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Increased expression of *OsSPX1* enhances cold/subfreezing tolerance in tobacco and *Arabidopsis thaliana*

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Summary

Low temperature is a major environmental stress for plants. Many important cultivated crops have limited capacity to survive below freezing/subfreezing temperatures. Low inorganic phosphate (Pi) is reportedly important in triggering cold acclimatization. SPX (SYG1/Pho81/XPR1: SYG1, suppressor of yeast gpal; Pho81, CDK inhibitor in yeast PHO pathway; XPR1, xenotropic and polytropic retrovirus receptor) domain proteins have been shown to be involved in the phosphate-related signal transduction and regulation pathways. Recently, *Arabidopsis AtSPX* family genes have been found to possess diverse functions in plant tolerance to phosphorus starvation, and *OsSPX1* is involved in phosphate homeostasis in rice and optimizes growth under phosphate-limited conditions through a negative feedback loop. In this study, our phylogenetic and gene expression profiling approaches identified six rice *OsSPX* genes up-regulated during cold stress. Transgenic tobacco plants with constitutive expression of *OsSPX1* were more tolerant to cold stress than were wild-type plants, and showed better seedling survival and reduced cellular electrolyte leakage. In addition, there was decreased total leaf Pi content and accumulation of free proline and sucrose in transgenic tobacco plants during cold stress. To further establish a cause-and-effect relationship between intracellular Pi level and cold acclimatization in transgenic plants, we generated transgenic *Arabidopsis* plants with constitutive expression of *OsSPX1*. Cold stress resulted in reduced leaf Pi levels in *Arabidopsis* transgenic relative to wild-type plants. From real-time reverse transcriptase-polymerase chain reaction analysis, several Pi starvation-related genes, such *as AtSPX1* (orthologue of *OsSPX1*), *PHO2, PLDZ2* and *ATSIZ1*, showed differential expression between wild-type and transgenic plants during cold stress. Our results indicate that *OsSPX1* may play an important role in linking cold stress and Pi starvation signal transduction pathways.

Keywords: cold tolerance, OsSPX1, real-time reverse transcriptase-polymerase chain reaction (RT-PCR), transgenic.

Introduction

Low temperature is a major environmental stress with which many plants have to cope during their entire life cycle. Plant sucrose synthesis and photosynthesis are inhibited by cold stress (Ciereszko *et al*., 2001), severely affecting plant growth and development (Stitt and Hurry, 2002). In plant leaf, multiple physiological changes can occur in response to low temperature, such as calcium ion fluxes (Albrecht *et al*., 2003; Chinnusamy *et al*., 2004) and changes in gene expression (Chinnusamy *et al*., 2007; Miura *et al*., 2007b), enzyme activity (Ciereszko *et al*., 2001; Savitch *et al*., 2001; Stitt and Hurry,

2002; Bhowmik *et al*., 2006) and compatible solutes (Wanner and Junttila, 1999; Hurry *et al*., 2000), etc. Through an analysis of *Arabidopsis* pho mutants, inorganic phosphate (Pi) has been shown to play an important role in the development of freezing tolerance and the acclimatization of photosynthesis to low temperature (Hurry *et al*., 2000).

SPX (SYG1/Pho81/XPR1) domain proteins have been shown to be involved in the phosphate-related signal transduction and regulation pathways. The SPX domain was defined after the SYG1/Pho81/XPR1 proteins [SYG1, suppressor of yeast gpal (Spain *et al*., 1995); Pho81, CDK inhibitor in yeast PHO pathway (Lenburg and O'Shea, 1996; Lee *et al*., 2000; Voicu *et al*., 2007); XPR1, xenotropic and polytropic retrovirus receptor (Battini *et al*., 1999; Tailor *et al*., 1999)], which is a domain of 180 residues in length at the N-termini of these proteins. Several studies of SPX (SYG1/Pho81/XPR1) domain proteins have shown that they may be involved in the Pi-related signal transduction and regulation pathways. For example, the N-terminus of yeast SYG1 binds to the G-protein β-subunit and inhibits the transduction of the mating pheromone signal (Spain *et al*., 1995). The N-termini of several proteins, such as the putative Pi-level sensors, Pho81 and NUC-2, may be involved in the regulation of Pi transport (Lenburg and O'Shea, 1996; Lee *et al*., 2000; Voicu *et al*., 2007). The human XPR1 functions as a Pi sensor and may be involved in G-protein-associated signal transduction (Battini *et al*., 1999; Tailor *et al*., 1999). There is limited information on how plant SPX domain proteins function in plant signalling pathways and networks. Normally, to overcome the low availability of Pi, plants have developed a series of adaptive responses to Pi starvation, such as the regulation of *Arabidopsis* miR399 and PHO2 (Fujii *et al*., 2005; Bari *et al*., 2006; Buhtz *et al*., 2008; Lin *et al*., 2008; Pant *et al*., 2008). One class of SPX domain protein was identified to be involved in ion transport in plants, and named PHO1 (At3g23430) and PHO1-like protein (Hamburger *et al*., 2002; Wang Y *et al*., 2004, 2008; Stefanovic *et al*., 2007). The *pho1* mutant was characterized by a severe deficiency in shoot Pi, but normal root Pi content. *PHO1* is a gene specifically involved in the loading of Pi to the xylem in roots and is expressed in the cells of the root vascular system (Hamburger *et al*., 2002). In addition, three members of the AtPHO1 family showed possible interactions with signalling pathways involved in Pi deficiency and responses to auxin, cytokinin and abscisic acid (ABA) (Ribot *et al*., 2008a). *AtPHO1;H10* expression has recently been identified as a key connection between ABA- and CORONATINE INSENSITIVE1 (COI1)-mediated pathways (Ribot *et al*., 2008b). Another PHO1 family gene, *SHORT HYPOCOTYL UNDER BLUE1* (*SHB1*), contains an N-terminal SPX domain and a C-terminal EXS domain, and has been reported to specifically regulate blue-light responses and/or possibly red- and far-red light responses in *Arabidopsis* (Kang and Ni, 2006). Furthermore, the *PHO1* gene family was identified in *Physcomitrella patens* and showed a response to Pi deficiency (Wang Y *et al*., 2008).

In addition, a barley (*Hordeum vulgare*) SPX domain protein was identified as Ids4 and induced in roots under iron deficiency (Nakanishi *et al*., 1993). *Arabidopsis AtSPX* family genes, another class of protein with an SPX domain, have diverse functions in plant tolerance to phosphorus starvation (Duan *et al*., 2008). There are three rice *OsSPX* genes identified as Pi starvation response genes in the rice seedling stage (Wang *et al*., 2006); in particular, *OsSPX1* is involved in phosphate homeostasis through a negative feedback loop under phosphate-limited conditions in rice (Wang C *et al*., 2008). Low phosphate (Pi) is reportedly important in triggering cold acclimatization from an analysis of *Arabidopsis pho1-2* and *pho2-1* mutants (Hurry *et al*., 2000). AtSIZ1 small ubiquitin-like modifier (SUMO) E3 ligase is a negative regulator of Pi starvation signalling and also functions in cold tolerance. Information on how plant *SPX* genes respond to cold stress is still limited. In this study, we employed phylogenetic and gene expression profiling analysis to identify and study the cold response of rice *OsSPX* genes. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was used to study *OsSPX* gene expression profiles during cold treatment. Transgenic tobacco plants with constitutive expression of *OsSPX1* were examined for changes in cellular electrolyte leakage, seedling survival, total leaf Pi content, free proline and sucrose during cold stress. Furthermore, transgenic *Arabidopsis* plants with constitutive expression of *OsSPX1* were generated to study the influences of *OsSPX1* on *Arabidopsis* Pi level during cold stress, and changes in the expression levels of Pi starvation-related genes, including *AtSPX1, SIZ1, PHO2* and *PLDZETA2* (*PHOSPHOLIPASE D ZETA 2*). Our findings indicate that *OsSPX1* may be an important link between signal transduction pathways related to Pi starvation and cold stress.

Results

Phylogenetic analysis for rice and *Arabidopsis* SPX domain proteins

Thirty-seven polypeptide sequences containing an SPX domain were collected from the Pfam database (http://pfam.sanger.ac.uk/). CLUSTALW was used to perform multiple alignments for the amino acid sequences from the N-terminal SPX domain region and MEGA 3.1 was applied for the phylogenetic analysis. The local bootstrap probabilities are shown on or

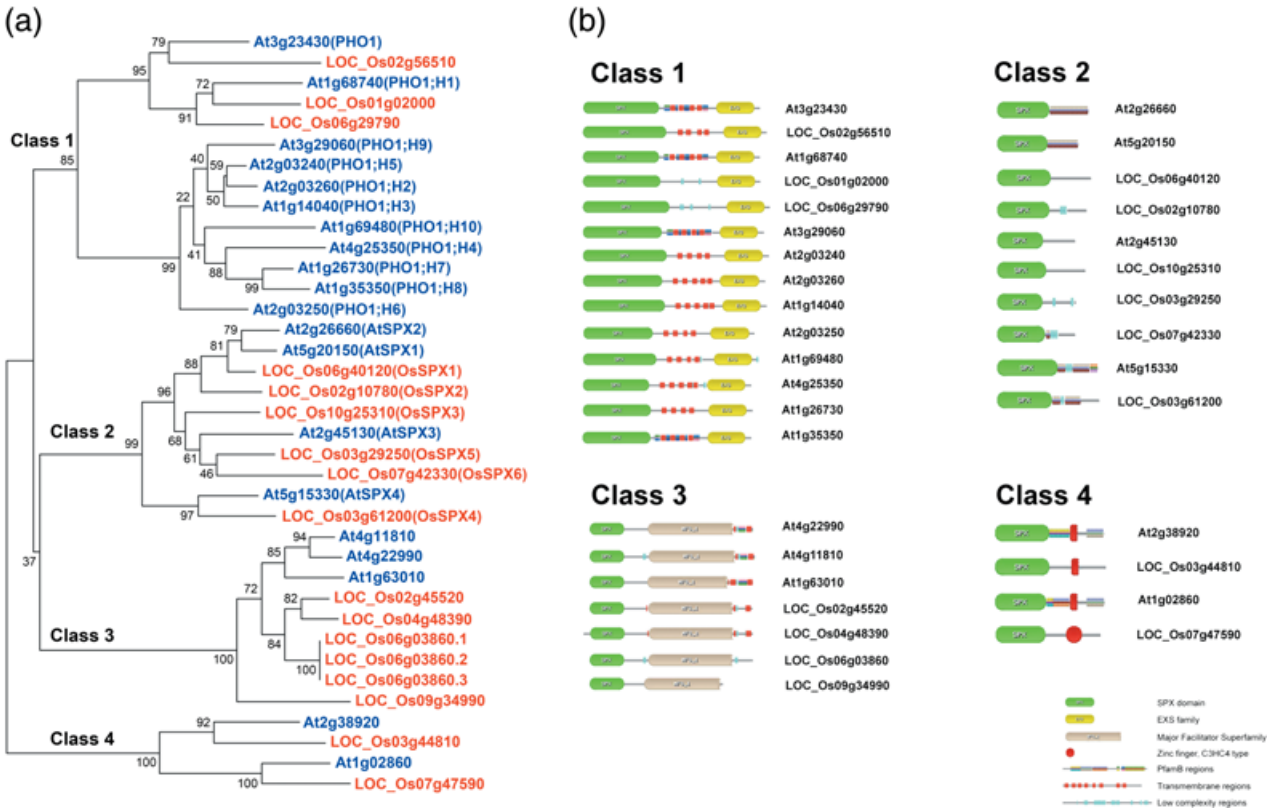

Figure 1 Classification of rice and *Arabidopsis* SPX domain proteins based on phylogenetic analysis and domain architectures. (a) Neighbour-joining tree for proteins with SPX domain from rice and *Arabidopsis*. The 37 SPX domain polypeptide sequences were aligned with CLUSTALW. Red labels represent rice genes and blue labels represent *Arabidopsis* genes. The phylogenetic analysis was performed using MEGA 3.1, and local bootstrap probabilities are shown on or below the branches. Bootstrap values were calculated from 1000 replicates. (b) The domain architectures for SPX proteins in rice and *Arabidopsis*. The proteins in Class 1 have SPX, EXS architecture; the proteins in Class 2 have SPX architecture; the proteins in Class 3 have SPX, MFS_1 architecture; the proteins in Class 4 have SPX, zf-C3HC4 architecture. The individual architecture of each protein is from the Pfam website, and the domain legend is in the bottom right-hand corner.

below the branches. Bootstrap values were calculated from 1000 replicates. Our phylogenetic analysis grouped 35 rice and *Arabidopsis* genes (37 polypeptide sequences) into four classes (Figure 1a). The four classes were differentiated by specific conserved domains: three rice and 11 *Arabidopsis* proteins in Class 1 (PHO1 and PHO1-like); six rice (OsSPX) and four *Arabidopsis* (AtSPX) proteins in Class 2; three *Arabidopsis* and six rice proteins (four rice genes) in Class 3; and two *Arabidopsis* and two rice proteins in Class 4.

The domain analyses for these rice and *Arabidopsis* SPX domain proteins were based on domain architectures from the Pfam website (Figure 1b). The proteins in Class 1 contained both an SPX domain in the N-terminus and an EXS [named after endoplasmic reticulum retention-defective 1 (ERD1)/XPR1/SYG1 proteins] domain in the C-terminus. The proteins in Class 2 only contained an SPX domain; here, we named the six rice proteins as OsSPX (LOC_Os06g40120, LOC_Os02g10780, LOC_Os10g25310, LOC_Os03g61200, LOC_Os03g29250 and LOC_Os07g42330; i.e. OsSPX1–OsSPX6, respectively) as the four *Arabidopsis* proteins in this class were already named AtSPX (Duan *et al*., 2008). The proteins in Class 3 have SPX and MFS_1 (belong to major facilitator superfamily clan) domains. The proteins in Class 4 have SPX and zf-C3HC4 (C3HC4-type zinc finger) domains.

Real-time RT-PCR analysis for all rice SPX domain genes under cold stress

Quantitative real-time RT-PCR analysis was conducted to comprehensively examine the differential expression of rice *SPX* domain genes under cold stress over a time course. One week after seed germination, the rice seedlings with 5-mm bud burst were treated at 4–5 °C. Bud burst and root tissues were harvested together after 6, 12, 24 and 48 h of cold treatment. Control seedlings without cold treatment were also harvested at each time point. There were 14 rice SPX

Table 1 Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for rice *SPX* genes under cold stress

Locus ID	Fold change (cold/control)				Class
	6 h	12 h	24 h	48 h	
LOC_Os01g02000	1.20 ± 0.0052	0.96 ± 0.0001	0.62 ± 0.0048	*0.48* ± 0.0027	1
LOC_Os02g56510	1.35 ± 0.0057	*0.25* ± 0.0064	*0.48* ± 0.0069	*0.35* ± 0.0029	1
LOC_Os06g29790	1.51 ± 0.0145	0.53 ± 0.0018	*0.44* ± 0.0095	0.76 ± 0.0038	1
LOC_Os02g10780	0.88 ± 0.0003	**5.46** ± 0.1800	**3.62** ± 0.1953	**13.41** ± 0.0000	2
LOC_Os03g29250	1.08 ± 0.0010	**4.91** ± 0.0593	**11.12** ± 0.5990	**6.81** ± 0.0270	2
LOC_Os03g61200	**4.85** ± 0.3287	**2.90** ± 0.0809	1.27 ± 0.0072	1.74 ± 0.0269	2
LOC_Os06g40120	0.87 ± 0.0009	1.61 ± 0.0185	**4.99** ± 0.1417	**5.09** ± 0.0240	2
LOC_Os07g42330	**2.76** ± 0.0235	**10.77** ± 0.5887	**36.31** ± 1.4879	**19.84** ± 1.4193	2
LOC_Os10g25310	**2.80** ± 0.0659	1.77 ± 0.0215	**10.10** ± 0.6101	**3.04** ± 0.1102	2
LOC_Os02g45520	0.70 ± 0.0033	*0.39* ± 0.0077	*0.36* ± 0.0043	*0.41* ± 0.0019	3
LOC_Os04g48390	0.67 ± 0.0091	0.76 ± 0.0026	*0.37* ± 0.0114	*0.33* ± 0.0104	3
LOC_Os06g03860	0.94 ± 0.0009	0.60 ± 0.0088	1.26 ± 0.0007	**3.23** ± 0.0627	3
LOC_Os03g44810	0.98 ± 0.0002	0.66 ± 0.0026	*0.40* ± 0.0122	1.79 ± 0.0053	4
LOC_Os07g47590	0.86 ± 0.0012	*0.23* ± 0.0043	*0.39* ± 0.0055	0.55 ± 0.0022	4

Bold and italic indicate up- and down-regulated gene expression under cold treatment.

domain genes selected for real-time RT-PCR analysis. The real-time RT-PCR results are given in Table 1 and the primer sequences in Table S1 (see Supporting Information). The relative quantification method ($\Delta\Delta C_T$) was used to evaluate the quantitative variation between replicates, and bold and italic indicate up- and down-regulated gene expression under cold treatment.

All six *OsSPX* genes in Class 2 were significantly up-regulated during cold stress (Table 1): LOC_Os06g40120 (*OsSPX1*) and LOC_Os02g10780 (*OsSPX2*) increased through all time points under cold treatment; LOC_Os10g25310 (*OsSPX3*), LOC_Os03g29250 (*OsSPX5*) and LOC_Os07g42330 (*OsSPX6*) reached their respective peaks at 24 h of cold treatment, which were more than 10-fold up-regulated; LOC_Os03g61200 (*OsSPX4*) showed a relatively early cold response (4.85-fold at 6 h and 2.90-fold at 12 h).

The majority of genes in the other classes, including Classes 1, 3 and 4, showed relatively lower expression under cold stress, except that LOC_Os06g03860 (48 h, 3.23 ± 0.0627) was slightly up-regulated after 48 h of cold stress.

Cold tolerance analysis of transgenic tobacco over-expressing *OsSPX1* gene

The real-time RT-PCR results indicated that most *OsSPX* (*OsSPX1*–*OsSPX6*) genes in Class 2 were dramatically up-regulated under cold stress. To clarify the physiological roles of *OsSPX* genes in cold stress responses in plants, we cloned full-length cDNAs for *OsSPX1* and applied a transgenic approach to study their potential function in the cold response. We generated transgenic tobacco (*Nicotiana tabacum* cv. Xanthinn) over-expressing the *OsSPX1* gene under the control of the ubiquitin promoter (Figure 2a). After leaf disc transformation with *Agrobacterium tumefaciens* carrying the binary vector, 13 independent hygromycin-resistant transgenic tobacco lines were generated for *OsSPX1*. The expression levels of the T_1 transgenes in these transgenic plants were analysed by RT-PCR. Independent lines over-expressing *OsSPX1* showed various levels of over-expression of each transgene (Figure 2b). Before treatment, there were no visible morphological changes between the wild-type (WT) and transgenic lines over-expressing *OsSPX1* at room temperature. With 24 h of 2 °C treatment, WT tobacco had wilted compared with lines 3, 4 and 11 of *Ubi::OsSPX1* transgenic tobacco (Figure 2c). Line 11 was wilted slightly when observed carefully.

Furthermore, two different cold treatment phases were conducted on transgenic and WT plants (Figure 3a). Before cold treatment, there were no visible morphological differences between WT and *Ubi::OsSPX1* transgenic plants (we mixed the different *Ubi::OsSPX1* transgenic lines together for treatment). After 0 °C treatment for 24 h, there was wilting in WT compared with transgenic tobacco. Then, all transgenic and WT plants were incubated at –2 °C for 3 h and suffered from freezing stress; transgenic *Ubi::OsSPX1* tobacco showed more tolerance to subfreezing than did WT. Transgenic tobacco showed better recovery than WT.

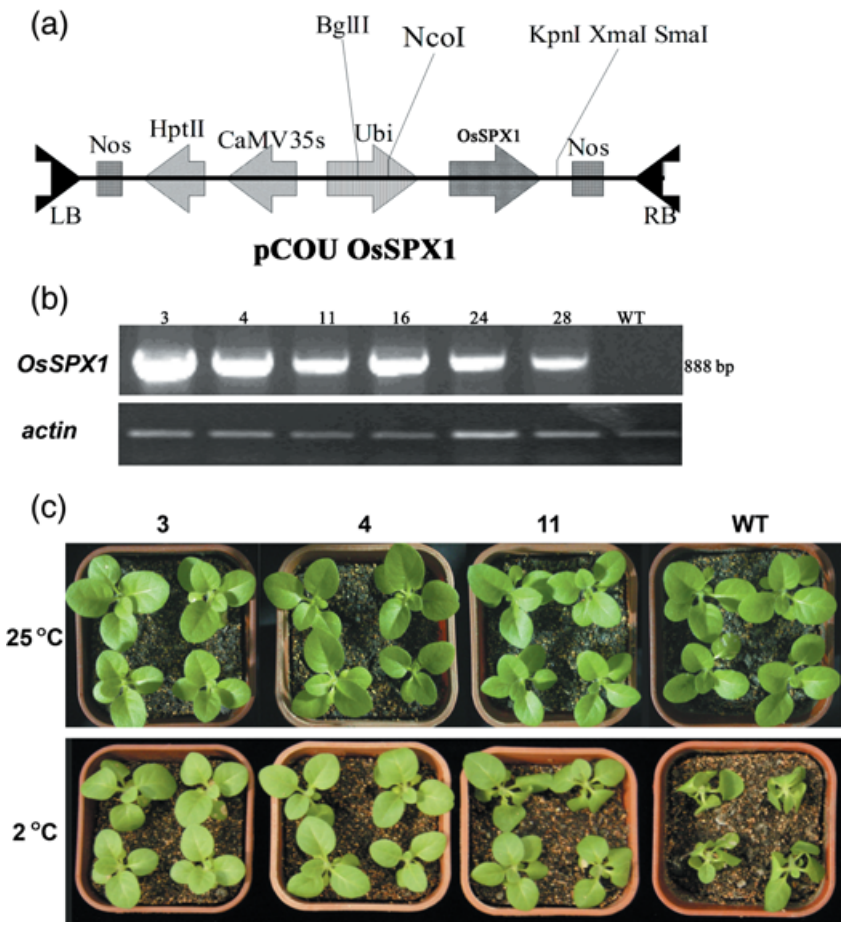

Figure 2 Analysis of *Ubi::OsSPX1* transgenic tobacco lines. (a) The binary vector pCOU OsSPX1 was used for transgenic tobacco transformation. (b) Reverse transcriptase-polymerase chain reaction (RT-PCR) results of tobacco *Ubi::OsSPX1* transgenic lines. (c) Comparison of three *Ubi::OsSPX1* transgenic lines (lines 3, 4 and 11) with the wild-type (WT) at room temperature (25 °C) and after 24-h cold (2 °C) treatment.

Moreover, we quantified the survival levels for WT and transgenic *Ubi::OsSPX1* tobacco seedling plants during subfreezing. After –2 °C treatment, the survival rate of transgenic *Ubi::OsSPX1* tobacco was 56.4% on average; it was 31.5% for WT plants. The difference was significant with three repeats (*P* value of about 0.02 with paired *t*-test).

Electrolyte leakage provides an estimate of cell damage at low temperatures. We employed increments in tissue electric conductivity to indicate the degree of membrane injury in plants exposed to various low temperatures (Figure 3b). When transgenic and WT tobacco plants were incubated at different temperatures for 3 h, electrolyte leakage of WT plants increased by approximately two-fold at –2 °C relative to that at 25 °C; transgenic plants over-expressing *OsSPX1* showed less electrolyte leakage at –2 °C compared with that of WT plants; there was no significant difference between transgenic and WT plants at –4 °C.

In addition to cellular electrolyte leakage, we also measured the changes in total Pi content in transgenic tobacco over-expressing *OsSPX1* and WT plants from 25 to 2 °C for 3 h. Transgenic *Ubi::OsSPX1* tobacco showed a similar Pi content to WT (Figure 3c). After WT and transgenic plants were taken from 25 °C and exposed to –2 °C for 3 h, the Pi content in all plants decreased, by 31.4% in WT and by 45.7% in *Ubi::OsSPX1*. Thus, the Pi content of transgenic tobacco was lower than that of WT after 3 h at –2 °C.

Compatible osmolytes, such as proline and various sugars, accumulate in many plants under cold stress; these osmolytes function as osmoprotectants (Wanner and Junttila, 1999; Taji *et al*., 2002). The free proline content of WT tobacco was low at 25 °C and increased with low-temperature treatments; proline was 2.8-fold higher at –2 °C than at 25 °C (Figure 3d). Transgenic tobacco plants over-expressing *OsSPX1* showed the same proline levels as WT at 25 °C; they showed higher proline accumulation under cold stress, with a 3.6-fold increase at –2 °C compared with that at 25 °C.

We also examined the accumulation of soluble sugars and sucrose in transgenic tobacco with –2 °C treatment for 3 h. There were no significant changes in the accumulation of soluble sugars between transgenic and WT plants (data not shown). However, transgenic plants accumulated higher levels of sucrose compared with WT at –2 °C (Figure 3e).

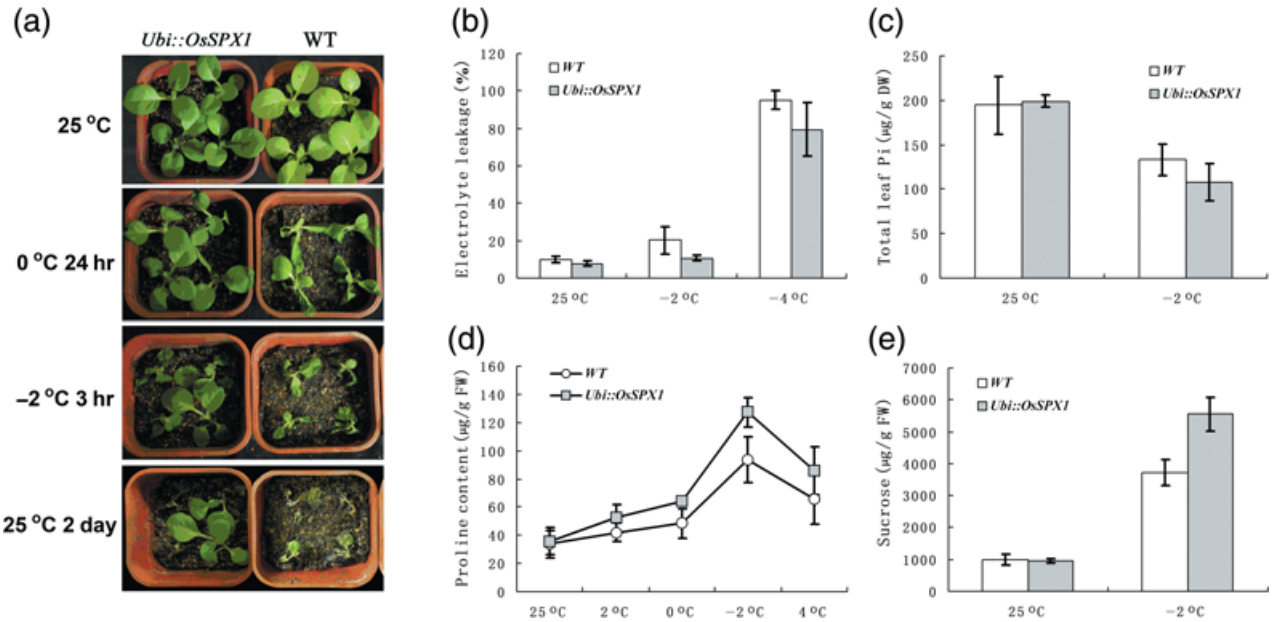

Figure 3 Phenotypic analysis of the *Ubi::OsSPX1* transgenic lines and wild-type (WT) tobacco plants during cold/subfreezing treatment. (a) Low-temperature treatment of 4-week-old transgenic and WT tobacco plants. *Ubi::OsSPX1* transgenic plants are on the left and WT plants on the right. (b) Electrolyte leakage of 4-week-old tobacco plants after 3 h of incubation at different temperatures. White and grey bars indicate WT and *Ubi::OsSPX1* transgenic plants, respectively. Results are the means ± standard deviation (SD) of three experiments. (c) Total leaf inorganic phosphate (Pi) contents of transgenic and WT tobacco plants. WT and transgenic tobacco plants over-expressing the *OsSPX1* gene were grown at 25 °C for 4 weeks and then exposed to –2 °C for 3 h. Total Pi from whole plants was measured. White bars, WT plants; grey bars, *Ubi::OsSPX1* transgenic plants. Results are the means ± SD of three experiments. (d) Free proline contents of WT plants and transgenic tobacco plants over-expressing the *OsSPX1* gene. Four-week-old tobacco plants were exposed to 2 °C for 24 h, 0 °C for 24 h, and –2 °C for 3 h, followed by 4 °C for 24 h. The free proline of whole plants was determined. White circles, WT plants; grey squares, *Ubi::OsSPX1* transgenic plants. Results are the means ± SD of three experiments. (e) Sugar contents of WT plants and transgenic tobacco plants over-expressing *OsSPX1*. WT and transgenic tobacco plants over-expressing the *OsSPX1* gene were grown at 25 °C for 4 weeks and then exposed to –2 °C for 3 h. Sucrose samples were prepared from whole plants and determined. White bars, WT plants; grey bars, *Ubi::OsSPX1* transgenic plants. Results are the means ± SD of three experiments.

Over-expression of *OsSPX1* enhanced cold tolerance and affected gene expression of Pi starvation-related genes during cold stress in *Arabidopsis*

To elucidate the possible molecular links between cold stress and Pi starvation, we generated transgenic *Arabidopsis* plants over-expressing the *OsSPX1* gene. Real-time RT-PCR results showed that *OsSPX1* was constitutively and more strongly expressed in *Ubi::OsSPX1* transgenic than WT plants at both room temperature and under cold stress (Figure 4a).

To confirm the effect of *OsSPX* over-expression on sub-freezing tolerance, T_3 transgenic and WT seedlings were exposed to –2 °C for 20 h. After 5 days of recovery under normal conditions, the seedlings of *Ubi::OsSPX1* transgenic plants (lines 1 and 2) showed much stronger freezing tolerance than WT (Figure 4b). The survival rates of *Ubi::OsSPX1* transgenic plants were relatively higher than those of WT (73.7% vs. 31.8% on average; *P* value of about 0.04 for three replicate experiments). In addition, subfreezing treatment was performed for the mutant line *atspx1* (SALK_039445), which showed more subfreezing tolerance than WT (Figure 4b). From the phylogenetic analysis result (Figure 1a), the *AtSPX1* gene may be the orthologue of the *OsSPX1* gene. The *AtSPX1* gene was up-regulated under cold stress in WT plants, but was knocked down in the *atspx1* mutant (Figure 4c).

Changes in Pi content in *Ubi::OsSPX1* transgenic and WT *Arabidopsis* plants were measured using leaf tissue under cold stress. Under normal conditions, the transgenic plants showed a lower Pi content than WT. After cold treatment (4 °C) for 3 days, the Pi content of transgenic plants decreased significantly, but there was no obvious change in WT plants (Figure 4d). Cold stress also reduced Pi levels in *Arabidopsis Ubi::OsSPX1* transgenic relative to WT plants.

Over-expression of the *OsSPX1* gene in *Arabidopsis* may affect the internal Pi content in the leaves of transgenic plants during cold stress. Several Pi starvation and cold-related genes were selected for expression profiling analysis, including *AtSPX1, PHO2, SIZ1* and *PLDZETA2* (Figure 4e). Real-time RT-PCR showed that the expression of *AtSPX1* was significantly lower in *Ubi::OsSPX1* transgenic relative to WT plants after

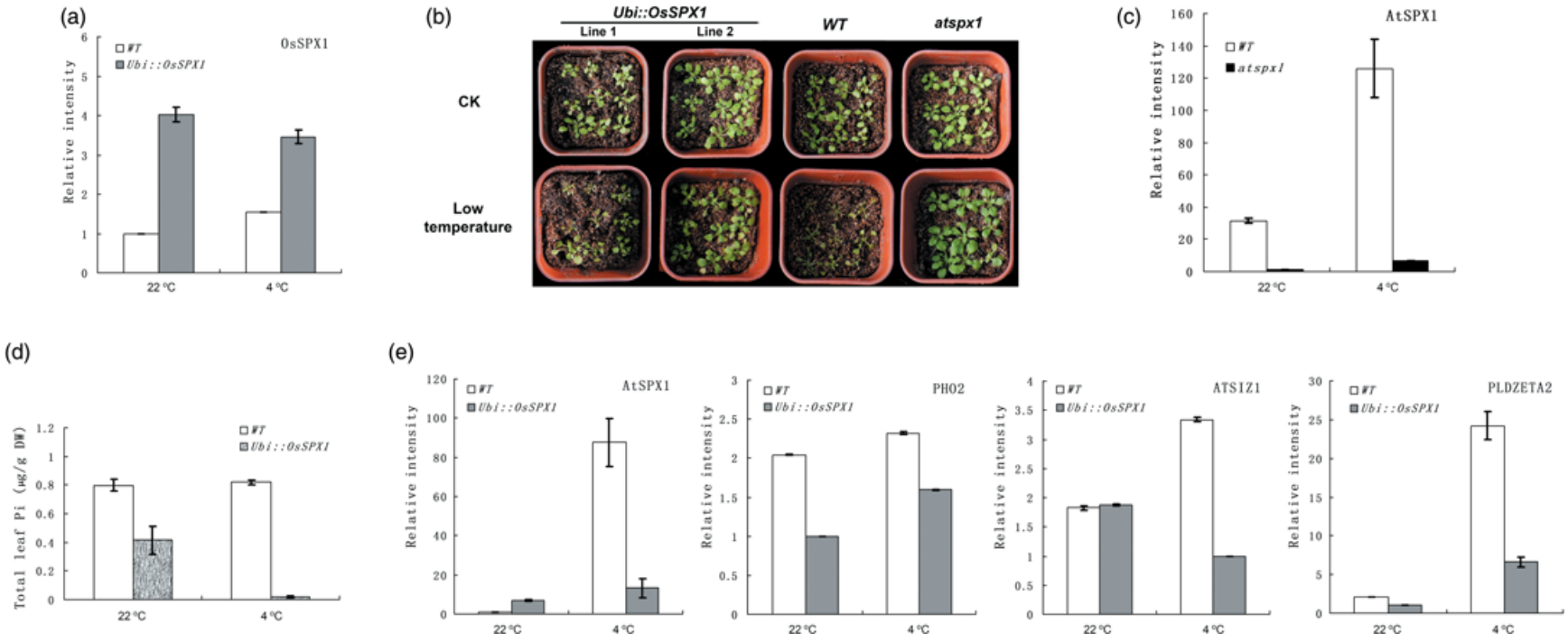

Figure 4 Analysis of *Ubi::OsSPX1 Arabidopsis* plants during low-temperature stress. (a) Expression of *OsSPX1* in *Arabidopsis* transgenic plants. (b) Low-temperature (–2 °C) treatment of 4-week-old *Ubi::OsSPX1* transgenic, *atspx1* mutant and wild-type (WT) plants. (c) Expression of AtSPXl in *atspxl* mutant and WT *Arabidopsis* plants under cold treatment. (d) Total leaf inorganic phosphate (Pi) contents of *Ubi::OsSPX1* transgenic and WT *Arabidopsis* plants under cold stress. (e) The expression pattern of selected genes related to Pi starvation in *Arabidopsis* transgenic and WT plants with 6 h of cold treatment. *PLDZETA2P2* (At3g05630); *ATSIZ1/SIZ1* (At5g60410); *PHO2* (At2g33770); *AtSPX1* (At5g20150). The error bars represent the standard deviations of three replicates.

6 h of cold treatment. The constitutive expression of *OsSPX1* in transgenic *Arabidopsis* led to the down-regulation of *PHO2* compared with WT. The expression of *AtSIZ1* was significantly reduced in transgenic relative to WT plants after 6 h of cold stress. Although *PLDZETA2* was up-regulated in both transgenic and WT plants under cold stress, the expression level was much lower in transgenic than in WT plants.

Discussion

All SPX domain proteins in rice and *Arabidopsis* were classified into four classes based on phylogenetic and domain analysis (Figure 1). There are a total of 11 members of the PHO1 family in *Arabidopsis*, but only four of the AtSPX family; there are a relatively large number of OsSPX members and fewer PHO1-like proteins in rice. *Arabidopsis AtSPX1,* a possible orthologue of rice *OsSPX1*, showed a 52-fold induction under Pi starvation (Bari *et al*., 2006). Recently, the AtSPX family was identified to be involved in the regulation of the Pi pathway. The expression levels of *AtSPX1* and *AtSPX3* were strongly induced by Pi starvation, *AtSPX2* was weakly induced and *AtSPX4* was suppressed. The AtSPX family may be a part of the phosphate signalling pathway controlled by PHR1 and SIZ1 (Duan *et al*., 2008). *Arabidopsis* microarray data mining for the stress response of *SPX* genes showed that *AtSPX1* was significantly up-regulated under cold and osmotic stress, but the expression levels of other members of the *AtSPX* family did not change (Table S3, see Supporting Information).

In rice, our real-time RT-PCR analysis showed that the majority of *OsSPX* genes were significantly up-regulated under cold stress (Table 1). OsSPX1 has been reported to be involved in phosphate homeostasis, and plays an essential role during the phosphate-starvation (PSI) signalling pathway in rice (Wang C *et al*., 2008). Through data mining and test mining, we identified three *OsSPX* genes (*OsSPX1*, *OsSPX2* and *OsSPX3*) as Pi starvation response genes (GSE6901 in GEO; Wang *et al*., 2006). Furthermore, we found that certain other cold stress response genes were also up-regulated by Pi deficiency in plants through data mining (Table S4, see Supporting Information), such as genes for phospho-enolpyruvate carboxylase kinase, glycerol-3-Pi transporter, Pi:H^+ symporter (*OsPT3*), glycerophosphoryl diester phospho-diesterase, inorganic pyrophosphatase, 1,2-diacylglycerol (*DAG*) and 3-β-galactosyltransferase (*MGD2*). Most of these genes are related to the signal transduction pathway to Pi starvation, and they may work together with *OsSPX* genes in the cross-talk between cold stress and Pi starvation.

Most significantly, transgenic *Ubi::OsSPX1* tobacco plants showed a strong cold/subfreezing tolerance phenotype (Figures 2 and 3). To clarify the pathways involved with the *OsSPX* genes and related to cold/subfreezing tolerance and Pi starvation in plants, we examined the changes in total Pi content in transgenic tobacco over-expressing *OsSPX1* and in WT tobacco when changed from 25 to –2 °C for 3 h. The Pi content in all tobacco plants decreased after cold treatment, with transgenic *Ubi::OsSPX1* tobacco showing a much lower Pi content than WT. There was decreased electrolyte leakage and the accumulation of free proline and sucrose in transgenic tobacco plants during cold stress (Figure 3). There is a close relationship between signal transduction pathways of phosphate and sucrose (Jain *et al*., 2007; Karthikeyan *et al*., 2007; Muller *et al*., 2007). It has been proposed that Pi starvation and cold stress might share common regulatory cascades, and that Pi may be involved in the acclimatization to cold stress, because some identified cold response genes are up- or down-regulated during Pi deficiency (Hammond *et al*., 2003).

To further identify the potential SPX pathway controlling the Pi level during cold stress, we generated *Arabidopsis* transgenic plants with over-expression of the *OsSPX1* gene (*Ubi::OsSPX1*). The transgenic plants showed a lower Pi content in leaf tissue than did WT plants, especially under cold stress (Figure 4c). To further establish a cause-and-effect relationship between the intracellular Pi level and cold acclimatization in *Arabidopsis OsSPX1* transgenic plants, we selected several Pi starvation-related genes (*AtSPX1, PHO2, SIZ1* and *PLDZETA2*) for expression profiling analysis; real-time RT-PCR indicated that their expression levels changed significantly between transgenic and WT plants during cold stress (Figure 4d); for example, the *AtSPX1* gene was up-regulated under cold stress in WT plants, but was repressed by the over-expression of the *OsSPX1* gene in *Arabidopsis*. Moreover, seedlings of both *Ubi::OsSPX1* transgenic and *Arabidopsis atspx1* mutant plants were more tolerant than WT to freezing. This may suggest that over-expression of the rice *OsSPX1* gene might interrupt *Arabidopsis* internal *AtSPX1* gene expression. In *Arabidopsis*, *AtSPX1* was induced by Pi starvation, and over-expression of *AtSPX1* increased the transcript levels of Pi-responsive genes, such as *ACP5*, *RNS1* and *PAP2* (Duan *et al*., 2008). *PHO2* encodes a ubiquitin-conjugating E2 enzyme as one of the key genes regulating Pi signalling pathways. *Arabidopsis pho2*-1 mutants were used to investigate whether low phosphate triggers cold acclimatization of photosynthetic carbon metabolism (Hurry *et al*., 2000). *AtSIZ1* encodes SUMO E3 ligase as a key controller of Pi starvation-dependent responses; it is a negative regulator of Pi starvation signalling and also functions in tolerance to freezing and other stresses (Miura *et al*., 2005, 2007a,b).

PLDZETA2 is a member of the PXPH-PLD subfamily of phospholipase D proteins with a major role in phosphatidic acid production during phosphate deprivation (Cruz-Ramirez *et al*., 2006). The study of *Arabidopsis OsSPX1* transgenic plants may provide an idea of why the over-expression of *OsSPX* genes in tobacco could lead to lower phosphate content and high freezing tolerance. *OsSPX* genes may be one of the key factors regulating the cross-talk between cold stress and phosphate starvation. Our findings may have uncovered some novel functions of plant SPX proteins, which may be involved in the cold signal transduction pathway.

Our study of the over-expression of the *OsSPX1* gene in rice is still proceeding. *OsSPX1* may play a role as a phosphate transporter and reallocate phosphate during cold stress, but may also act as a phosphate sensor and show different responses in different plant species during cold stress. The six *OsSPX* genes may have diverse functions in cold and phosphate starvation-related signal transduction pathways. The study of plant *SPX* gene functions may promote an analysis of the possible mechanisms by which plants sense Pi and signal Pi reallocation. The work on *OsSPX1*-involved signal transduction pathways will be greatly beneficial for the protection against phosphate starvation and cold stress, improving plant growth and crop yield.

Experimental procedures

Plant material and growth conditions

Rice (*Oryza sativa* L. cv. Nipponbare) seeds were surface sterilized in 5% sodium hypochlorite for 20 min, washed in distilled water three to four times, and then germinated in a glasshouse (28 °C day/25 °C night, 12-h light/12-h dark cycle and 83% relative humidity). About 1 week after germination, seedlings with 5-mm bud burst were placed at 4–5 °C.

Tobacco (*Nicotiana tabacum* L. cv. Xanthinn) seeds were surface sterilized with 2% sodium hypochlorite for 15 min, washed five times in sterile water, and germinated on half-strength Murashige and Skoog (MS) medium (16-h light/8-h dark at 25 °C). Four- to 6-week-old sterile WT tobacco plants were used for transformation. Four- to six-leaved tobacco plants were used for cold treatment.

Arabidopsis thaliana (Col-0, transgenic lines and *atspx1* mutant line) seeds were surface sterilized with 2% sodium hypochlorite, washed in sterile water five times, sown on MS-agar Petri plates, and placed in the dark at 4 °C for 3 days. Seedlings were incubated in a growth chamber (16-h light/8-h dark at 22 °C). Plants were continued on MS medium or transferred to soil, depending on the requirement for the experiments.

Cold treatments and RNA isolation

Rice bud burst tissues were harvested after 6, 12, 24 and 48 h of stress treatment, frozen in liquid nitrogen and stored at –80 °C for further analysis. Control plants were harvested at the same time as stressed plants.

Two-week-old *Arabidopsis* seedlings grown on MS-agar Petri plates were used for cold treatment. The plants were divided into two groups. One group was placed back into the growth chamber as the growing temperature control and the other group was placed at 4 °C for cold treatment. After 6 h, cold-treated and control plants were harvested and quickly placed into liquid nitrogen for total RNA extraction.

Total RNA was extracted using TRizol (Invitrogen) and purified by using Qiagen RNeasy kit.

Real-time quantitative RT-PCR

Reverse transcription was performed with total RNA (2 μg) using M-MLV (Invitrogen, Carlsbad, CA, USA). The cDNA samples were diluted to 8 ng/μL. Triplicate quantitative assays were performed on 1 μL of each cDNA dilution using SYBR Green Master Mix (Applied Biosystems, PN 4309155) with an ABI 7900 sequence detection system, according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). The gene-specific primers were designed using PRIMEREXPRESS software (Applied Biosystems, Foster City, CA, USA). The amplification of 18S rRNA was used as an internal control to normalize all data (forward primer, 5′-CGGCTAC CACATCCAAGGAA-3′; reverse primer, 5′-TGTCACTACCTCCCCGTGTCA-3′). The gene-specific primers are given in Tables S1 and S2 (see Supporting Information). AACT was used to evaluate the quantitative variation between replicates.

Construction of transgenic tobacco and *Arabidopsis* lines

The *OsSPX1* gene was cloned into the binary vector pCOU controlled by the ubiquitin promoter form pCOU OsSPX1. The recombinant plasmids were then introduced into *Agrobacterium tumefaciens* EHA105 strain following the freeze–thaw method. Transgenic tobacco lines were obtained by leaf disc transformation (Horsch and Klee, 1986), and transgenic *Arabidopsis* plants were obtained by the floral dipping method (Clough and Bent, 1998). The concentration of the selected antibiotic, hygromycin B, was 25 mg/L.

RT-PCR

Total RNA extracted from transgenic tobacco plants was denatured at 70 °C for 5 min and reverse transcribed at 42 °C for 60 min using AMV reverse transcriptase (Promega, Madison, WI, USA). PCR amplification was performed using the *OsSPX1* primers (P1, 5′-ATGAAGTTTGGGAA GAGCCTGAG-3′; P2, 5′-TCATTTGGCGGCCT-GCTCAATC-3′) corresponding to an 888-bp fragment. The amplification programme consisted of 5 min at 94 °C for initial denaturation, 30 cycles for 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, and 10 min at 72 °C for extension.

Cultivation and cold stress tolerance of the transgenic tobacco plants

T_1 seeds of transgenic tobacco plants were surface sterilized with 2% sodium hypochlorite for 15 min, washed five times in sterile water and

then sown on plates of half-strength MS medium containing 25 mg/L hygromycin B. The plates were incubated under fluorescent illumination (16-h light/8-h dark at 25 °C) for 10 days. The seedlings were then transferred to a 1 : 1 mixture of vermiculite and soil. At 3–4 weeks of age, the plants were exposed to cold stress treatments in which the temperature was gradually dropped to 2 °C for 24 h, then 0 °C for 24 h, then –2 °C for 3 h, and finally 25 °C for 2 days of recovery.

Cultivation and cold tolerance of the *Arabidopsis Ubi::OsSPX1* transgenic and *atspx1* mutant plants

Seeds of two independent transgenic lines, *atspx1* mutant and WT plants grown on soil were cultured in a growth chamber, and 4-week-old *Arabidopsis* plants in the glasshouse were exposed to –2 °C for 20 h. The phenotype of transgenic lines and WT was investigated after 5 days of recovery in the growth chamber.

Electrolyte leakage assay

The percentage of electrolyte leakage was measured to evaluate the degree of cold injury in transgenic and WT tobacco. Following the exposure of 4-week-old tobacco plants to different temperatures (2, 0 and –2 °C) for 3 h, 0.3 g leaf samples from each group were washed with deionized water and immersed in 7 mL of deionized water with 150 r.p.m. shaking for 16 h. The electrical conductivity of the initial sample (S_1) was detected using a DDSJ-308 A detector (Shanghai, China). The samples were then boiled for 10 min and the ultimate conductivity was measured (S_2, maximum conductivity of tissues) after cooling to room temperature. The relative degree of leakage (L) was calculated using the ratio S_1/S_2.

Proline and sugar measurements

Free proline contents of transgenic tobacco plants were measured. Fresh leaf tissue (0.5 g) was extracted in 5 mL of 3% sulphosalicylic acid at 95 °C for 15 min. After filtration, 2 mL of supernatant was transferred to a new tube containing 2 mL of acetic acid and 2 mL of acidified ninhydrin reagent. After 30 min of incubation at 95 °C, 5 mL of toluene was added to the tube with full shaking to extract red products. The absorbance of the toluene layer was determined at 532 nm.

Soluble sugars were extracted in 80% ethanol with constant stirring at 80 °C for 2 h, ethanol was evaporated and water was added. For the measurement of the soluble sugar content, 0.15% anthrone solution was added to each sample. The mixture was heated at 95 °C for 15 min and placed at room temperature. The absorbance of the reaction solution was determined at 620 nm. For the measurement of the sucrose content, 30% KOH was added to each sample at 95 °C for 10 min before the addition of 0.15% anthrone solution. The absorbance of the mixture was determined at 620 nm.

Pi measurement

To measure total Pi, leaf samples (0.3–0.5 g dry weight) (m) were digested with 4 mL of H_2SO_4 and 8–10 mL of H_2O_2 at 400 °C for about 5 h, and H_2O was added to 100 mL (V_1). From the supernatant solution, 2–5 mL (V_2) was added to H_2O to 30 mL, with one to two drops of 2,6-dinitrophenol; 6 M NaOH was added until neutralization, when a yellow colour appeared; one drop of 2 mol/L H_2SO_4 was used to remove the yellow colour, and 5 mL of Mo-Sb spectrochrometry colour reagent were added and mixed well; finally, H_2O was added to a constant volume (V_3). After 30 min of incubation, the absorbance at 700 nm was measured, and the amount of Pi was quantified from Pi standards: total Pi (mg/g) = $c(P) \times (V_1/m) \times (V_3/V_2)$.

Statistical analysis of microarray data

The CEL files of each experiment in the microarray data sets were downloaded from the GEO website (http://www.ncbi.nlm.nih.gov/geo/). All CEL files were re-processed by Affymetrix GCOS software to produce the CHP file, and the target mean value (TGT) was rescaled as 100 for each chip. Student's t-test was performed for mean comparisons between stress and control conditions. The algorithm is incorporated into Microsoft Excel (Microsoft, Redmond, WA, USA).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Primers of real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for rice *SPX* genes under cold stress.

Table S2 Primers of real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for *Arabidopsis* genes under cold stress.

Table S3 Stress response of *SPX* genes in *Arabidopsis* based on microarray data analysis.

Table S4 Selected rice genes up-regulated by cold stress and phosphate starvation.

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