo J.N., Pais I.P., Martins L.D., Simoes-Costa M.C., ..., DaMatta F.M. (2013) Sustained Photosynthetic Performance of *Coffea* spp. under Long-Term Enhanced [CO₂]. *Plos One*, **8**.

Ray S., Agarwal P., Arora R., Kapoor S. & Tyagi A.K. (2007) Expression analysis of calcium-dependent protein kinase gene family during reproductive development and abiotic stress conditions in rice (*Oryza sativa* L. ssp. indica). *Molecular Genetics and Genomics*, **278**, 493-505.

Reid E.E., Thompson P., Lyttle C.R. & Dennis D.T. (1977) Pyruvate dehydrogenase complex from higher plant mitochondria and proplastids. *Plant Physiology*, **59**, 842-848.

Ruan Y.L. (2014) Sucrose metabolism: gateway to diverse carbon use and sugar signaling. *Annual Review of Plant Biology*, **65**, 33-67.

Saijo Y., Hata S., Kyoizuka J., Shimamoto K. & Izui K. (2000) Over-expression of a single Ca²⁺-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. *The Plant Journal*, **23**, 319-327.

Satake T. & Hayase H. (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. *Proceedings of the Crop Science Society of Japan*, **39**, 468-473.

Schulz P., Herde M. & Romeis T. (2013) Calcium-dependent protein kinases: hubs in plant stress signaling and development. *Plant Physiology*, **163**, 523-530.

- Sebastià C.H., Hardin S.C., Clouse S.D., Kieber J.J. & Huber S.C. (2004) Identification of a new motif for CDPK phosphorylation in vitro that suggests ACC synthase may be a CDPK substrate. *Archives of Biochemistry and Biophysics*, **428**, 81-91.
- Shen W., Reyes M.I. & Hanley-Bowdoin L. (2009) Arabidopsis protein kinases GRIK1 and GRIK2 specifically activate SnRK1 by phosphorylating its activation loop. *Plant Physiology*, **150**, 996-1005.
- Su W., Huber S.C. & Crawford N.M. (1996) Identification in vitro of a post-translational regulatory site in the hinge 1 region of Arabidopsis nitrate reductase. *The Plant Cell*, **8**, 519-527.
- Sugden C., Donaghy P.G., Halford N.G. & Hardie D.G. (1999) Two SNF1-related protein kinases from spinach leaf phosphorylate and inactivate 3-hydroxy-3-methylglutaryl-coenzyme A reductase, nitrate reductase, and sucrose phosphate synthase in vitro. *Plant Physiology*, **120**, 257-274.
- Sun J., Zhang J., Larue C.T. & Huber S.C. (2011) Decrease in leaf sucrose synthesis leads to increased leaf starch turnover and decreased RuBP regeneration-limited photosynthesis but not Rubisco-limited photosynthesis in Arabidopsis null mutants of SPSA1. *Plant, Cell & Environment*, **34**, 592-604.
- Suzuki A. & Knaff D.B. (2005) Glutamate synthase: structural, mechanistic and regulatory properties, and role in the amino acid metabolism. *Photosynthesis Research*, **83**, 191-217.

- Taneja D. & Das N. (2014) Molecular cloning, sequence analyses, and expression studies of sucrose-phosphate synthase in the potato (*Solanum tuberosum* L.) cultivars. *Acta Physiologiae Plantarum*, **36**, 2253-2269.
- Theocharis A., Clement C. & Barka E.A. (2012) Physiological and molecular changes in plants grown at low temperatures. *Planta*, **235**, 1091-1105.
- Tornroth-Horsefield S., Wang Y., Hedfalk K., Johanson U., Karlsson M., Tajkhorshid E.,..., Kjellbom P. (2006) Structural mechanism of plant aquaporin gating. *Nature*, **439**, 688-694.
- Toroser D., Athwal G.S. & Huber S.C. (1998) Site-specific regulatory interaction between spinach leaf sucrose-phosphate synthase and 14-3-3 proteins. *FEBS Letters*, **435**, 110-114.
- Toroser D., McMichael R.Jr., Krause K.P., Kurreck J., Sonnewald U., Stitt M. & Huber S.C. (1999) Site-directed mutagenesis of serine 158 demonstrates its role in spinach leaf sucrose-phosphate synthase modulation. *The Plant Journal*, **17**, 407-413.
- Tovar-Mendez A., Miernyk J.A. & Randall D.D. (2003) Regulation of pyruvate dehydrogenase complex activity in plant cells. *European Journal of Biochemistry*, **270**, 1043-1049.
- Tsai A.Y.-L. & Gazzarrini S. (2012) AKIN10 and FUSCA3 interact to control lateral organ development and phase transitions in Arabidopsis. *The Plant Journal*, **69**, 809-821.

- Uno Y., Rodriguez Milla M.A., Maher E. & Cushman J.C. (2009) Identification of proteins that interact with catalytically active calcium-dependent protein kinases from Arabidopsis. *Molecular Genetics and Genomics*, **281**, 375-390.
- Volkert K., Debast S., Voll L.M., Voll H., Schiessl I., Hofmann J., ..., Bornke F. (2014) Loss of the two major leaf isoforms of sucrose-phosphate synthase in Arabidopsis thaliana limits sucrose synthesis and nocturnal starch degradation but does not alter carbon partitioning during photosynthesis. *Journal of Experimental Botany*, **65**, 5217-5229.
- Wan B., Lin Y. & Mou T. (2007) Expression of rice Ca(2+)-dependent protein kinases (CDPKs) genes under different environmental stresses. *FEBS Letters*, **581**, 1179-1189.
- Weckwerth P., Ehlert B. & Romeis T. (2015) ZmCPK1, a calcium-independent kinase member of the Zea mays CDPK gene family, functions as a negative regulator in cold stress signalling. *Plant, Cell & Environment*, **38**, 544-558.
- Wernimont A.K., Artz J.D., Finerty P.Jr., Lin Y.H., Amani M., Allali-Hassani A., ..., Hui R. (2010) Structures of apicomplexan calcium-dependent protein kinases reveal mechanism of activation by calcium. *Nature Structural & Molecular Biology*, **17**, 596-601.
- Whiteman S.A., Nuhse T.S., Ashford D.A., Sanders D. & Maathuis F.J. (2008) A proteomic and phosphoproteomic analysis of Oryza sativa plasma membrane and vacuolar membrane. *The Plant Journal*, **56**, 146-156.
- Wilkinson S., Clephan A.L. & Davies W.J. (2001) Rapid low temperature-induced stomatal closure occurs in cold-tolerant Commelina communis leaves but not in

cold-sensitive tobacco leaves, via a mechanism that involves apoplastic calcium but not abscisic acid. *Plant Physiology*, **126**, 1566-1578.

Wolschin F., Lehmann U., Glinski M. & Weckwerth W. (2005) An integrated strategy for identification and relative quantification of site-specific protein phosphorylation using liquid chromatography coupled to MS2/MS3. *Rapid Communications in Mass Spectrometry*, **19**, 3626-3632.

Yao Q., Ge H., Wu S., Zhang N., Chen W., Xu C., ..., Xu D. (2014) P(3)DB 3.0: From plant phosphorylation sites to protein networks. *Nucleic Acids Research*, **42**, D1206-1213.

Ye C., Fukai S., Godwin I., Reinke R., Snell P., Schiller J. & Basnayake J. (2009a) Cold tolerance in rice varieties at different growth stages. *Crop & Pasture Science*, **60**, 328-338.

Ye S., Wang L., Xie W., Wan B., Li X. & Lin Y. (2009b) Expression profile of calcium-dependent protein kinase (CDPKs) genes during the whole lifespan and under phytohormone treatment conditions in rice (*Oryza sativa* L. ssp. indica). *Plant Molecular Biology*, **70**, 311-325.

Yoshida S.F.D., Cock J.H. & Gomez K.A. (1976) Laboratory manual for physiological studies of rice. International Rice Research Institute (IRRI), Manila (The Philippines).

Xie K., Chen J., Wang Q. & Yang Y. (2014) Direct phosphorylation and activation of a mitogen-activated protein kinase by a calcium-dependent protein kinase in rice. *Plant Cell* **26**, 3077-3089.

Zhang W., Wan B., Zhou F., Chen H., Li X. & Lin Y. (2014) Up- and Down-regulated Expression of OsCPK25/26 Results in Increased Number of Stamens in Rice. *Plant Molecular Biology Reporter*, **32**, 1114-1128.

Zhang X.Q. & Chollet R. (1997) Seryl-phosphorylation of soybean nodule sucrose synthase (nodulin-100) by a Ca^{2+} -dependent protein kinase. *FEBS Letters*, **410**, 126-130.

Zhao M.G., Chen L., Zhang L.L. & Zhang W.H. (2009) Nitric reductase-dependent nitric oxide production is involved in cold acclimation and freezing tolerance in *Arabidopsis*. *Plant Physiology*, **151**, 755-767.

Table 1. Putative OsCPK17 target phosphopeptides and corresponding proteins, identified by phosphoproteomic analysis, in WT and *oscpk17* seedlings both in control growth conditions (28°C) and 30 min after exposure to cold stress (10°C). The predicted phosphorylated residues, within the identified phosphopeptides, are underlined. (Ref: UniProt Knowledgebase).

Accession (UniProtKB)	Description	Protein	Gene	Phosphopeptide sequence	Position in protein
GLTB_ORYSJ	Ferredoxin-dependent glutamate synthase, chloroplastic	OsFd-GOGAT	<i>OsFd-GOGAT</i>	TDILKAKHV <u>S</u> LAK	S1300
PIP21_ORYSJ or PIP26_ORYSJ	Plasma membrane intrinsic protein 2-1 or Plasma membrane intrinsic protein 2-6	OsPIP2;1 or OsPIP2;6	<i>OsPIP2;1</i> or <i>OsPIP2;6</i>	ALG <u>S</u> FR	S285 or S275
SPSA4_ORYSJ	Sucrose-phosphate synthase 4	OsSPS4	<i>OsSPS4</i> <i>OsSPS8</i>	IG <u>S</u> TD AIEAWASQHK	S170
SPSA5_ORYSJ	Sucrose-phosphate synthase 5	OsSPS5	<i>OsSPS5</i> <i>OsSPS11</i>	IV <u>S</u> DEEEVTTDR	S105
NIA1_ORYSJ	Nitrate reductase [NADH] 1	OsNR1	<i>OsNIA1</i>	RST <u>S</u> IPFMNTTDGK	T533
ODPA1_ORYSJ	Pyruvate dehydrogenase E1 component subunit alpha-1, mitochondrial	OsPDHE1-A		YHGH <u>S</u> M <u>S</u> DPGSTYR	S293 or S295

Figure 1. Characterization of *OsCPK17* rice transgenic lines. (A) Schematic diagram of *OsCPK17* gene component (Locus Os07g06740), adapted from MSU Rice Genome Annotation Project (RGAP), showing the T-DNA insertion position in the T-DNA insertion line (4A-50582), represented by a triangle. Exons and introns are indicated by boxes and dotted lines, respectively. White boxes represent the untranslated regions (UTRs). ATG and TAA indicate start and stop codons. Grey arrows represent the localization of primers used for *oscpk17* genotyping. (B) *OsCPK17* expression in 13 day-old seedlings of WT and *OsCPK17* independent transgenic lines (T4 generation), analyzed by RT-PCR. Ubiquitin-conjugating enzyme E2 (*Ubc*) was used as a control. (C) Representation of the construct used to develop *OsCPK17* overexpressing lines. *OsCPK17* full coding sequence was cloned under the control of maize ubiquitin1 promoter (*Ubi1*), in frame with 6 MYC tags in the C-terminal. (D) Representation of the construct used to develop *OsCPK17*

silencing lines. A fragment of the last intron plus a portion of 3'UTR of OsCPK17 was cloned in the pANDA vector, under the control of maize ubiquitin1 promotor (Ubi1). (E) OsCPK17-MYC accumulation in protein extracts in shoots of overexpressing lines in different generations (T1-T4). Total protein extracts (20 µg) were probed with anti-MYC. Coomassie staining is shown as a loading control. Experiments were repeated at least three times with similar results. (F-H) Box-plots showing the phenotypic comparison of WT and transgenic plants grown in soil pots, under normal growth conditions, in a greenhouse. Several parameters were monitored, including: (F) plant height, (G) number of spikelets per panicle, and (H) spikelet fertility. Spikelet fertility was calculated as the percentage of filled spikelets per panicle. Boxes show means and interquantile ranges (n=20), and the asterisks denotes statistically significant differences between WT and transgenic plants (**p<0.01 and ***p<0.001; Student's t-test).

Figure 2. In vitro kinase activity of OsCPK17. Recombinant OsCPK17 protein was purified and in vitro kinase activity was determined using radiolabeled ATP and Histone III-S as a substrate, in the absence (EGTA) or presence of calcium. The activity of denatured OsCPK17 was included as a negative control. Radiolabeled substrate was quantified by scintillation counting. Data represent means $\pm$ SE of two independent experiments.

Figure 3. Analysis of OsCPK17 transgenic lines in cold stress conditions at the seedling stage. The plants were grown in hydroponic conditions, in a growth chamber under 50-70% relative humidity, with a 12h photoperiod. (A) Diagram representing the experimental design for the cold stress assay. Three-leaf stage seedlings (13 days after germination, DAG) were subjected to a pre-treatment incubation period of one day at 10°C, before being transferred to 4°C for 18 days. (B) Cold injury symptoms in the second leaves of WT, *oscpk17*, OX1 and RNAi2 plants, after 18 days under cold stress conditions. OX2 and RNAi29 plants showed similar phenotypes to the ones shown for OX1 and RNAi2, respectively. (C) The percentage of electrolyte leakage in the second fully expanded leaf was monitored to evaluate the degree of cold stress injury along the assay. Data represent means $\pm$ SE ($n \geq 6$).

Figure 4. Analysis of *OsCPK17* expression in shoots of WT and transgenic lines in response to cold stress and evaluation of the transcription regulation of cold stress-responsive genes. Thirteen-day-old seedlings (DAG) were submitted to an acclimation period of one day at 10°C and then transferred to 4°C. Times indicated

represent time after low temperature imposition. (A) Expression profiling of OsCPK17 in WT seedling's shoots. The accumulation of OsCPK17 transcripts was analyzed by Real-time quantitative PCR (qPCR). At each time point, the transcript level was normalized against the expression of constitutively expressed rice Eukaryotic Elongation Factor 1- α and Expressed Protein EP. The expression levels are presented as relative units with the levels of WT at 0h being taken as 1. Data represent means $\pm$ SE (n=3). All values are statistically significantly different at $p < 0.01$ (Student's t-test).

(B) OsCPK17-MYC protein accumulation in OX1 seedlings under cold stress was analyzed by immunoblotting with anti-MYC. Ponceau loading control shows a band corresponding to Rubisco large subunit. (C) Expression patterns of rice cold stress-responsive genes in WT (full circles) and *oscpk17* (empty circles) lines in response to cold stress conditions. The accumulation of transcripts of OsDREB1b, OsMYB4, OsNAC6, OsP5CS1 and OsLip9 were analyzed by semi-quantitative RT-PCR and calculated by quantification of the band intensities, using ImageJ. At each time point, the transcript level was normalized against the expression of constitutively expressed rice Eukaryotic Elongation Factor 1- α and Expressed Protein EP. The expression levels are presented as relative units, with the levels of WT at 0h being taken as 1. Data represent means $\pm$ SE (n $\geq$ 3). Asterisks denote statistically significant differences between WT and *oscpk17* lines (** $p < 0.01$, Student's t-test).

Figure 5. Putative targets of OsCPK17. Quantitative phosphoproteomic analysis of WT (full circles) and *oscpk17* (empty circles) seedlings exposed to cold stress conditions (10°C) allowed the high confidence identification of six phosphosites (see Table 1) with statistically significant different abundances between the genotypes in cold conditions ($p < 0.05$, Student's t-test). The corresponding protein names are indicated in each graph. Whole protein extracts of shoots, collected before (0h) and after incubation in cold stress conditions (30 min), were analyzed by LC-MS/MS using an LTQ Orbitrap Elite mass spectrometer.

Figure 6. Validation of OsCPK17 putative targets. (A) Protein abundance in the plant extracts analyzed by LC-MS/MS (see Fig. 5). The levels of each protein were calculated by the sum of the individual amount of all peptides identified for that protein with false discovery rate of 1%. Data represent means $\pm$ SE (n=3). (B) Gene expression patterns in WT (full circles) and oscpk17 (empty circles) rice lines in response to cold stress conditions (see Fig. 3A). Seedling shoots were collected for analysis at the indicated times. The accumulation of transcripts was analyzed by semi-quantitative RT-PCR and calculated by quantification of the band intensities, using ImageJ. At each time point, the transcript level was normalized against the expression of constitutively expressed rice Eukaryotic Elongation Factor 1- α and Expressed Protein. The expression levels are presented as relative units, with the levels of WT at 0h being taken as 1. Data represent means $\pm$ SE (n=3). Asterisks denote statistically significant differences between WT and oscpk17 lines (**p<0.01, Student's t-test). (C) In vitro phosphorylation of synthetic peptides (Table S2) by OsCPK17. Recombinant OsCPK17 protein was purified and in vitro kinase activity was determined using radiolabeled ATP (black bars). A reaction without substrate (no subs) and the activity of denatured OsCPK17 over each substrate (dotted bars) were included as negative controls. Radiolabeled substrates

were quantified by scintillation counting. Data represent means $\pm$ SE (n=2). D) OsPIP2;1/OsPIP2;6 synthetic peptides carrying mutations in the two possible residues phosphorylated by OsCPK17. Phosphorylation conditions were as described in (C).

Figure 7. Regulation of sucrose content in WT and OsCPK17 transgenic lines (oscpk17 and OX1) in cold stress conditions. Thirteen-day-old seedlings (DAG) were submitted to an acclimation period of one day at 10°C and then transferred to 4°C. Times indicated represent time after low temperature imposition. Sucrose content in shoots was measured by HPLC. Data represent means $\pm$ SE (n=3). Means denoted by the same letter did not differ significantly at $p < 0.01$ (Student's t-test) between samples collected at the same timepoint. Differences among samples from the same genotype, along cold incubation period (except among OX1 0h and 30 min) were statistically significant at $p < 0.001$ (Student's t-test).

Figure 8. Schematic representation of the putative OsCPK17 regulatory network, in the initial phase of rice response to cold stress (30 min of cold stress imposition). Blue arrows represent cold-activated mechanisms. Blue unbroken and broken arrows represent putative direct or indirect (unconfirmed) phosphorylations mediated by OsCPK17, respectively. The possible consequences of those phosphorylations under cold stress are considered in the discussion. Grey broken arrows indicate multiple intermediary steps. F6P: Fructose-6-phosphate, Gln: glutamine, Glu: glutamate, GOGAT: ferredoxin-dependent glutamate synthase, GS: glutamine synthetase, Mannitol-1P: mannitol-1-phosphate, Mannose-6P: mannose-6-phosphate, NIR: nitrite reductase, NO: Nitric oxide, NR1: nitrate reductase 1, P: phosphorylation, PDH: pyruvate dehydrogenase (phosphorylation of the E1 component subunit), Pi: inorganic phosphate, Pip: Plasma membrane Intrinsic Protein 2;1 or 2;6, Pyr: pyruvate, SPP: sucrose –phosphate phosphatase, SPSs: sucrose-phosphate synthase 4 and 5, Suc: sucrose, Suc-P: sucrose-phosphate, UDP-G: Uridine diphosphate glucose.