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Research

Comparative metabolomic analysis reveals a reactive oxygen species-dominated dynamic model underlying chilling environment adaptation and tolerance in rice

Jingyu Zhang[1], Wei Luo[1], Yuan Zhao[1], Yunyuan Xu[1], Shuhui Song[2] and Kang Chong[1]

[1]Key Laboratory of Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China; [2]Big Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing 100101, China

Authors for correspondence:
Jingyu Zhang
Tel: +86 10 62836695
Email: jingyuzhang@ibcas.ac.cn

Kang Chong
Tel: +86 10 62836517
Email: chongk@ibcas.ac.cn

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Summary

• Cold, a major environmental stress for plants, has been studied intensively for decades. Its response system has been revealed, especially at the transcriptional level. The mechanisms underlying recovery growth and environmental adaptation, however, remain unknown.

• Taking advantage of a naturally existing system, two subspecies of Asian cultivated rice (*Oryza sativa*) with significant divergence in chilling tolerance, we analyzed representative *japonica* and *indica* varieties, Nipponbare and 93-11, using comparative metabolomic analysis at six time points covering chilling treatment and recovery.

• In total, 223 known metabolites were detected. During chilling treatment, significant biochemical changes were centered on antioxidation. During recovery, a wide-ranging chilling response was observed. Large-scale amino acid accumulation occurred, consistent with the appearance of chilling injury. At the mid-treatment stage, the accumulation of antioxidation-related compounds appeared earlier in Nipponbare than in 93-11, consistent with the higher reactive oxygen species (ROS) levels in *japonica* vs *indica* varieties. A significant contribution of ROS-mediated gene regulation, rather than the C-repeat binding factor/dehydration-responsive-element binding factor (CBF/DREB) regulon, to the more vigorous transcriptional stress response in Nipponbare was revealed by RNA-seq. Accordingly, during recovery, the induction of stress-tolerant-related metabolites was more active in the chilling-tolerant variety Nipponbare. Senescence-related compounds accumulated only in the chilling-sensitive variety 93-11.

• Our study uncovers the dynamic metabolic models underlying chilling response and recovery, and reveals a ROS-dominated rice adaptation mechanism to low-temperature environments.

Introduction

Low temperature drastically impairs plant growth and significantly restricts the productivity and spatial distribution of crop plants. The response of plants to low-temperature stress is a highly complex process involving multiple levels of regulation. After decades of intensive studies, we have begun to understand the cold-responsive system in plants, especially at the transcriptional level (Chinnusamy *et al.*, 2007; Knight & Knight, 2012; Miura & Furumoto, 2013). However, little, is known about how the plant adjusts its response system to adapt to low environmental temperatures. The cold-responsive transcriptional pathways, including the well-known C-repeat binding factor (CBF)/dehydration-responsive-element binding factor (DREB) regulon, have been outlined, but no obvious connection has been detected between the expression levels of transcription factors and the degree of cold tolerance (Mao & Chen, 2012; Zhao *et al.*, 2015).

Chilling (0–15°C) is the environmental stress typically experienced by plants from tropical and subtropical regions, such as rice, one of the most important food crops in the world. Contrasting environmental temperature is the key factor driving the divergence between two subspecies of Asian cultivated rice (*Oryza sativa*): *japonica* (*O. sativa* ssp. *japonica*) and *indica* (*O. sativa* ssp. *indica*) (Kovach *et al.*, 2007; Sang & Ge, 2007). The *japonica* varieties are usually grown in higher altitude or latitude regions and exhibit a certain level of chilling tolerance. On the contrary, *indica* varieties, typically grown in tropical and low-altitude areas, are sensitive to chilling stress. The divergence of the two rice subspecies in chilling tolerance makes them an excellent model to study the adaptation of plants to a low-temperature environment.

Chilling is regarded as a major climatic problem for agricultural production in tropical and subtropical regions. Chilling stress causes obvious damage to plants, including wilting, discoloration, drying of leaf edges, accelerated aging, incomplete

ripening and even the death of plants. Interestingly, unlike other kinds of abiotic stress, a drop in temperature (above zero) for a short period of time does not lead to any visible change in plant phenotype. Chilling injury actually appears during subsequent recovery growth. Wilting and even death are observed for chilling-sensitive plants at this stage. Although this phenomenon has been known for years, its underlying mechanism remains largely unknown. In addition, to date, most of the studies on chilling stress have concentrated on the stage during which the plants are exposed to low environmental temperature; recovery growth has received little attention, except for a few proteomic analyses (Badowiec *et al.*, 2013; Badowiec & Weidner, 2014).

Metabolites are the real players in plant stress responses. Changes in metabolites and their levels are regarded as the ultimate response of plants to environmental stresses (Balmer *et al.*, 2013). Despite the detailed analysis of cold stress at the transcriptional level, relatively little is known at the metabolic level. Metabolomic analysis can shed new light on the adaptation mechanism to a low-temperature environment. In addition, the kinetic characteristics of the cold response have received little attention and the regulation mechanism remains elusive, although a series of important metabolic changes, including the accumulation of sugars and amino acids, have been detected in the cold response in recent metabolic analysis studying the effect of environmental factors (Maruyama *et al.*, 2014). Here, taking advantage of a naturally existing experimental system for chilling stress – two rice subspecies with significantly different chilling tolerance – we performed a comparative metabolomic analysis at a series of time points, covering both the chilling treatment and recovery stage, to detect global dynamic changes and to uncover the metabolic models underlying the chilling response and its adaptation mechanism to different thermal environments. Our results deepen our understanding of plant chilling tolerance and environmental adaptation, and provide useful information for chilling tolerance improvement in crop plants.

Materials and Methods

Plant material and chilling treatment

Rice (*Oryza sativa* L.) seeds of *japonica* variety Nipponbare and *indica* variety 93-11 were grown in plastic pots filled with nutrient soil in a glasshouse (28°C : 25°C, day : night). Fifteen-day-old seedlings were subjected to chilling treatment at 2 ± 1°C for 10, 33 and 57 h. The rice seedlings, with 57 h of chilling treatment, were transferred back to the glasshouse and grown for 24, 48 and 72 h for recovery. Survival rates were calculated after 7 d of recovery and are shown as the percentage of seedlings that were alive. The experiment was performed with three biological replicates.

Metabolomic profiling and statistical analysis

Global unbiased metabolic profiling was performed by Metabolon (www.metabolon.com) based on three independent platforms: ultra-high-performance liquid chromatography/tandem mass spectrometry (UHLC/MS/MS^2) optimized for basic species, UHLC/MS/MS^2 optimized for acidic species and gas chromatography/mass spectrometry (GC/MS) (Evans *et al.*, 2009).

Six biological replicates (except for five biological replicates collected at 24 and 48 h in stage II) were collected for each rice cultivar at every time point. To eliminate the growth effect, both chilling-treated samples and corresponding untreated controls were prepared for every time point. Samples were analyzed as described previously (Oliver *et al.*, 2011). Metabolite identification was performed by automated comparison of their detected ion features with a reference library of chemical standard entries including retention time, molecular weight (m/z), preferred adducts, in-source fragments and their associated MS/MS^2 spectra. Process blanks (water only) and solvent blanks were used to remove artifactual peaks.

Data analysis was carried out as described previously (Evans *et al.*, 2009). The missing value for a given metabolite was assigned the observed minimum detection value, based on the assumption that the missing value was below the detection limit. The raw area count for each metabolite was rescaled through the division of each sample value by the median value for this specific metabolite to obtain the scaled amount for better data visualization.

Statistical analysis was performed with the commercial software package JMP (SAS, http://www.jmp.com) and open-source software package R (http://cran.r-project.org/). The fold change for each compound was calculated as the ratio of the scaled amount of chilling-treated sample compared with that of the untreated sample. Welch's two-sample t-tests were used to determine whether the level of each metabolite was significantly ($P < 0.05$) increased or decreased. The false discovery rate (FDR) was used to correct for multiple comparisons of all identified metabolites (Storey, 2002). The FDR for a given set of metabolites was estimated by the Q value (see Supporting Information Table S2).

A heat map was generated with MeV v.4.9 to show the fold change for the identified compounds under chilling stress (Saeed *et al.*, 2003). Principal component analysis (PCA) was performed with MetaGeneAlyse and IBM SPSS v.19 (http://www-01.ibm.com/software/analytics/spss/) (Daub *et al.*, 2003). To assess metabolite–metabolite correlations, Pearson's product-moment correlation was employed and the level of significance was measured with the P-value using R statistical software.

RNA-seq and statistical analysis

Total RNA was extracted from Nipponbare and 93-11 seedlings with and without chilling treatment using the mirVana miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA). Sequencing libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Beverly, MA, USA) following the manufacturer's recommendations. Briefly, the mRNAs were enriched using oligo(dT) magnetic beads and broken into short fragments. First-strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (New England Biolabs). Second-strand cDNA synthesis was subsequently performed using DNA Polymerase I (Life Technologies). NEBNext Adaptor was ligated to the

fragments, which were purified with the Agencourt AMPure XP Kit (Beckman Coulter, Brea, CA, USA) to preferentially select cDNA fragments of 150–200 bp. PCR amplification was performed with size-selected and adaptor-ligated cDNA with Phusion High-Fidelity DNA Polymerase (New England Biolabs). PCR products were purified and sequenced on an Illumina HiSeq 2000 platform.

After low-quality reads had been removed, an index of the reference genome was built using Bowtie v.2.0.6 and clean reads were aligned to the reference genome (MSU Rice Genome Annotation Project Release 7, http://rice.plantbiology.msu.edu/) using TopHat v.2.0.9 (Langmead & Salzberg, 2012; Kim *et al.*, 2013). HTSeq v.0.5.4 was used to count the read numbers mapped to each gene (Anders *et al.*, 2015). Differential expression analysis of chilling-treated and untreated samples was performed with a $\log_2$ fold change of $\pm$ 1.5 as the threshold.

Heat maps were generated with MeV v.4.9 to show gene expression levels (Saeed *et al.*, 2003). Gene ontology analysis was performed using agriGO (Du *et al.*, 2010). Kobas v.2.0 was employed to analyze the statistical enrichment of differentially expressed genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Xie *et al.*, 2011). Significant enrichment was detected with $Q < 0.05$.

Histochemical analysis

The formation of hydrogen peroxide was detected by 3,3′-diaminobenzidine (DAB) staining as described previously (Ueda *et al.*, 2013). To detect superoxide anion radicals, rice leaves from triplicate biological replicates of the above samples were first cut into sections (*c.* 1 cm in length) and exposed to nitroblue tetrazolium (NBT) staining as described previously (Wohlgemuth *et al.*, 2002).

Quantification of gibberellic acid, salicylic acid and ethylene

The quantification of endogenous gibberellic acid and salicylic acid (SA) was performed as described previously (Chen *et al.*, 2012). Leaves from Nipponbare and 93-11 seedlings were harvested at the indicated time points and analyzed using a Shimadzu Prominence nano-flow liquid chromatography (nano-LC-ESI-Q-TOF-MS) system. Samples from *c.* 20 plants were analyzed for each time point.

Ethylene quantification was performed as described previously (Iwai *et al.*, 2006). Briefly, fully expanded leaves were collected at different time points, cut into pieces and incubated in a sealed 20-ml glass vial containing 100 µl of water. Ethylene production was determined with a gas chromatograph on an Agilent 7890A (Agilent Technologies, Santa Clara, CA, USA). Samples from *c.* 15 plants were analyzed for each time point.

All analyses were performed in three biological replicates.

Accession numbers

The raw reads and expression estimates for RNA-seq were deposited in the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) with accession number GSE67373.

Results

The *japonica* variety Nipponbare and *indica* variety 93-11, with high and low chilling tolerance, respectively, exhibited chilling-induced injury during recovery growth

After chilling treatment (2 ± 1°C), all the Nipponbare plants remained alive, but only *c.* 9% of the 93-11 plants survived, indicating that Nipponbare and 93-11, representative varieties of *japonica* and *indica*, are tolerant and sensitive to chilling stress, respectively (Figs 1, S1). Six points in the time course, covering both chilling treatment (stage I: 10, 33 and 57 h) and recovery growth (stage II: 24, 48 and 72 h) processes, were chosen for metabolomic analysis to gain a global view of the dynamic changes of chilling stress (Fig. 1a). No obvious phenotypic changes were found for Nipponbare and 93-11 during chilling treatment. During subsequent recovery growth, the leaves of 93-11 began to curl at 24 h. The percentage of curled leaves increased over time and most were wilted at 72 h. For Nipponbare, however, no obvious phenotypic change was detected at 24 or 48 h. At 72 h, wilting and curling were observed, but only for a small portion of the leaves (Figs 1b, S1).

Different patterns of metabolic changes during chilling treatment and recovery growth

We performed unbiased global metabolic profiling based on UHLC/MS/MS^2 and GC/MS. A total of 223 metabolites that matched known biochemicals were detected, and *c.* 90% showed chilling-induced changes at different time points (Tables S1, S2). To eliminate the growth effect, both chilling-treated and untreated samples were collected for every time point and metabolomic data from chilling-treated samples were first compared with those from corresponding untreated controls; the relative contents obtained were used for further analysis.

Based on global metabolomic profiling, different metabolic patterns were identified for chilling treatment and recovery growth. During chilling treatment, obvious changes (more than five-fold) were detected for specific compounds (Fig. 2). No metabolic collapse was observed. In recovery growth, however, obvious changes were detected in the content of various types of metabolite, including amino acids, peptides, carbohydrates, nucleotides, lipids, cofactors and secondary metabolites, especially at the initial stage in Nipponbare, suggesting a wide-ranging response to chilling stress (Fig. 2). Obvious accumulation was observed for a wide variety of amino acids in both rice varieties at different stages, implying the imbalance of protein metabolism (Fig. 2). Interestingly, in 93-11, large-scale amino acid accumulation appeared throughout the recovery stage, together with the damage caused by chilling stress. For Nipponbare, however, neither large-scale amino acid accumulation nor obvious phenotypic change was observed at 24 and 48 h. At 72 h, large-scale amino acid accumulation was observed, together with

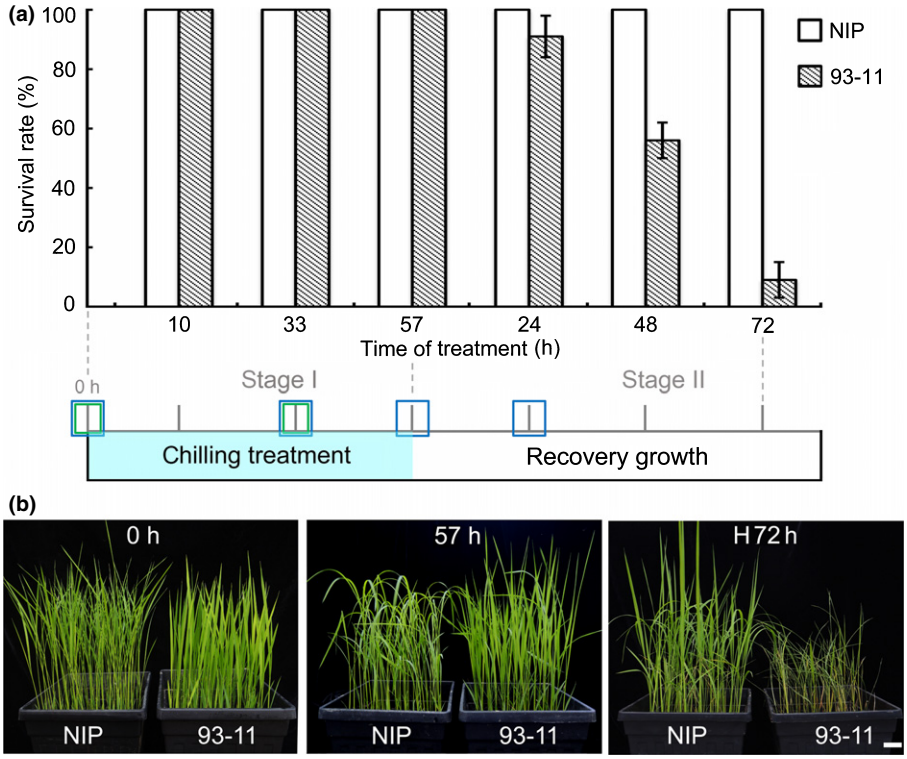

Fig. 1 Different chilling tolerance between *Oryza sativa* ssp. *japonica* and *indica* varieties, Nipponbare (NIP) and 93-11, and experimental design. (a) The *japonica* variety NIP and *indica* variety 93-11 exhibit high and low chilling tolerance, respectively. Survival rates after chilling treatment are indicated as the mean of three independent replicate experiments ± SD. Metabolomic analysis of NIP and 93-11 was performed at six different time points covering chilling treatment (stage I: 10, 33 and 57 h) and recovery growth (stage II: 24, 48 and 72 h). RNA-seq and phytohormone quantification are denoted by green and blue squares, respectively. (b) Representative images of NIP and 93-11 for untreated samples (0 h) and plants exposed to 57 h of chilling treatment (57 h) and recovered for 72 h (H72 h). See Supporting Information Fig. S1 for more information. Bar, 2.5 cm.

the phenotypic changes caused by chilling stress. A positive connection was detected between large-scale amino acid accumulation and chilling injury, suggesting that large-scale amino acid accumulation could be used as a biomarker for chilling injury (Figs 2, S1).

Metabolomic differences between representative *japonica* and *indica* varieties, Nipponbare and 93-11, during chilling treatment and recovery growth

With metabolomic data at six time points, we were able to gain a complete view of the dynamic changes in central carbohydrate metabolism and related amino acid biosynthetic pathways during chilling stress. A general increase in the content of glycolysis intermediates was observed in both varieties during chilling treatment, suggesting the activation of glycolysis. During recovery growth, this type of increase was not observed in Nipponbare, suggesting the return of glycolysis activity to a normal level. In 93-11, however, glucose-6-phosphate still showed an increased amount at the end of recovery growth, implying that chilling injury is too severe in this variety and metabolic reprogramming is probably disturbed (Fig. S2). A general increase was also observed for the content of amino acids in both rice varieties (Fig. S2).

Principal component analysis (PCA) was performed to obtain an overview of the sample distribution for metabolomic analysis performed at a series of time points. Different trends in changes were detected for chilling treatment and recovery growth. During chilling treatment, chilling-treated samples separated from untreated samples in both Nipponbare and 93-11, indicating the effect of chilling treatment. In recovery growth, the distribution of samples was associated with the level of chilling tolerance. In the chilling-tolerant variety Nipponbare, chilling-treated samples grouped together with untreated samples, suggesting recovery at the metabolic level. For the chilling-sensitive variety 93-11, however, the chilling-treated samples were separated from untreated samples, suggesting the irreversible effect of chilling stress in this variety (Fig. 3). Moreover, the 33-h and 57-h samples were found to exhibit the most significant divergence from others in Nipponbare and 93-11, respectively, indicating that dramatic metabolic changes occurred at different time points in these two varieties (Fig. 3).

To further analyze the metabolite dataset, we performed correlation analysis for metabolomic data at six time points employing Pearson's product-moment correlation to reveal relationships among metabolites. For Nipponbare, clear overall positive and negative correlations were observed for different types of metabolite, suggesting strong overall regulation. By and large, positive correlations were detected between amino acids and peptides, which also showed positive correlations with themselves. Negative correlations were detected between carbohydrates and amino acids, as well as peptides. Positive and negative correlations were also detected between nucleotides and amino acids, as well as nucleotides and carbohydrates, with some exceptions. For 93-11, however, no clear correlation was detected among different types of metabolite, suggesting the lack of overall regulation (Fig. 4).

Antioxidation-related metabolic changes were observed in *japonica* variety Nipponbare, but not in *indica* variety 93-11, at the middle stage of chilling treatment

Metabolic profiling indicated that, during the chilling response, the accumulation of amino acids, such as proline, asparagine and

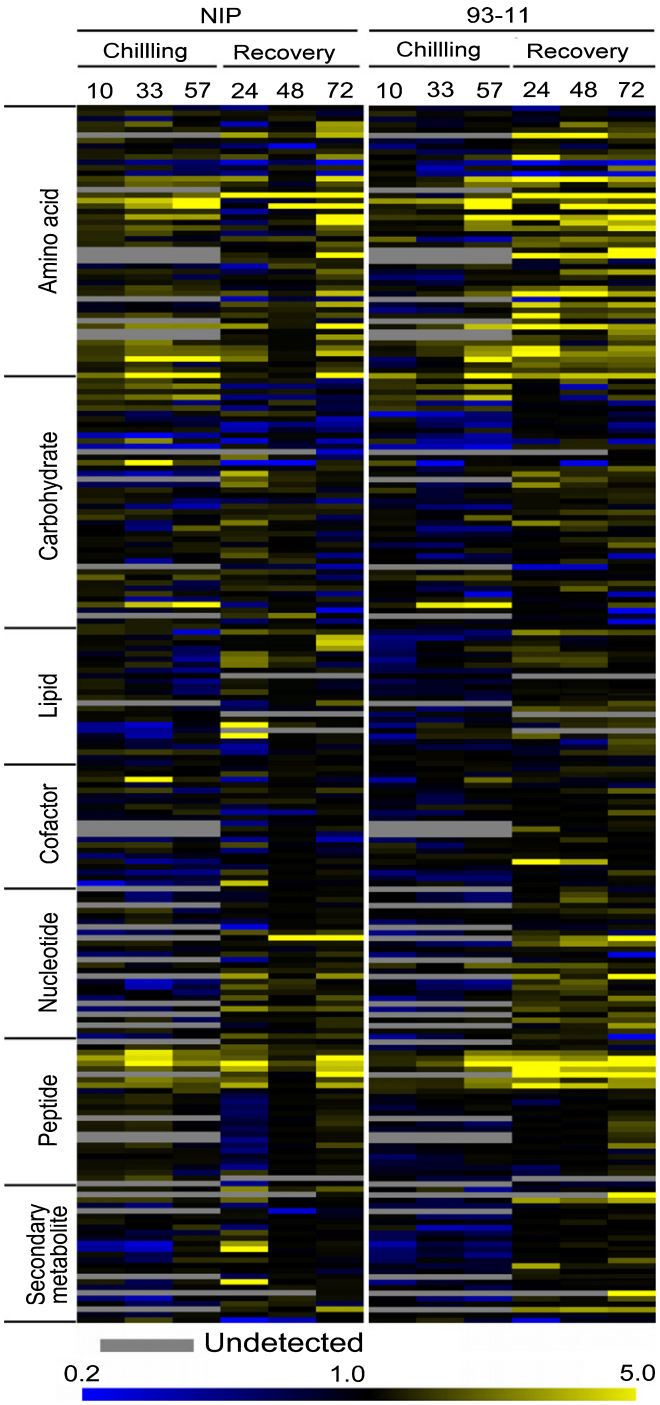

Fig. 2 Heat map representation of chilling-induced changes in the contents of all detected metabolites in rice (*Oryza sativa*) varieties Nipponbare (NIP) (*japonica*) and 93-11 (*indica*) during chilling treatment and recovery growth. The six time points are denoted by numbers. The level of a given metabolite is judged to increase or decrease ($P < 0.05$) by comparing the scaled amount in chilling-treated samples with that in untreated samples. Metabolites are categorized according to compound classes. *P* values are shown in Supporting Information Table S2.

β-alanine, and carbohydrates, including fructose, glucose and their phosphorylated forms, as well as mannose-6-phosphate, was observed in both Nipponbare and 93-11, consistent with their positive roles in cold stress (Tables 1, S2) (Guy *et al.*, 2008). These well-known stress responses, detected in both *japonica* and *indica* varieties, are obviously not responsible for their different chilling tolerance.

With the elimination of the growth effect, dynamic changes under chilling treatment were detected in the metabolome of Nipponbare and 93-11. No significant (more than five-fold) metabolic changes were observed at the beginning of chilling treatment (10 h), suggesting that this time point is too early for obvious metabolic responses. At the middle stage of chilling treatment (33 h), significant metabolic changes were observed in Nipponbare, but not in 93-11 (Table 1). Most of these changes in Nipponbare were connected with antioxidation and related metabolic adjustment. The accumulation of glutathione (GSSG), a metabolite involved in the glutathione–ascorbate cycle, was observed (Noctor *et al.*, 2012). The content of γ-glutamylleucine, a glutamyl amino acid involved in glutathione recycling, also increased significantly (Table 1) (Ohkama-Ohtsu *et al.*, 2008). An increase was also observed in the content of γ-glutamylisoleucine and γ-glutamylglutamine (Table S2). The contents of 5-oxoproline, glycine and glutamate, intermediates of glutathione metabolism, and threonate, an ascorbate metabolite, also increased (Table S2) (Debolt *et al.*, 2007; Ohkama-Ohtsu *et al.*, 2008). These results suggest that the glutathione–ascorbate cycle, as well as glutathione metabolism, is activated. Interestingly, an increased level of oxalate was also detected, a compound involved in ascorbate metabolism, as well as the regulation of plant oxidative status with a somewhat disputed mechanism (Table 1) (Cessna *et al.*, 2000; Hu *et al.*, 2003; Debolt *et al.*, 2007). A significant increase was detected in the content of nicotinamide mononucleotide (NMN), an intermediate in nicotinamide adenine dinucleotide (NAD) synthesis (Table 1) (Jayaram *et al.*, 2011). NAD is involved in plant cellular redox homeostasis and plays a crucial role in antioxidant metabolism (Hashida *et al.*, 2009). Putrescine, a polyamine playing a role in plant cold tolerance and possessing antioxidant activities through the prevention of lipid peroxidation, also accumulated significantly in Nipponbare (Table 1) (Verma & Mishra, 2005; Cuevas *et al.*, 2008). This compound also contributes to plant stress tolerance through the regulation of its oxidative status (Tanou *et al.*, 2014). These types of induction, however, were not observed in 93-11 at this time point. At the end of chilling treatment (57 h), only some of these compounds, including metabolites related to the glutathione–ascorbate cycle and putrescine, were induced in 93-11 (Table 1).

We also compared the baseline levels of the detected metabolites between Nipponbare and 93-11. Higher levels of two unsaturated fatty acids, linoleate and linolenate, were detected in Nipponbare, which may contribute to its higher chilling tolerance (Upchurch, 2008). No other obvious difference associated with chilling tolerance was detected, implying the limited contribution of metabolite baseline levels (Table S2).

Higher level of ROS accumulated in *japonica* varieties than in *indica* varieties at the middle stage of chilling treatment

As already described, during the middle stage of chilling treatment (33 h), the levels of antioxidation-related compounds

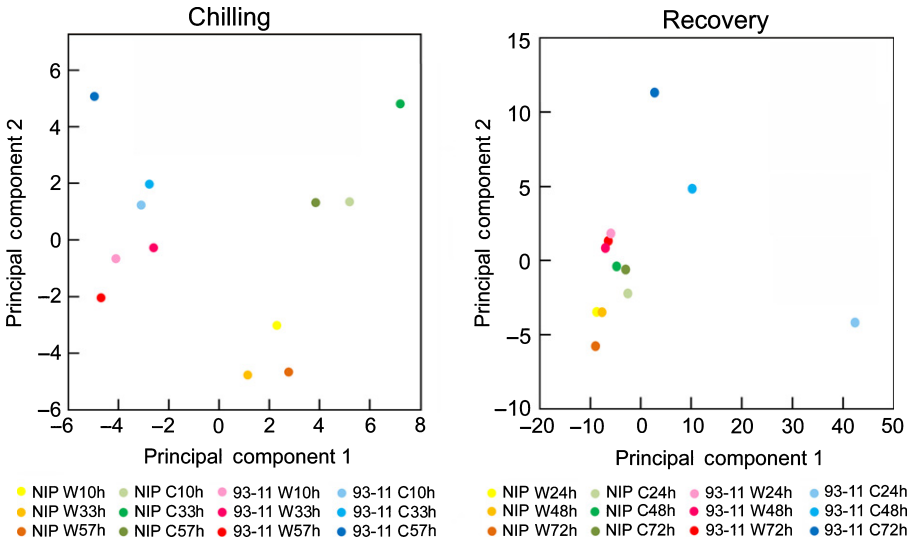

Fig. 3 Principal component analysis of metabolic profiles of rice (*Oryza sativa*) varieties Nipponbare (NIP) (*japonica*) and 93-11 (*indica*) with and without chilling treatment. During chilling treatment: C10h, C33h and C57h, samples with chilling treatment; W10h, W33h and W57h, samples without chilling treatment. During recovery: C24h, C48h and C72h, samples with chilling treatment; W24h, W48h and W72h, samples without chilling treatment. The first two principal components explain 62.68% and 46.07% of the variance separating NIP from 93-11 in the chilling treatment and recovery stage, respectively.

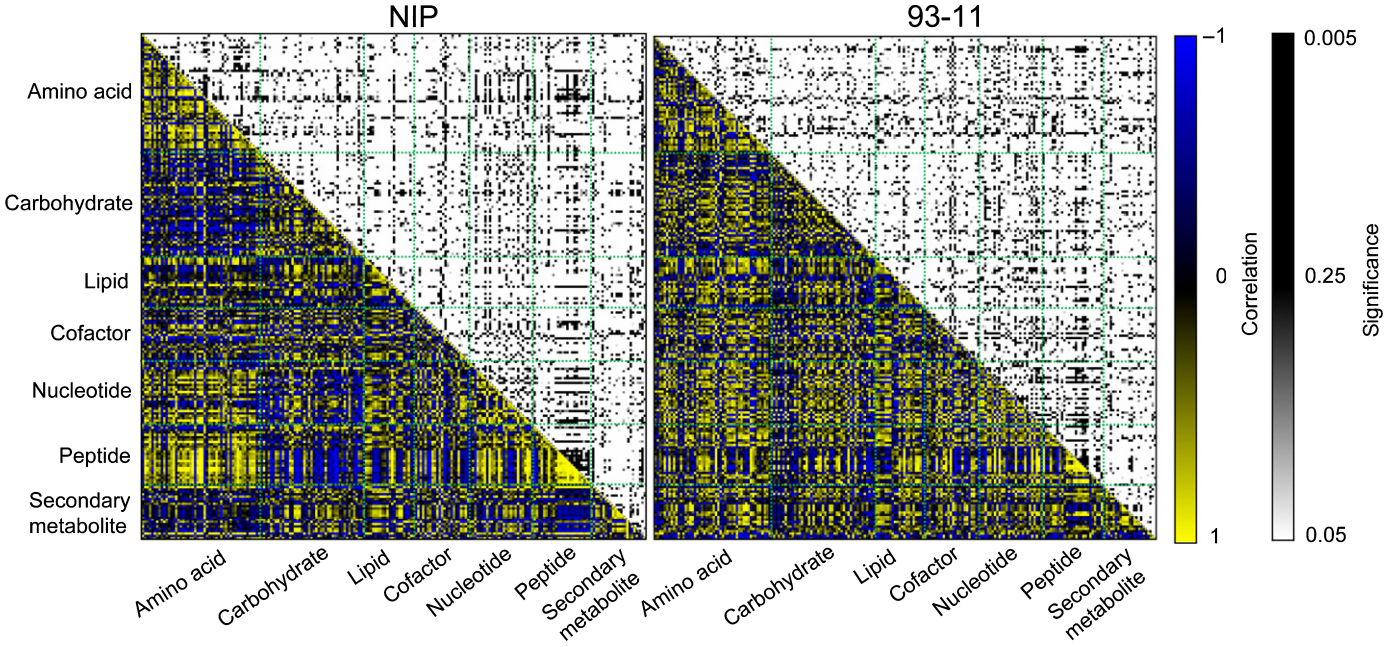

Fig. 4 Comparison of metabolite–metabolite correlation in rice (*Oryza sativa*) varieties Nipponbare (NIP) (*japonica*) and 93-11 (*indica*). Heat maps of metabolite–metabolite correlation and significance are shown. In the colored area, rectangles represent Pearson's product-moment correlation values of metabolite pairs (see correlation color key). In the black and white area, rectangles represent the respective *P* values (see significance color key). *x* and *y* axes show metabolites grouped by compound classes.

clearly increased in Nipponbare, but not in 93-11, suggesting stronger accumulation of ROS in the former cultivar (Table 1). We therefore performed histochemical analysis, NBT staining and diaminobenzidine tetrahydrochloride (DAB 4HCl) staining to detect ROS accumulation. The results indicated that strong ROS accumulation appeared at the middle stage in Nipponbare, but only at the end of chilling treatment in 93-11 (Fig. S3). At the middle stage of chilling treatment, obvious differences were detected between the *japonica* variety Nipponbare and *indica* variety 93-11. Strong ROS accumulation was detected in Nipponbare, whereas only low levels of ROS were observed in 93-11 (Fig. 5a). To test the universality of this finding, 10 representative *japonica* and *indica* varieties were randomly selected and analyzed by NBT staining (Table S3). To exclude the influence of senescence-related ROS, *indica* cultivars with extremely low chilling tolerance were not included. All the *japonica* varieties, with high survival rates, showed strong ROS accumulation, but the accumulation of ROS was quite weak in *indica* varieties, which exhibited low survival rates under chilling stress (Fig. 5b).

Stronger ROS-mediated gene expression regulation was observed in *japonica* variety Nipponbare

Clear metabolic differences were detected between Nipponbare and 93-11 at the middle stage of chilling treatment (33 h) (Fig. 2). We therefore performed RNA-seq at this time point to

Table 1 List of metabolites with obvious changes in abundance (relative content) during chilling treatment and recovery in rice (*Oryza sativa*)

Pathway	Biochemical name	NIP			93-11		
		Chilling treatment					
		10 h	33 h	57 h	10 h	33 h	57 h
Amino acid	Glutathione (GSSG)	2.85	**11.53**	4.96	3.44	1.92	**13.06**
	Putrescine	1.96	**5.14**	**6.76**	0.70	1.08	4.90
	Asparagine	1.92	3.59	**5.38**	1.36	1.66	**5.08**
	β-Alanine	1.73	3.64	3.55	1.92	1.86	**5.13**
Peptide	γ-Glutamylleucine	2.68	**5.08**	3.35	1.65	2.43	**5.30**
Carbohydrate	Oxalate	2.29	**13.84**	2.07	2.27	**0.21**	1.14
	Mannose-6-phosphate	2.23	4.08	**9.71**	1.50	4.66	**7.50**
	Isocitrate	0.42	**0.01**	0.51	0.86	0.70	0.47
Cofactor	Nicotinamide mononucleotide	1.64	**6.02**	1.52	0.51	1.06	2.70
		Recovery growth					
		24 h	48 h	72 h	24 h	48 h	72 h
Amino acid	Glutathione (GSSG)	1.61	1.14	**9.09**	3.45	4.13	4.10
	2-Aminoadipate	**16.76**	**8.61**	**12.92**	**129.05**	**57.01**	**6.46**
	Tryptophan	3.32	1.16	4.54	**11.13**	**10.01**	3.79
	Asparagine	1.00	**5.13**	**11.86**	4.72	**10.40**	**9.71**
	Valine	2.40	1.23	4.07	**5.36**	3.19	3.04
	Proline	1.94	1.34	2.24	**6.09**	2.16	2.62
	β-Alanine	0.95	1.17	4.90	**5.91**	3.65	**8.66**
	Homoserine	0.70	1.56	**9.94**	1.14	4.33	3.71
	Isoleucine	3.34	1.37	**5.49**	**7.71**	4.56	3.54
	Histidine	2.31	1.34	4.02	**6.78**	**5.52**	3.61
	Quinate	0.33	0.75	**0.18**	**0.08**	0.40	0.24
	Shikimate	0.59	1.06	0.42	**0.18**	0.46	0.33
	1,3-Diaminopropane	1.00	1.00	1.86	1.28	1.00	**20.02**
	S-methylmethionine	3.61	1.91	3.90	4.78	**5.06**	2.54
	2-Aminobutyrate	1.52	1.12	**10.91**	**10.55**	4.49	**7.79**
Peptide	γ-Glutamylisoleucine	2.40	1.30	**6.48**	3.79	3.18	**6.17**
	γ-Glutamylleucine	**6.65**	2.19	4.20	**10.66**	4.97	**7.20**
	γ-Glutamylphenylalanine	3.75	1.60	3.95	**8.43**	3.80	3.61
	γ-Glutamyltryptophan	1.92	1.09	**5.35**	**10.54**	**10.85**	**6.92**
Carbohydrate	Oxalate	**0.09**	**0.07**	1.48	0.10	0.24	0.69
	Raffinose	1.48	0.75	0.28	1.49	0.84	**0.14**
Nucleotide	Uridine-2′,3′-cyclic monophosphate	2.44	1.65	1.97	1.69	1.47	**0.15**
	Xanthine	3.47	1.05	3.23	3.22	1.42	**6.56**
	Allantoin	1.05	**5.24**	**11.39**	1.89	3.09	**5.23**
Lipid	1-Palmitoylglycerophosphoethanolamine	**5.17**	0.70	1.25	2.50	1.23	1.76
	1-Palmitoylglycerophosphocholine	**10.56**	1.57	0.67	0.83	1.61	1.88
Cofactor	γ-Tocopherol	1.04	1.63	1.31	**7.96**	3.77	0.80
Secondary metabolism	Luteolin-6-*C*-glucoside	**5.08**	0.96	1.18	5.80	1.34	0.98
	Tryptamine	–	–	1.28	–	–	**21.36**
	Ferulate	**6.71**	2.73	1.25	7.09	1.67	1.51
	p-Coumaroylserotonin	–	–	1.18	–	–	**15.72**

Metabolites with obvious changes in relative abundance (compared with untreated seedlings) in Nipponbare (NIP) and 93-11 are shown under chilling treatment (10 h, 33 h and 57 h) and during recovery growth (24 h, 48 h and 72 h). Bold type indicates significant changes (> 5-fold, $P < 0.05$).

obtain a global view of changes at the transcriptional level. The results obtained were verified by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) (Fig. S4). Similar numbers of genes showed chilling-induced changes in Nipponbare and 93-11 (Fig. S5).

ROS can act as signaling molecules mediating the systemic regulation of gene expression (Apel & Hirt, 2004). Previous studies have identified a group of rice genes that are responsive to both oxidative and chilling stress at an early stage (Mittal *et al.*, 2012). RNA-seq at 33 h indicated that most of these genes showed the same trend of changes in Nipponbare during chilling treatment. This type of change, however, was relatively weak in 93-11, implying weaker ROS-elicited transcriptional regulation in this variety (Fig. 6a). RNA-seq also showed that the expression levels of ROS metabolism-related genes, especially for genes encoding enzymes involved in glutathione and oxalate metabolism, were obviously higher in Nipponbare than in 93-11, which is consistent with the accumulation of glutathione and oxalate in the former variety (Table 1; Fig. 6b). Further analysis with qRT-PCR at different time points led to a similar conclusion (Fig. S6).

For transcriptional factors, the induction of bZIP, which is regarded as a major factor mediating oxidative signals in plants, was obviously stronger for chilling-tolerant variety Nipponbare than for chilling-sensitive variety 93-11 (Fig. 6c) (Yun *et al.*, 2010). A similar observation was noted for WRKY, another type of transcriptional factor involved in ROS-induced gene

regulation, although to a lesser extent (Fig. S7) (Yun *et al.*, 2010). The CBF/DREB regulon is regarded as a critical player in cold-responsive gene regulation (Zhou *et al.*, 2011). Interestingly, the induction of genes encoding CBF/DREB transcriptional factors was stronger in chilling-sensitive variety 93-11 than in chilling-tolerant variety Nipponbare, which is in accordance with previous reports on the inconsistency between the expression levels of *CBF/DREB*s and the degree of cold tolerance (Fig. 6c) (Mao & Chen, 2012). The analysis of the expression of *CBF/DREB*s at different time points further supports this conclusion (Fig. S6). A similar observation was made for another type of transcriptional factor, NAC, which has been shown not to be significantly responsive to ROS (Fig. S7). No obvious difference was observed for other types of transcriptional factors between these two rice varieties (Fig. S7). These results demonstrate the contribution of ROS-mediated gene expression regulation, but not the CBF/DREB regulon, to the difference in chilling tolerance between Nipponbare and 93-11.

To further analyze the ROS metabolism system in *japonica* and *indica,* we performed single nucleotide polymorphism (SNP) analysis for genes encoding enzymes involved in ROS production and scavenging in 75 *japonica* and 25 *indica* accessions. Some important ROS metabolism-related genes were found to possess subspecies-specific non-synonymous SNPs in functional domains, which lead to amino acid alterations (Fig. 7, Tables S5, S6) (Moyer *et al.*, 2008; Edwards *et al.*, 2012; Ma *et al.*, 2015).

More vigorous stress responses were induced in *japonica* variety Nipponbare at the transcriptional level

Gene ontology (GO) analysis was performed for chilling-responsive genes detected by RNA-seq and a somewhat similar enrichment pattern was observed in Nipponbare and 93-11. Furthermore, GO analysis was performed for genes with different expression levels in Nipponbare compared with those in 93-11 under chilling stress. Significant enrichment was observed for

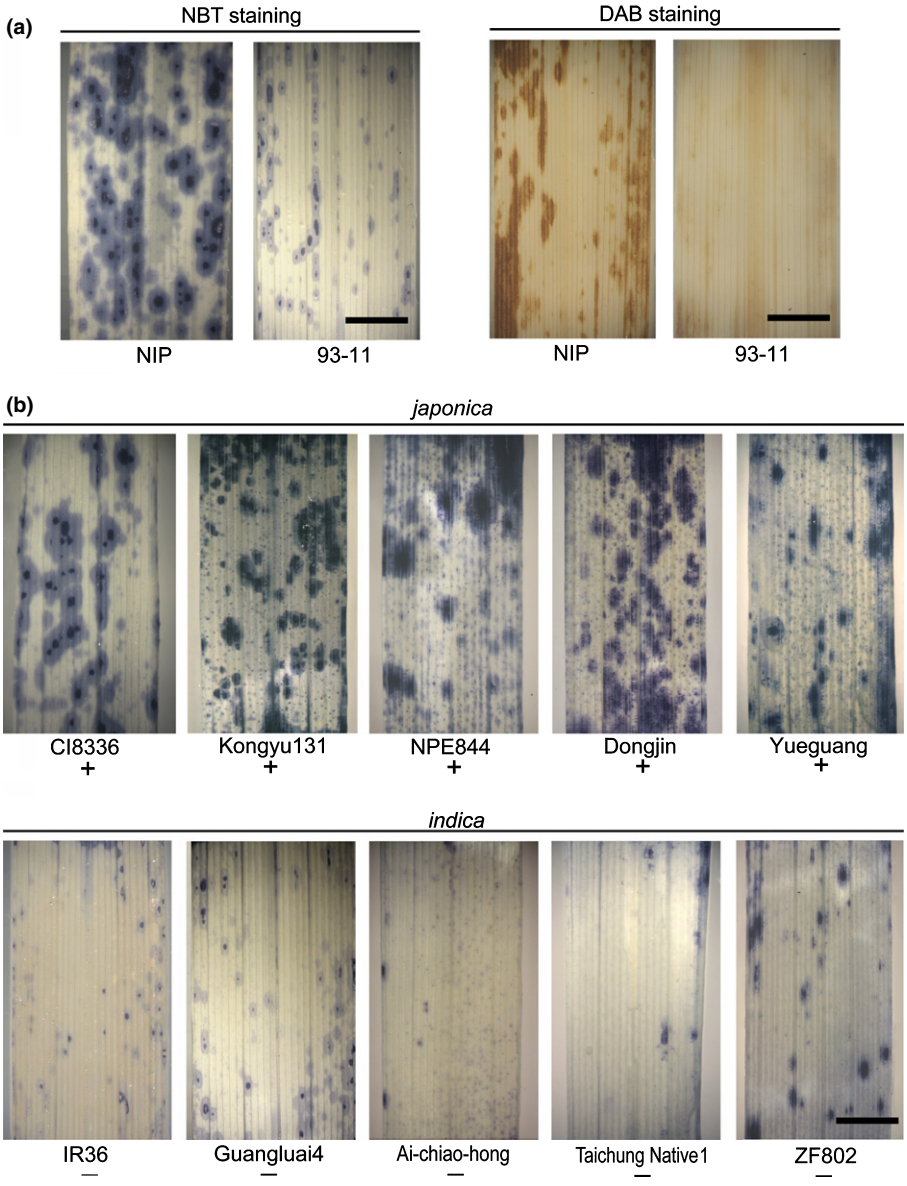

Fig. 5 Reactive oxygen species (ROS) accumulation is stronger in *japonica* varieties than in *indica* varieties during chilling stress. (a) ROS accumulation in Nipponbare (NIP) and 93-11 was detected at 33 h in stage I with nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining. Bar, 0.2 cm. (b) NBT staining for representative *japonica* and *indica* varieties after chilling treatment for 33 h. The background information for rice varieties is shown in Supporting Information Table S3. The rice (*Oryza sativa*) varieties with survival rates of <10% are considered to be chilling sensitive and are indicated by '−'; the rice varieties with survival rates of 100% are considered to be chilling tolerant and are indicated by '+'. For the staining assay, at least three independent experiments were performed and representative images are shown. Bar, 0.2 cm.

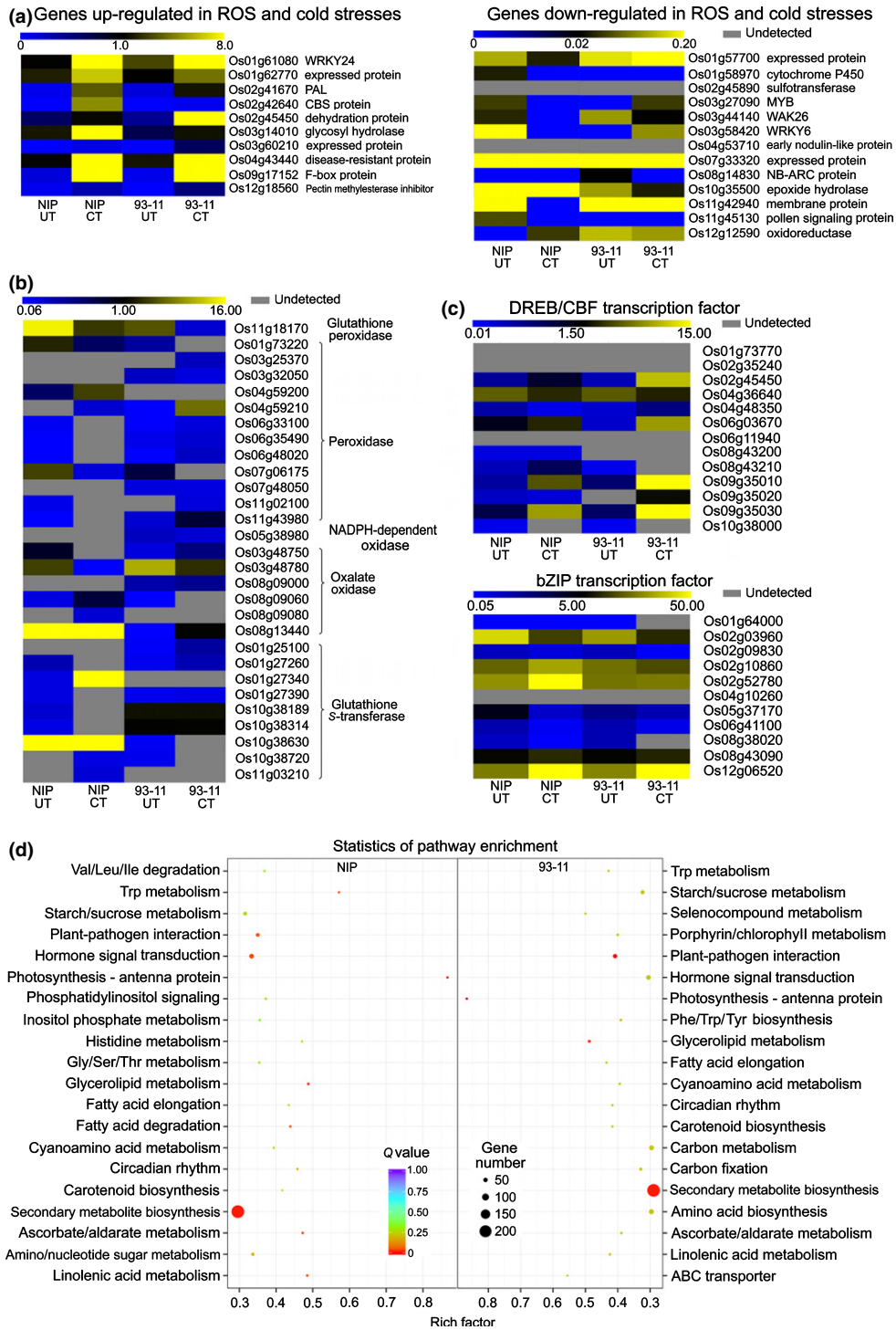

Fig. 6 RNA-seq analysis of rice (*Oryza sativa*) varieties Nipponbare (NIP) (*japonica*) and 93-11 (*indica*). RNA-seq was performed at 33 h in stage I for chilling-treated (CT) and untreated (UT) samples. (a) Heat maps illustrating the expression levels of genes, responsive to both oxidative and cold stresses at the early stage, in NIP and 93-11 (Mittal *et al.*, 2012). (b) Heat map for the abundance of the top 29 reactive oxygen species (ROS) metabolism-related genes with the most significant changes in chilling stress. See Supporting Information Table S4 for more information. (c) Heat maps illustrating the expression levels of genes encoding transcription factor C-repeat binding factor/dehydration-responsive-element binding factor (CBF/DREB) and bZIPs in chilling stress. The *CBF/DREB* and *bZIP*s genes were obtained from MSU Rice Genome Annotation Project Release 7 (http://rice.plantbiology.msu.edu/). (d) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the effect of chilling stress in NIP (left) and 93-11 (right). KEGG pathway analyses were applied to genes expressed differently in chilling-treated and untreated samples. The *y*-axis represents the pathway category and the *x*-axis represents the degree of enrichment (Rich factor, number of differently expressed genes in a given pathway divided by the total number of genes involved in that pathway) of the pathways. The color of the circle indicates the *Q* value (corrected *P* value, shown in Table S7) and the size of the circle indicates the number of involved genes. Significant enrichment was detected with $Q < 0.05$. The top 20 pathways with the smallest *Q* values are shown for NIP and 93-11, respectively.

'responses to stimulus' in the biological process category, suggesting Nipponbare-specific stress responses at the transcriptional level (Fig. S8).

To obtain a complete view of the significantly altered pathways at the transcriptional level in chilling stress, we performed pathway enrichment analysis for RNA-seq data using the KEGG database. Overall, more pathways were activated by chilling in Nipponbare than in 93-11, consistent with the stronger ROS-mediated gene expression regulation in this cultivar (Fig. 6d; Table S7). The pathways for hormone signal transduction and metabolism of ascorbate/aldarate, tryptophan and linolenic acid, as well as fatty acid degradation, were significantly enriched ($Q < 0.05$) in Nipponbare, indicating that the stress response is in an active state in this variety at the transcriptional level. This enrichment, however, was not observed in 93-11 (Fig. 6d). In addition, Nipponbare-specific enrichment of ascorbate/aldarate metabolism at the transcriptional level corresponds to the increased content of glutathione at the metabolic level, which also suggests the contribution of transcriptional regulation to antioxidation (Table 1, Fig. 6d).

More active metabolic response to chilling stress was detected in *japonica* variety Nipponbare during recovery growth

Consistent with the more vigorous transcriptional chilling responses in Nipponbare, more metabolites conferring stress tolerance accumulated in this variety during recovery growth. At the initial stage (24 h), the accumulation of lysophospholipids (1-palmitoylglycerophosphoethanolamine and 1-palmitoylglycerophosphocholine), cellular membrane compounds that always accumulate during the stress response and probably play a role in stress tolerance, was detected in Nipponbare (Oliver *et al.*, 2011; Vu *et al.*, 2012; Okazaki & Saito, 2014). In addition, significant induction ($P < 0.05$) of two anti-stress-related secondary metabolites, ferulate and luteolin-6-*C*-glucoside (isoorientin), was observed (Lim *et al.*, 2007; Cheng *et al.*, 2013). These inductions, however, were not significant in 93-11. Interestingly, the accumulation of γ-tocopherol, a compound playing a role in the plant stress response, was observed in 93-11, but not in Nipponbare, indicating that this compound does not contribute to the higher chilling tolerance of Nipponbare (Table 1) (Abbasi *et al.*, 2007). In chilling-sensitive variety 93-11, significant metabolic changes included the large-scale accumulation of amino acids, suggesting the disturbance of protein metabolism (Table 1) (Goyer *et al.*, 2004; Araujo *et al.*, 2011).

At the middle stage of recovery growth (48 h), the metabolome of Nipponbare was relatively stable. Significant changes were only observed for some amino acids and allantoin, a compound with anti-stress function (Watanabe *et al.*, 2014). In 93-11, large-scale accumulation of amino acids was still observed (Table 1).

At the end of recovery growth (72 h), the content of free fatty acids, including 2-hydroxymyristate, 2-hydroxypalmitate and

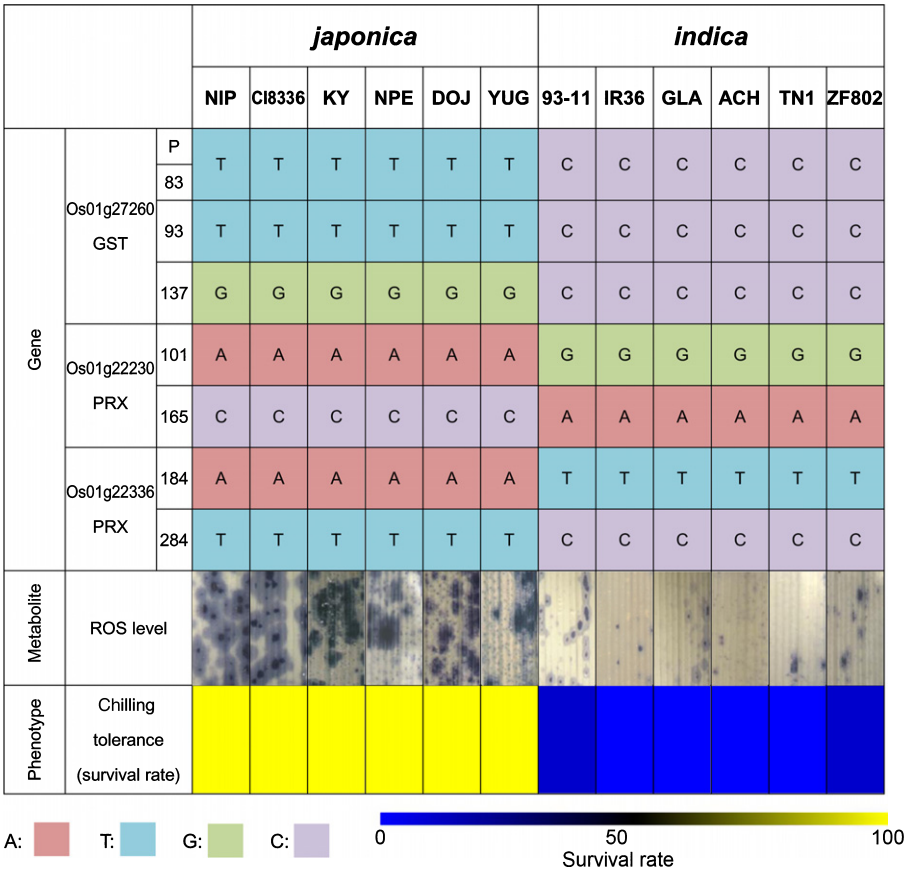

Fig. 7 Summary of the adaptation of reactive oxygen species (ROS) metabolism in two rice (*Oryza sativa*) varieties, Nipponbare (*japonica*) and 93-11 (*indica*), to a low-temperature environment. Non-synonymous single nucleotide polymorphism (SNP) variations between *japonica* and *indica* varieties in the functional domain of ROS metabolism-related genes are shown. Only genes with more than one non-synonymous SNP in the functional domain are shown. See Supporting Information Tables S5 and S6 for more information. The metabolite ROS level was detected with nitroblue tetrazolium (NBT) staining. Phenotype chilling tolerance was evaluated using survival rates after chilling treatment. The background information for rice varieties is shown in Table S3. P, position; GST, glutathione *S*-transferases; PRX, peroxidase; NIP, Nipponbare; KY, Kongyu131; NPE, NPE844; DOJ, Dongjin; YUG, Yueguang; GLA, Guangluai4; ACH, Ai-chiao-hong; TN1, Taichung Native 1.

2-hydroxystearate, increased in Nipponbare, but not in 93-11, although their base levels were lower in the former variety (Table S2). Fatty acids play a role in the plant stress response and some have anti-cell death function (Zhang *et al.*, 2003; Kachroo & Kachroo, 2009). In 93-11, the large-scale accumulation of amino acids was still observed. Moreover, the accumulation of compounds relating to polyamine degradation and senescence, including 1,3-diaminopropane and *p*-coumaroylserotonin, was only observed in 93-11, consistent with the serious chilling damage observed in this variety (Table 1; Figs 1b, S1) (Tavladoraki *et al.*, 2006). 93-11-specific accumulation was also observed for tryptamine, which gives rise to serotonin, another senescence-related compound (Table 1) (Kang *et al.*, 2009, 2011).

Interestingly, increased content was also observed for glutathione (GSSG) and glutamyl-peptides in 93-11 across the recovery stage, but only at the final time point in Nipponbare, which is in accordance with the appearance of chilling injury and suggests senescence-related ROS (Table 1; Figs 1b, S1) (Lee *et al.*, 2012; Rogers, 2012).

Salicylic acid displayed different chilling-responsive patterns between *japonica* and *indica* varieties, Nipponbare and 93-11

We performed the quantification of the stress-related phytohormones jasmonic acid (JA) and SA, and the dormancy-related phytohormone ethylene, in Nipponbare and 93-11 at different stages of chilling stress. Significant differences between Nipponbare and 93-11 were detected in the content of SA, but not JA and ethylene. Furthermore, endogenous SA levels displayed opposite trends of changes in Nipponbare and 93-11, but the levels of JA and ethylene showed similar trends of changes in these two rice varieties (Fig. 8).

Discussion

The adaptability of rice to cold environments depends on the divergence between the two subspecies of Asian cultivated rice, *japonica* and *indica*, during domestication (Kovach *et al.*, 2007; Sang & Ge, 2007). It was of interest to determine what types of chilling response mechanism were obtained in these two rice subspecies after long-term selection and adaptation, which would provide an opportunity to examine the plant adaptation mechanism to a low-temperature environment. Using comparative metabolomic analysis of these two rice subspecies at a series of time points, we have uncovered the dynamic metabolic models underlying their different chilling tolerance and have revealed a ROS-dominated environmental adaptation.

Chilling injury appears during recovery growth rather than during chilling treatment. Taking advantage of this characteristic of chilling stress, it is easy to determine whether metabolic changes observed at different time points are related to stress perception, or represent a signal associated with chilling damage, which enables us to obtain a clear view of the chilling response in rice and to perform a detailed comparison between *japonica* and *indica* varieties. In addition, to obtain a clear view of chilling-induced metabolic changes, the growth effect

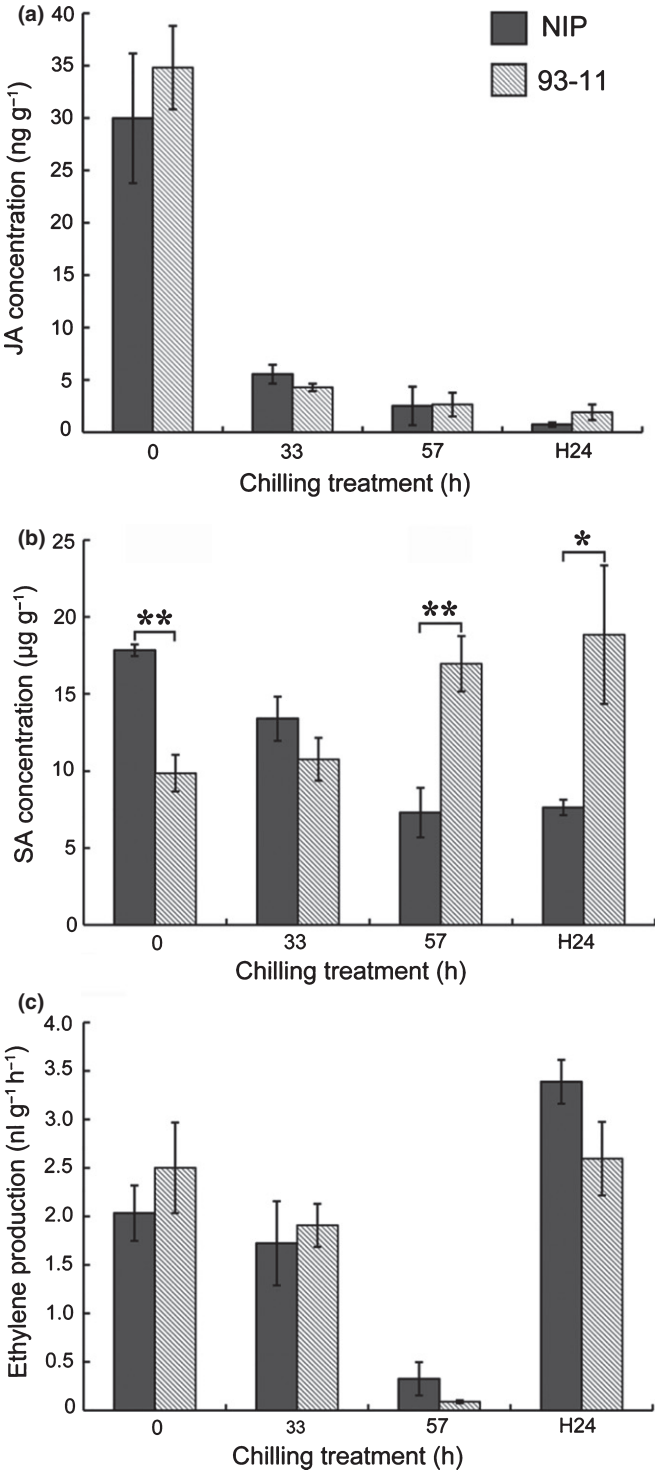

Fig. 8 The effect of chilling stress on phytohormone levels in rice (*Oryza sativa*) varieties Nipponbare (NIP) (*japonica*) and 93-11 (*indica*). The endogenous levels of (a) jasmonic acid (JA), (b) salicylic acid (SA) and (c) ethylene were measured for NIP and 93-11 seedlings exposed to chilling treatment for 0, 33 and 57 h, followed by recovery for 24 h (H24). The data are means of at least three replicates ± SD. Significant difference from Student's unpaired two-tailed *t*-test: *, $P < 0.05$; **, $P < 0.005$.

was eliminated by preparing an untreated control at every time point.

Distinct metabolic mechanism underlying chilling and recovery

Chilling, unlike other types of abiotic stress, causes phenotypic changes in plants, not during exposure to low temperature, but in subsequent recovery growth, which draws our attention to the recovery stage (Figs 1b, S1). Metabolic changes are obviously different during chilling treatment and recovery growth, and some well-known anti-stress compounds are produced in recovery growth rather than during chilling treatment (Figs 2, 3). During chilling treatment, significant metabolic changes, mainly of antioxidation-related compounds, are limited to a certain group of metabolites (Table 1). At this stage, the metabolic balance is still maintained and no metabolic collapse is observed, which is in accordance with the lack of chilling damage symptoms (Figs 2, S1). During recovery growth, significant changes are observed in the contents of various types of metabolite, suggesting a metabolome-wide stress response. The production of certain metabolites with anti-stress function is induced at this stage (Table 1). The large-scale accumulation of amino acids is observed, suggesting an imbalance of protein metabolism (Fig. 2). Accordingly, phenotypic changes appear (Figs 1b, S1). A positive correlation between large-scale amino acid accumulation and chilling-induced phenotypic damage was detected on comparative analysis of chilling-sensitive and chilling-tolerant rice varieties, suggesting a close association between protein metabolism and chilling injury (Figs 2, S1).

Metabolic fluxes are regulated by enzymes and their activities decrease at low temperature, which, in turn, probably leads to the inactivity of the entire metabolome (Illanes, 2008). However, the contents of antioxidation-related compounds and some stress tolerance-related metabolites altered significantly during chilling treatment, which may result from other levels of regulation, such as transcriptional level adjustment (Table 1; Figs 6b, S6). During the recovery stage, chilling-induced suppression is released and the metabolome probably returns to the active state. Thus, various types of metabolite conferring chilling tolerance are produced and a wide-ranging chilling response is observed (Table 1). There is also an effect of chilling stress on protein metabolism and large-scale amino acid accumulation occurs, which is in accordance with the appearance of damage in the plant phenotype (Figs 2, S1).

Phytohormones also contribute to the state shift between these two different stages of chilling stress. For ethylene, a type of phytohormone which plays a key role in the release of seed dormancy and seasonal dormancy (Corbineau *et al.*, 2014; Yordanov *et al.*, 2014), its content decreases during chilling treatment, but returns to the pre-stress level during recovery growth (Fig. 8c). Thus, one reasonable explanation is that the ethylene-mediated resistance of dormancy is attenuated during chilling treatment to achieve the inactive state, but returns to the normal level during recovery growth to release the dormancy-like state caused by chilling stress.

Different chilling response models have evolved in *japonica* and *indica* varieties in their adaptation to different thermal environments

To adapt to different types of growth environment, the two rice subspecies, *japonica* and *indica*, have evolved high and low chilling tolerance after years of natural and artificial selection. Interestingly, there is not a large significant difference (more than five-fold) between their metabolomes under normal growth conditions, suggesting that their different chilling tolerance largely comes from different chilling responses (Table S2).

Under chilling treatment, the most significant metabolic difference between *japonica* and *indica* varieties, Nipponbare and 93-11, is the earlier induction of ROS in the former variety (Table 1; Figs 5, S3). ROS accumulation not only activates ROS-scavenging responses, especially the glutathione–ascorbate cycle, to protect the plant from oxidative damage, but also induces corresponding transcriptional regulation (Table 1; Figs 5, 6). The induction of ROS- and cold stress-responsive genes and the ROS-responsive transcriptional factor bZIP is much stronger in *japonica* variety Nipponbare than in *indica* variety 93-11, indicating the contribution of ROS-mediated gene regulation to the higher chilling tolerance of *japonica* variety Nipponbare (Figs 6a–c, S6). Accordingly, more active transcriptional stress responses are induced in *japonica* variety Nipponbare than in *indica* variety 93-11 (Fig. 6d). It can be deduced from the relatively weaker induction of the expression of *CBF/DREB* genes in Nipponbare that the contribution of the well-known CBF/DREB regulon to the higher chilling tolerance of this *japonica* variety is negligible, although it cannot be eliminated completely (Figs 6c, S6). Although, in 93-11, antioxidation-related compounds are induced at the end of chilling treatment, suggesting the induction of ROS at this stage, it is probably too late for the plant to make a full response to chilling stress (Table 1).

At the beginning of recovery growth, more chilling tolerance-related metabolites are induced in Nipponbare, which is in accordance with the stronger chilling response at the transcriptional level in this *japonica* variety (Table 1; Fig. 6d). Nipponbare-specific induction of certain antioxidation-related secondary metabolites corresponds, to some extent, to the Nipponbare-specific transcriptional activation of tryptophan metabolism, which is considered to be a prelude to the production of secondary metabolites (Table 1; Fig. 6d) (Zhao *et al.*, 1998). Ultimately, no senescence-related compounds are induced in *japonica* variety Nipponbare, which benefits from the protection of antioxidants and chilling tolerance-related compounds produced at different stages (Table 1). By contrast, compounds related to senescence are induced in *indica* variety 93-11, consistent with its poor survival rate in chilling stress (Table 1; Figs 1b, S1).

ROS production is the initial metabolic reaction which shows a significant difference between *japonica* and *indica* varieties in chilling stress. With different ROS-mediated regulation, the two rice subspecies proceed through different response channels, resulting in high and low chilling tolerance, respectively (Fig. 9). In addition, strong overall regulation for different types of metabolite also contributes to the higher chilling tolerance of

indica variety Nipponbare, as suggested by metabolic correlation analysis (Fig. 4).

We also measured the phytohormone levels in Nipponbare and 93-11, and a significant difference was detected for SA (Fig. 8b). A low concentration of SA induces a low level of ROS accumulation and enhances plant antioxidant capacity, but a high concentration of SA induces ROS over-accumulation and even an oxidative burst (Miura & Tada, 2014). Consistently, chilling induces strong ROS accumulation in *japonica* variety Nipponbare, which may lead to a decrease in SA level to prevent ROS over-accumulation. The content of SA increases in *indica* variety 93-11 during chilling stress, which may make some possible contribution to ROS induction (Figs 5, 8b).

Contributions of amino acids to the different chilling tolerance between *japonica* and *indica* varieties, Nipponbare and 93-11

The accumulation of amino acids, observed in cold and several types of abiotic stress, has been speculated to play a role in plant tolerance to environmental stresses (Caldana *et al.*, 2011; Oliver *et al.*, 2011; Shingaki-Wells *et al.*, 2011; Maruyama *et al.*, 2014). In our study, obvious accumulation of amino acids can be detected throughout the whole stage of recovery growth in chilling-sensitive variety 93-11, but occurs only at the final time point in chilling-tolerant variety Nipponbare (Figs 1b, S1). The coincidence of the appearance of chilling injury and amino acid accumulation suggests that the latter probably originates from the disturbance of protein metabolism (Figs 2, S1). Moreover, metabolite correlation analysis revealed strong overall regulation for amino acids in Nipponbare, but not in 93-11 (Fig. 4). These results suggest the contribution of the strong overall regulation of amino acids, rather than their amount, to the higher chilling tolerance of *japonica* variety Nipponbare.

The accumulation of amino acids may originate from the activation of protein degradation or the inhibition of protein synthesis. Amino acid accumulation appeared when chilling injury was observed (Figs 2, S1). The whole protein metabolism is probably disturbed at this stage and both degradation and synthetic pathways may contribute to the accumulation of amino acids.

ROS-dominated mechanism underlying the adaptation to a low-temperature environment in rice

Comparative metabolomic analysis as well as related RNA-seq assay indicate that the rice plant largely relies on ROS-mediated gene regulation, instead of the well-known CBF/DREB regulon, to adjust its chilling response system to adapt to different environmental temperatures, indicating that the plant chooses the adaptation regulation point at the metabolic level instead of the transcriptional level. ROS have been identified as the regulation switch in environmental adaptation (Fig. 9). As a type of metabolite, ROS are very sensitive to environmental changes (Baxter *et al.*, 2014). Through the regulation of ROS signals, which are amplified during transmission, level by level, the rice plant can achieve an efficient and environmentally sensitive adjustment of its chilling response system.

Although ROS are known to be involved in numerous biological processes, their exact role remains to be uncovered (Baxter *et al.*, 2014). Here, we have revealed their role in environmental adaptation (Fig. 9). Moreover, the ROS metabolism system exhibits obvious differences between *japonica* and *indica*. The

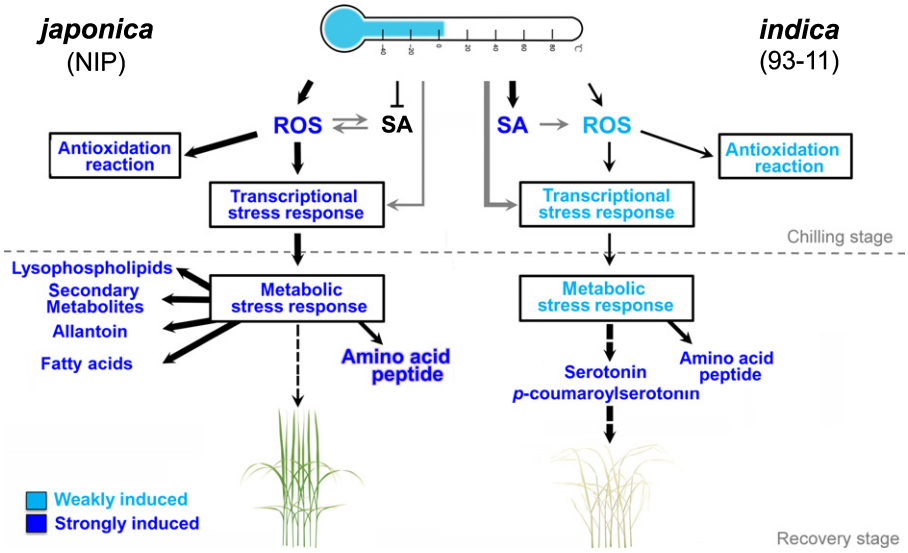

Fig. 9 Possible chilling response models for rice (*Oryza sativa*) ssp. *japonica* and *indica*. In response to chilling stress, stronger reactive oxygen species (ROS) accumulation was induced in *japonica* than in *indica* varieties, which activated both antioxidation reactions and transcriptional regulation. More stress tolerance-related metabolic reactions were activated in *japonica* variety Nipponbare during recovery. The accumulation of senescence-related compounds, serotonin and *p*-coumaroylserotonin, was only induced in *indica* variety 93-11. Finally, the *japonica* and *indica* varieties exhibited high and low survival rates, respectively. Highlighted words, metabolites with strong overall regulation; arrows, overall upstream/downstream relationships in the chilling response pathway; thick and thin arrows, strong and weak induction of stress responses, respectively; broken-line arrows, gaps in the pathway; gray arrows, relationships that require further analysis. SA, salicylic acid.

expression levels of ROS metabolism-related genes, especially for genes of enzymes involved in glutathione and oxalate metabolism, are obviously higher in Nipponbare than in 93-11 (Figs 6b, S6). After analysis of *japonica* and *indica* accessions, subspecies-specific non-synonymous SNPs were found in the functional domains of ROS metabolism-related genes, which lead to amino acid alterations and probably corresponding differences in enzyme activity (Fig. 7; Tables S5, S6). These differences, at both transcriptional and genomic levels, suggest that the ROS metabolism system may have been the target of selection during the divergence of these two subspecies. The *japonica* variety is mainly adapted to growth conditions with relatively low annual temperature, and cold is its major stress. However, *indica* is mainly adapted to hot and humid growth conditions and must cope with strong light and pests. In plants the timing and location of ROS production is strictly regulated, to satisfy the demand of different types of regulation (Dickinson & Chang, 2011). The *japonica* and *indica* varieties may have evolved different ROS production mechanisms to deal with different types of stress, and these mechanisms are probably not interchangeable, which explains the slow ROS induction and low chilling tolerance of *indica* varieties.

ROS are known to play a role in various types of biological process (Baxter *et al.*, 2014). In this study, both ROS functioning as signaling molecules and ROS related to senescence were detected in a series of metabolomic analyses (Table 1). Metabolic and phenotypical analyses of rice plants suggest that under cold treatment ROS induced by chilling probably function as signaling molecules triggering further defense response. On the other hand, they may also be aggressors in plants during recovery stage due to over-accumulation and excessive stress response (Table 1; Fig. S1).

Through comparative metabolomic analysis at several time points, we have uncovered the adaptation mechanism to different thermal environments in rice and have revealed the dynamic model underlying the chilling response. Interestingly, under chilling treatment, most significant biochemical changes centered on antioxidation, and some well-known anti-stress compounds were produced only during recovery growth. The biomarker for chilling injury has also been identified. Of course, metabolic changes that occur during chilling stress are not necessarily correlated with the stress response. Although both chilling-tolerant and chilling-sensitive rice varieties were analyzed in this study to discriminate stress-related from unrelated changes, genetic materials will be used in the future to obtain more specific information. Our results not only help us to obtain a deeper understanding of the adaptation of plants to unfavorable environments, but also have considerable potential in cold-resistant genetic engineering.

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Author contributions

K.C. and J.Z. designed the experiments; J.Z., W.L. and Y.Z. performed the experiments; S.S. performed the SNP analysis; J.Z., Y.X. and K.C. analyzed the data; J.Z. and K.C. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Phenotypes of Nipponbare (NIP) and 93-11 under chilling treatment and during recovery growth.

Fig. S2 Effect of chilling on central carbohydrate metabolism and related amino acid metabolism in Nipponbare (NIP) and 93-11.

Fig. S3 Reactive oxygen species (ROS) accumulation in Nipponbare and 93-11.

Fig. S4 Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) validation of gene expression data provided by RNA-seq analysis.

Fig. S5 Volcano plot showing the $\log_2$(fold change) and $-\log_{10}(Q$ value) of gene expression after chilling treatment in Nipponbare and 93-11.

Fig. S6 Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis of the expression of *CBF/DREB* and ROS-related genes with significantly different expression (more than six-fold) between Nipponbare (NIP) and 93-11.

Fig. S7 Heat maps illustrating the expression levels of transcription factors in Nipponbare (NIP) and 93-11.

Fig. S8 Gene ontology (GO) analysis of genes with significant changes in expression level in chilling stress.

Table S1 Metabolites detected in this study

Table S2 Metabolomic profiles for Nipponbare and 93-11

Table S3 Background information for *japonica* and *indica* varieties used in this study

Table S4 Transcriptional changes of genes involved in reactive oxygen species (ROS) metabolism in Nipponbare and 93-11 after 33 h of chilling treatment

Table S5 Single nucleotide polymorphisms (SNPs) in reactive oxygen species (ROS) metabolism-related genes

Table S6 Non-synonymous single nucleotide polymorphism (SNP) variation between *japonica* and *indica* in the functional domain of reactive oxygen species (ROS) metabolism-related genes

Table S7 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the effect of chilling stress in Nipponbare and 93-11 at the transcriptional level

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