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Metabolomic Analysis of Arabidopsis *ost1-4* Mutant Revealed the Cold Response Regulation Mechanisms by OPEN STOMATA 1 (OST1) at Metabolic Level

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Abstract: Cold stress is one of the major abiotic stresses that limits the growth and development of plants. Improving the cold tolerance of plants is essential to enhance crop productivity in the changing environment. OPEN STOMATA 1 (OST1), also known as sucrose non-fermenting 1 related protein kinases 2.6/2E (SnRK2.6/SnRK2E), has been reported to involved in cold stress response in plants. This interesting protein is confined to expressed in guard cells and vascular system. However, the detailed mechanism of how OST1 regulates cold stress, especially at the metabolomic level is largely unknown. In this study, metabolomic profiling of *ost1* mutant and WT Arabidopsis plants under cold stress was investigated. The results showed that *ost1-4* mutants displayed cold sensitive phenotypes compared with the WT plant, as evidenced by higher MDA content and electrolyte leakage and lower photosynthetic characteristics. Next, the metabolic changes between *ost1-4* and WT plants in response to cold stress was analyzed by using the GC-TOF-MS system. The results showed that numbers of metabolites were identified to be related to OST1 regulated cold stress response. A large portion of the metabolites were carbohydrates and organic acids. The KEGG enrichment analysis revealed that the alanine, aspartate and glutamate metabolism, cyanoamino acid metabolism and citrate cycle (TCA cycle) were presumptive pathways that most related to OST1 regulated cold stress response. Gene expression such as *AtGDHs*, *AtPPC1* and *AtAK1* was also in line with the metabolic changes in the presumed pathways. Overall, this study provides fundamental knowledge for understanding the underlying metabolic mechanisms of OST1 mediated cold stress response in plants.

Keywords: OST1; metabolomic analysis; cold stress; physiological response; metabolic regulation

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1. Introduction

In their natural environment, plants are predominantly affected by different abiotic stresses. Cold stress is one of the main abiotic stresses that affect plant growth, development and productivity worldwide. Previous investigations revealed that cold stress could alter various plant processes and results in inhibition of germination, growth of seedlings and leaves, change in biochemical composition, plasma membrane fluidity and disturbed ion

homeostasis of cells [1,2]. Due to their genetic makeup, different plants have different adaptive capacities to cold stress. In the long process of evolution, plants have developed complex mechanisms at genetic and physiological levels to cope with the negative effects of cold stress [3]. When exposed to cold stress, plant will increase the activities of antioxidant enzymes [4] and close the stomata [5] to protect the cells from being injured. Besides, expressions of many genes are regulated under cold stress conditions. It is reported that *GRFs* (*GROWTH REGULATORY FACTOR*) can regulate plant growth in cold stress via interact with DELLAs [6]. DREBs/CBFs (dehydration-response element-binding protein 1/C-repeat binding factors) signaling pathway plays a central role in cold stress response of plant, and it is connected with cold response related genes such as CAMTAs, PIFs and RVEs [7]. Omics analysis has revealed that cold stress response in plant refers to a complex transcriptional regulatory network [8]. Therefore, the mechanism of cold stress response involves extensive reprograming of gene expression and metabolism.

Open Stomatal 1 (OST1) kinase, also known as sucrose non-fermenting 1 related protein kinases 2.6/2E (SnRK2.6/SnRK2E), was reported to play important roles in abiotic stresses response of plant, i.e., drought [9] and low CO_2 [10] stresses. This interesting protein is confined to expressed in guard cells and vascular system [11]. It was a key kinase to mediate stomatal closure of the plants [12]. In recent years, OST1 was reported to play roles in cold stress response [4]. When exposed to cold stress, OST1 phosphorylated other proteins such as BASIC TRANSCRIPTION FACTOR 3 (BTF3), BTF3L (BTF3-like) and Inducer of CBF EXPRESSION 1 (ICE1) to enhance cold tolerance of the plant [13,14]. In plants, ICE1-CBF-COR is a well-known signaling pathway in cold response [15]. The OST1 protein acts upstream of *CBFs* during cold stress via phosphorylation of the core component of ICE1. Besides, OST1 is also involved in suppressing *HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1* (*HOS1*) mediated ICE1 degradation to enhance the freezing tolerance of Arabidopsis [16]. Therefore, OST1 should play an important role in cold stress response of plant.

Studies have shown that stress-induced metabolites could play a remarkable role in plants stress tolerance [17]. Under cold stress, several protective substances such as soluble sugars, proline and cold-resistance proteins are accumulated in plants [18]. However, the detail mechanism that how OST1 regulate cold stress response in plants, especially at metabolomics level is still largely unclear. Nowadays, global metabolite profiling has been performed to identify metabolites and products of metabolic pathways in Arabidopsis [19], Siberian spruce (*Picea obovata*) [20], perennial ryegrass (*Lolium perenne*) [21] under stress conditions, and a mass of compounds had been identified to be involved in stress response in different plants [22,23]. Although Arabidopsis *ost1* mutant was characterized as a low temperature sensitive genotype, its metabolic profile in response to cold stress was not well documented. Therefore, given the crucial role of *OST1* and metabolites in plants stress tolerance, understanding of the *OST1* regulated metabolic changes in response to cold stress could be helpful to revealing the regulation mechanisms of stress response in plant. This study was aimed to identify *OST1* regulated metabolites under cold stress in Arabidopsis and understand the cold stress response mechanism at metabolome level by the GC-TOF/MS platform.

2. Materials and Methods

2.1. Plant Material, Growth Condition and Treatment

The Arabidopsis *thaliana* ecotype Columbia (Col-0) was used as a wild-type (WT). The *ost1-4* (GK516B05) mutant used in this study was obtained from Prof. Shuhua Yang (China Agricultural University). After surface sterilized with 75% ethanol, Arabidopsis seeds were sown on the Murashige and Skoog (MS) medium which contained 3% sucrose and 0.8% (*w*/*v*) agar. The seeds were kept at 4 °C for two days in the dark and then transferred into the growth chamber with 22 °C, 60% relative humidity, and 16 h light/8 h dark. One-week-old seedlings were transplanted to soil. For cold treatment, four-week-old seedlings were exposed to 4 °C. For metabolomic analysis and gene expression, the

rosette leaves were collected after 3 h cold treatment. For physiological index measurement, the leaves were collected after 3 d cold treatment. Six biological replicates were set for metabolomic analysis and three biological replicates were set for gene expression analysis and other measurements.

2.2. Physiological Indexes Measurements

Malondialdehyde (MDA) content and relative electrical leakage (EL) of plants were measured according to Fan et al. (2014) [24]. Briefly, MDA content was determined after preparation of crude enzyme solution. The crude enzyme was extracted according to our previous study. The MDA content was measured with thiobarbituric acid (TBA) method. Relative electrical leakage was obtained by measurement of electrical conductivity after and before autoclave treatment, and the ratio of the two values was the relative EL.

2.3. Chlorophyll a Fluorescence (OJIP) Kinetics Measurement

Chlorophyll a fluorescence was analyzed by the pulse-amplitude modulation (PAM) portable chlorophyll fluorometer (PAM-2500) according to Fan et al. (2015) [25]. Briefly, WT and *ost1-4* mutant leaves were kept in the dark for 30 min and the OJIP transients were measured at 3000 µmol photons m^{-2} s^{-1}. The Chl a fluorescence between 10 µs and 320 ms was recorded.

2.4. Metabolites Extraction

A total of 50 mg leaf sample was transferred into a 2 mL EP tube, and 450 µL pre-cold extraction mixture (Vmethanol/VdH_2O = 3:1) with 10 µL internal standard (L-2-Chlorophenylalanine, 1 mg/mL stock) were added.

Samples were vortexed for 30 s and then homogenized with a ball mill at 35 Hz for 4 min, after that the mixture was treated with ultrasonication in ice water for 5 min. After centrifugation at 4 °C for 15 min at 12,000 rpm, 200 µL supernatant was transferred to a new tube. For the quality control (QC) sample preparation, 60 µL of each sample was combined together.

The samples were evaporated in a vacuum concentrator, and then incubated in 30 µL of Methoxyamination hydrochloride (20 mg/mL in pyridine) for 30 min at 80 °C. For derivatization, 40 µL of BSTFA regent (1% TMCS, v/v) was applied at 70 °C for 1.5 h.

Samples were cooled to 25 °C and 5 µL of the FAMEs (in chloroform) was added to the QC sample. All of the samples were analyzed by gas chromatograph coupled with a time-of-flight mass spectrometer (GC-TOF-MS).

2.5. GC-TOF/MS Analysis

The gas chromatograph (Agilent 7890, Wilmington, DE, USA) coupled with a time-of-flight mass spectrometer was applied for the GC-TOF/MS analysis, and a DB-5MS capillary column was used in this system.

Exactly, 1 µL aliquot of samples was injected in splitles mode. The carrier gas used in this study was helium. The front inlet purge flow and the gas flow rate through the column were set as 3 mL min^{-1} and 1 mL min^{-1}, respectively. The initial temperature was set as 50 °C and kept this temperature for 1 min. Then, the temperature was raised at a rate of 10 °C min^{-1} until to 310 °C and kept for 8 min. The temperatures of injection and transfer line were set as 280 °C, and the ion source temperature was 250 °C. The energy in electron impact mode was −70 eV. The data of the mass spectrometry were obtained in full-scan mode with the m/z range of 50–500 at a rate of 12.5 spectra per second after a solvent delay of 6.25 min.

2.6. Data Preprocessing and Annotations

Raw data analysis, such as peak extraction, baseline adjustment, deconvolution, alignment and integration were performed with the Chroma TOF (V 4.3x, LECO, MI, USA) software. The database of LECO-Fiehn Rtx5 was used to identify the metabolites by match-

ing the mass spectrum and retention index. The peaks that detected in less than half of the QC samples or RSD > 30% in the QC samples were filtered out. To ensure the reliability of metabolite identification the metabolites were selected by similarity value (SV) above 700. The identification was regarded as a putative annotation, for SV was between 200 and 700, while samples with the SV value below 200 were discarded.

For principal component analysis (PCA), the data of peak number, sample name and normalized peak area were inputted into SIMCA software (V15.0.2, Sartorius Stedim Data Analytics AB, Umea, Sweden) to analyze the distribution of the original data. Furthermore, the orthogonal projections to latent structures-discriminant analysis (OPLS-DA) to get more reliable difference between groups and understand the variables responsible for classification. To identify the significantly different metabolites (SDMs), the OPLS-DA model was used with the first principal component of variable importance in the projection values (VIP > 1), and combined with the p value of Student's t-test ($p < 0.05$). The fold change (FC) values of the metabolites were calculated by comparison the mean values of the peaks. The pathways of the metabolites were searched by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database.

2.7. RNA Extraction and Gene Expression Analysis

Total RNA was extracted from leaves with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and digested with DNase I (Beyotime, Shanghai, China). The cDNA was synthesized by M-MLV cDNA synthesis kit (Promega, Shanghai, China). The qRT-PCR was conducted with a SYBR Green PCR Master Mix kit (Monad, Wuhan, China) on ABI StepOneplus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific Corp. MA, USA). The relative value of each gene expression was calculated as Chen et al. (2010) [26]. The primers used in this study were listed in Table S1.

2.8. Data Analysis

The physiological data were analyzed by SPSS Statistics 16.0 (IBM, Chicago, IL, USA), the significant differences ($p < 0.05$) were determined by one-way ANOVA, Tukey's method.

3. Results

3.1. Arabidopisis ost1-4 Mutant Was Sensitive to Cold Stress

To evaluate the cold tolerance of *Arabidopisis ost1-4* mutant, we measured MDA content, relative EL and chlorophyll *a* fluorescence in *ost1-4* and WT Arabidopsis plants. The results showed that both MDA content and relative EL remarkably increased in both WT and *ost1-4* plants after cold treatment. However, the increasement was quite higher in *ost1-4* mutant relative to the WT (Figure 1A,B). In addition, cold stress dramatically disturbed the OJIP curve and significantly reduced the F_V/F_M in both genotypes. Comparatively, the photosynthesis indexes were remarkably lower in *ost1-4* mutant than that of the WT (Figure 1C,D). These results suggested that mutation of *OST1* in Arabidopsis induced cold stress sensitivity of the plant.

3.2. Overall Metabolic Response to Cold Stress in ost1-4 Arabidopsis

Initially, a total of 622 chromatography peaks were obtained. After data filtering and missing value recoding, 607 peaks were retained (Table S2). Metabolites matching analysis revealed that among the 607 peaks 257 had no match with metabolites and 118 peaks were unknown (more than one peak matching to one metabolite) which should be filtered out for further analysis. Finally, 232 peaks that matched with metabolites were successfully identified. Generally, the 232 metabolites were divided into 34 categories (Table S3). The top three categories with the most metabolites were carbohydrates and carbohydrate conjugates, carboxylic acids and derivatives as well as amino acids and derivatives which had 59, 41 and 20 metabolites, respectively (Figure 2A).

Next, principal component analysis (PCA) was performed on the metabolic data of both *ost1-4* mutant and WT plants. As shown in Figure 2B. WT and *ost1-4* mutant showed

distinct clustering trend in the first and second principal component. Briefly, metabolites of non-cold treated WT and *ost1-4* plants were assigned into the second quadrant and the fourth quadrant, respectively. After cold treatment, the metabolites of *ost1* mutant and WT plants were placed in the same first quadrant, but clearly separated into two groups. This result suggested that the metabolites in *ost1-4* mutant were different from that of the WT regardless of the growth condition. Moreover, the OPLS-DA model was employed to differentiate groups by evaluating metabolic patterns. The OPLS-DA score plot showed that the metabolites could be separated clearly between *ost1-4* mutant and WT no matter whether it was exposed to cold stress (Figure 2C,D). The values of classification parameters in different comparison groups were shown in Table 1.

Table 1. Classification parameter values of PCA and OPLS-DA model.

Title	Type	A	N	R^2X	R^2Y	Q^2
TOTAL with QC	PCA	7	27	0.527		
$ost1\text{-}4_{CK}$vsWT_{CK}	OPLS-DA	1+1+0	12	0.573	0.999	0.981
$ost1\text{-}4_{cold}$vsWT_{Cold}	OPLS-DA	1+1+0	12	0.53	0.999	0.965

A = number of principal components of the model; N = sample size; R^2X = explanatory properties of the model for the X variable; R^2Y = explanatory properties of the model for the Y variable; Q^2 = predictability of the model.

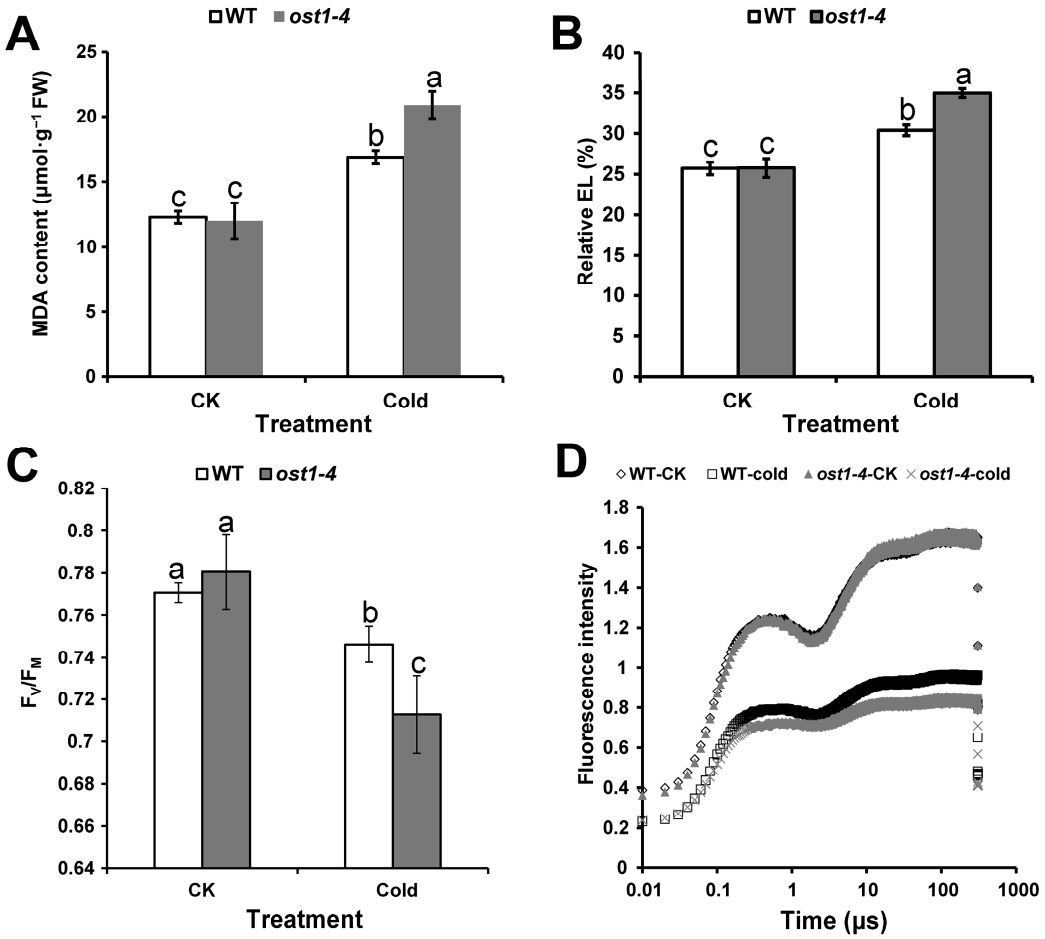

Figure 1. Physiological change of *ost1-4* mutant and WT Arabidopsis under cold stress. (**A**) MDA content; (**B**) relative EL; (**C**) F_V/F_M; (**D**) OJIP curve; CK = control; cold = cold treatment; WT = wild type; *ost1-4* = *ost1-4* mutant line. Means with the same letters represent no significant difference ($p > 0.05$).

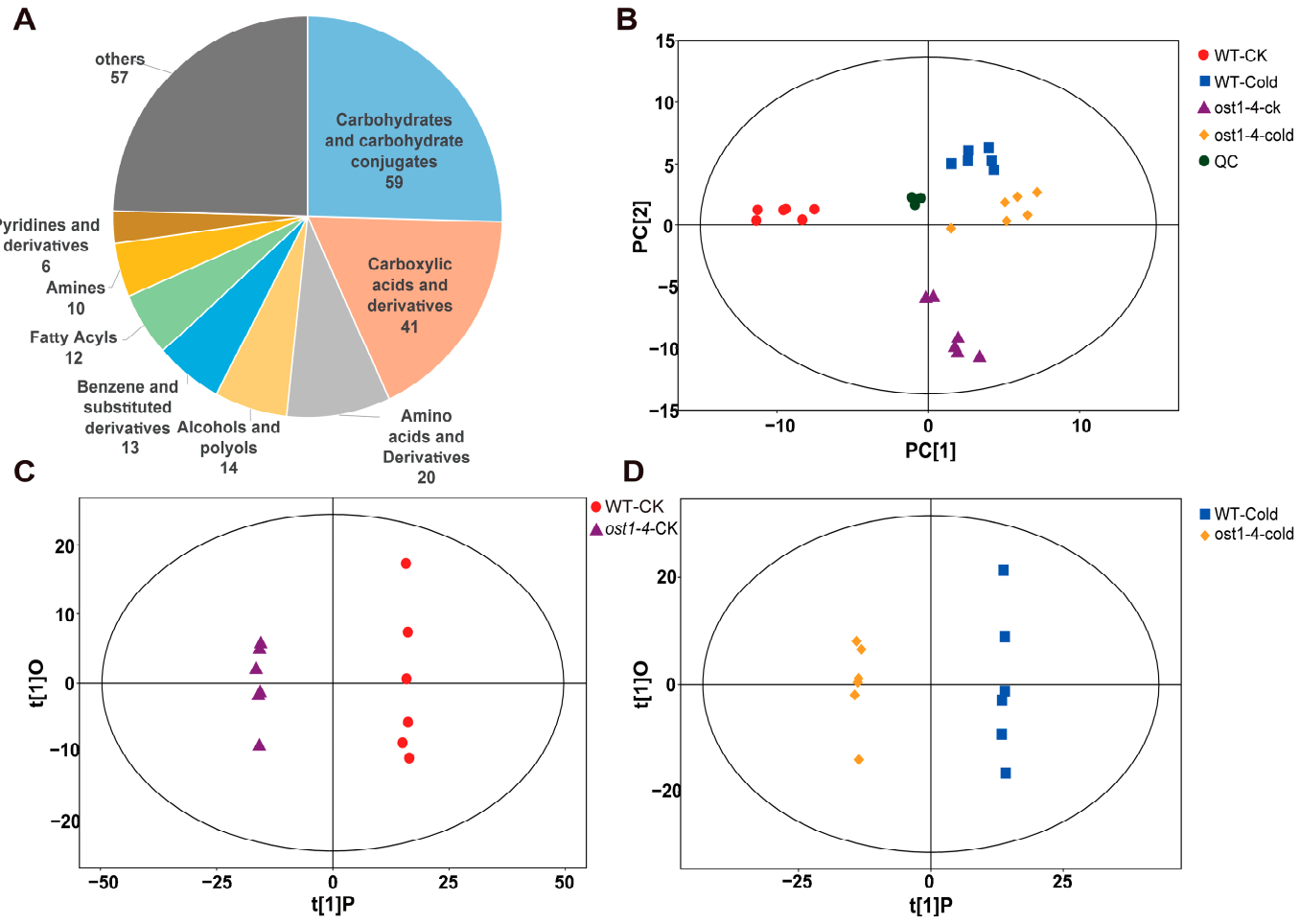

Figure 2. Total statistical analysis of the metabolite profiles. (**A**) classification statistics; (**B**) PCA analysis; (**C**) OPLS-DA analysis between *ost1-4* mutant and WT under normal condition; (**D**) OPLS-DA analysis between *ost1-4* mutant and WT under cold stress. The R2X value of the PCA model was 52.3%, and all the samples from both genotypes fall inside the Hotelling's T2 tolerance ellipse which represents 95% confidence limit. CK = control; cold = cold treatment; WT = wild type; *ost1-4* = *ost1-4* mutant line.

3.3. Changes of Metabolite Profiles in WT and ost1-4 Mutant

Further analysis was performed to identify SDMs in both genotypes. A total of 114 SDMs were identified in comparison *ost1-4*$_{CK}$vsWT$_{CK}$, of which 58 and 55 SDMs were up- and down- regulated, respectively. However, unexpectedly, less SDMs were identified in the comparison *ost1-4*$_{cold}$vsWT$_{cold}$, of which 65 and 37 SDMs were up- and down-regulated, respectively (Figure 3A).

Furthermore, heat map was constructed using the hierarchical clustering analysis to explore the pattern of metabolite changes between the *ost1-4* mutant and WT genotypes. Under normal condition, dozens of metabolites such as sucrose, galactose, ascorbate and aspartic acid were decreased in *ost1-4* mutant compared to the WT, while several metabolites such as pyruvic acid, citric acid, malonic acid and malic acid were increased in *ost1-4* mutant compared to the WT. When exposed to cold stress, profiles of many metabolites changed in both genotypes. The results showed that after cold exposure, the content of some metabolites, including aspartic acid, asparagine, 2,4-diaminobutyric acid, citrulline, citric acid and mannitol, were significantly lower in *ost1-4* mutant than that in WT. Interestingly, these metabolites were much higher in *ost1-4* mutant than that in WT under normal condition. On the contrary, the metabolites such as erythrose, linoleic acid and lipoic acid got higher in *ost1-4* mutant than that in WT, while content of these substances

were lower in *ost1-4* mutant than that in WT under normal condition (Figures 3B and S1, the data was showed in Table S4). Besides, contents of several metabolites, i.e., proline, phosphate and pipecolinic acid, decreased further in *ost1-4* mutant after cold treatment (Figure 3B).

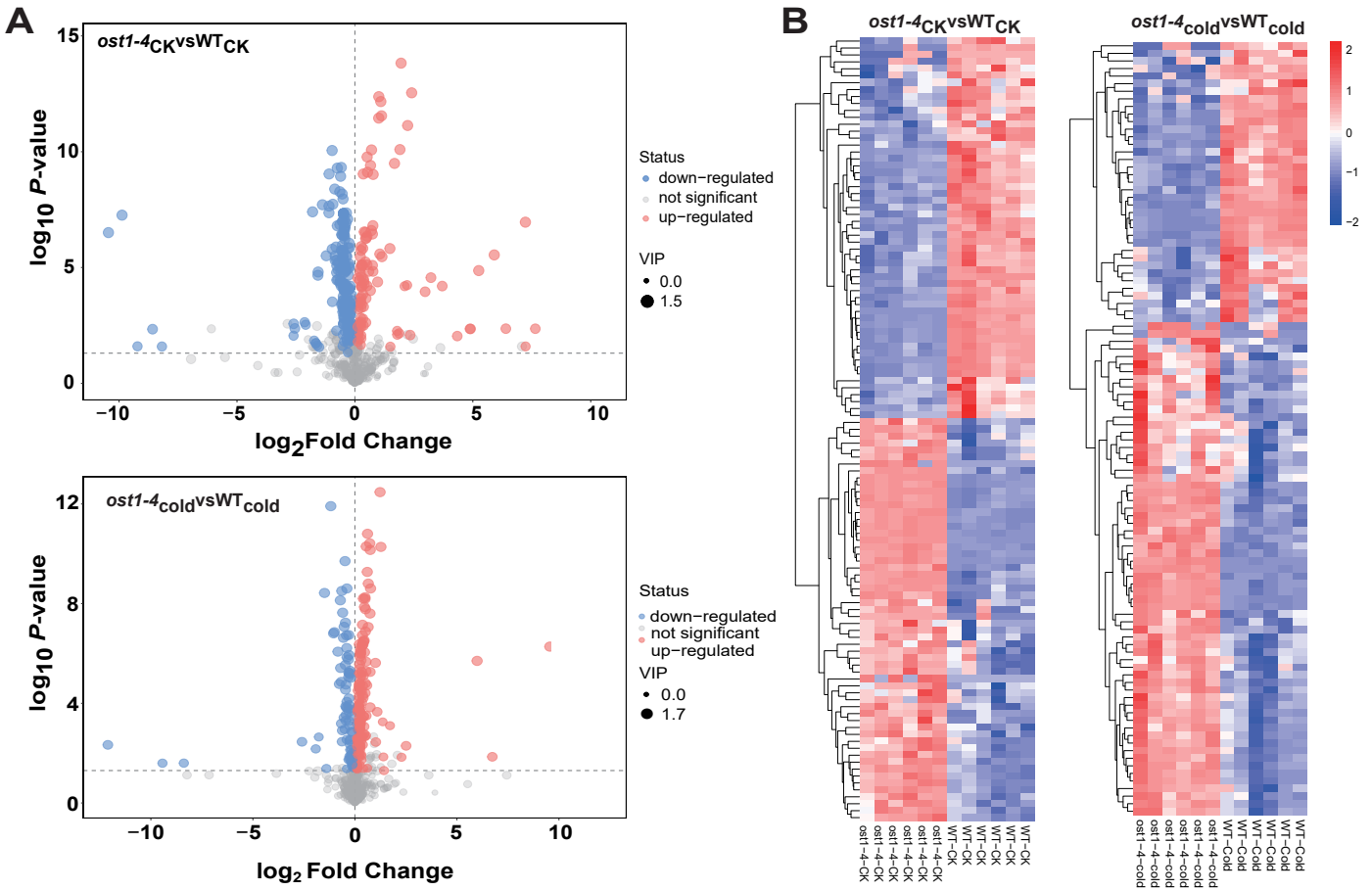

Figure 3. SDMs analysis between *ost1-4* mutant and WT under different conditions. (**A**) volcano plot of the metabolite; (**B**) Hierarchical clustering analysis for the SDMs. The relative metabolite content was depicted with color scale. Red means upregulated, blue means downregulated. CK = control; cold = cold treatment; WT = wild type; *ost1-4* = *ost1-4* mutant line.

3.4. Metabolic Pathways Affected by Cold Stress in WT and ost1-4 Mutant

To explore the potential metabolic pathways affected by cold stress in WT and *ost1* mutant, the SDMs of both genotypes were subjected to MetaboAnalyst, Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway database (http://www.kegg.jp/kegg/pathway.html, accessed on 11 February 2020) and PubChem analysis revealed that 40 and 34 pathways were found in comparisons *ost1-4*$_{CK}$vsWT$_{CK}$ and *ost1-4*$_{cold}$vsWT$_{cold}$, respectively. To facilitate the later analysis, the data were further screened using the cutoff −lnp>2 or pathway impact score > 0.25, and found 10 and 11 most relevant metabolic pathway in the two comparisons, respectively. In detail, pathways such as alanine, aspartate and glutamate metabolism, cyanoamino acid metabolism, citrate cycle (TCA cycle) and linoleic acid metabolism were identified in comparison of *ost1-4*$_{CK}$vsWT$_{CK}$. Besides, pathways including glycine, serine and threonine metabolism, glycerolipid metabolism and sphingolipid metabolism got more significant after cold treatment (Figure 4, Table S5).

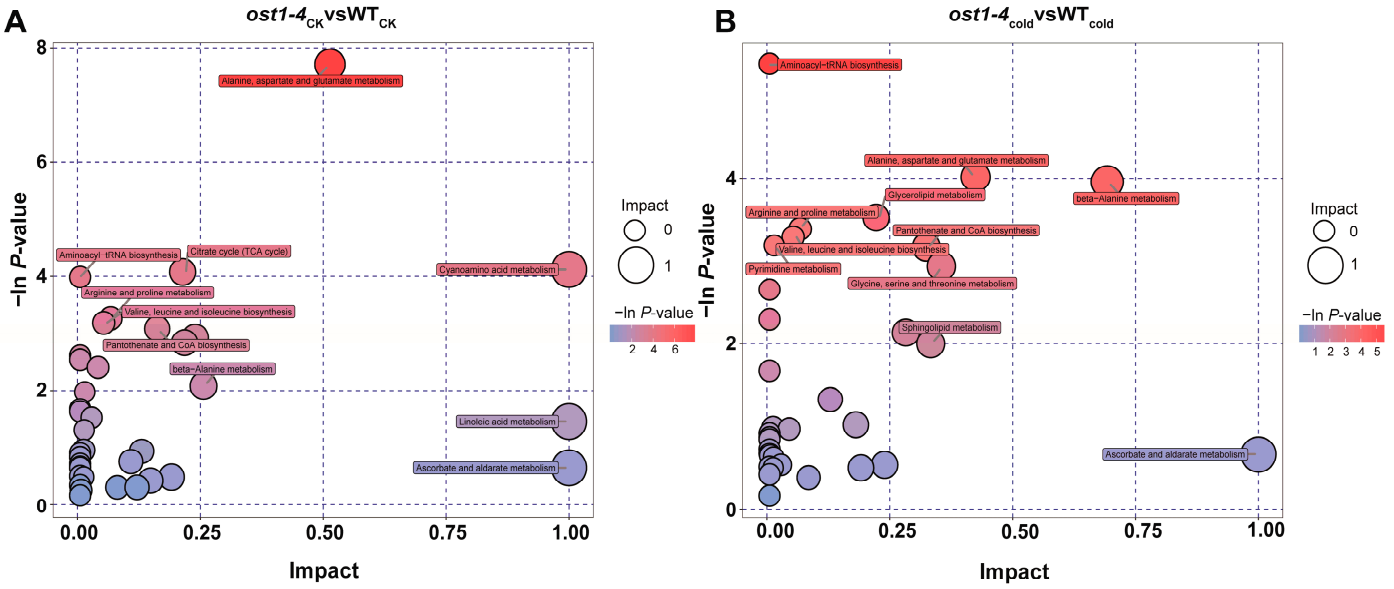

Figure 4. Metabolome view map of significant metabolic pathways identified between *ost1-4* mutant and WT. (**A**) Significant pathways between different genotypes under normal condition; (**B**) Significant pathways between different genotypes after cold treatment. The *x*-axis represents pathway enrichment, and the *y*-axis represents pathway impact. Large sizes and dark colors represent major pathway enrichment and high pathway impact values, respectively. CK = control; cold = cold treatment; WT = wild type; *ost1-4* = *ost1-4* mutant line.

In order to further study the most important metabolites regulated by OST1, the profiles of metabolites in different pathways were analyzed. Under normal condition, contents of *N*-Acetyl-D-galactosamine, aspartic acid, 3-hydroxynorvaline, mannitol and sophorose were upregulated more than 5-folds in *ost1-4* compared to WT. While, contents of erythrose, linoleic acid, glutamic acid and ascorbate were downregulated remarkably in *ost1-4* compare to WT. Interestingly, metabolites contents changed remarkably in the plants after cold treatment. Contents of digitoxose, lactic acid, raffinose and pelargonic acid were more than 2-fold higher in *ost1-4* than that in WT plant. While, contents of 1,5-anhydroglucitol, sulfuric acid, mannitol, malonic acid and citrulline were less than 0.5-fold lower in *ost1-4* than that in WT plant (Figure 3B; Table S2). The KEGG analysis showed that these substances especially *N*-Acetyl-D-galactosamine, aspartic acid, glutamic acid, ascorbate, lactic acid, raffinose, mannitol and citrulline were key compounds in different pathways such as alanine, aspartate and glutamate metabolism, ascorbate and aldarate metabolism and arginine and proline metabolism. These results implied the complex regulation mechanisms of OST1 in cold stress response of plant. In detail, for alanine, aspartate and glutamate metabolism pathway, aspartate and glutamine were two central metabolites. Interestingly, the contents of aspartic acid and asparagine decreased significantly in *ost1-4* mutant after cold treatment, while both of them got increased in WT plant. Not the same as aspartate, the contents of glutamine increased after cold treatment in both genotypes. However, the change fluctuations were lower in *ost1-4* mutant than that in WT plant. Citrate cycle, also be known as TCA cycle, was a typical pathway for cellular energy metabolism and carbon cycling. Under normal condition, contents of pyruvic acid, malic acid and α-ketoglutaric acid were higher in *ost1-4* mutant than that in WT plant. On the contrary, the content of succinic acid was lower in *ost1-4* mutant than that in WT plant. After cold treatment, the citric acid content was higher in WT plant after cold treatment than that under normal condition. But it was dramatically decreased in *ost1-4* mutant after cold treatment compare to that of normal condition. Meanwhile, the contents of other metabolites in citrate cycle such as malic acid, fumaric acid, succinic acid and α-ketoglutaric acid were also decreased in *ost1-4* mutant after cold treatment compare to that of normal

condition. However, the contents of citric acid and fumaric acid in *ost1-4* mutant were still higher than that in WT plant under cold stress.

3.5. Gene Expression Analysis in ost1-4 Mutant

Pathway analysis suggested that pathways of alanine, aspartate and glutamate metabolism, cyanoamino acid metabolism, and citrate cycle (TCA cycle) were related to OST1 regulation. Moreover, contents of metabolites in these pathways changed significantly in *ost1-4* mutant before and after cold treatment. To explore the regulation mechanisms further, genes expression related to these pathways were measured. The results showed that both of the candidate *AtGDH* (glutamate dehydrogenase) genes that encoded glutamate dehydrogenease were upregulated remarkably after cold treatment for 1 h in *ost1-4* mutant compared to WT plant (Figure 5A,B). After cold treatment, the genes were upregulated faster and higher in *ost1-4* mutant than that in WT plant. Similar profiles were also detected in the expressions of *AtPPC1* (phosphoenolpyruvate carboxylase 1) (Figure 5C). However, the expression of *AtAK1* (aspartate kinase 1) was much higher in *ost1-4* mutant than that in WT plant at normal temperature. After cold treatment for 6 h it was upregulated in WT plant, but severely downregulated in *ost1-4* mutant after 3 h of cold treatment. (Figure 5D).

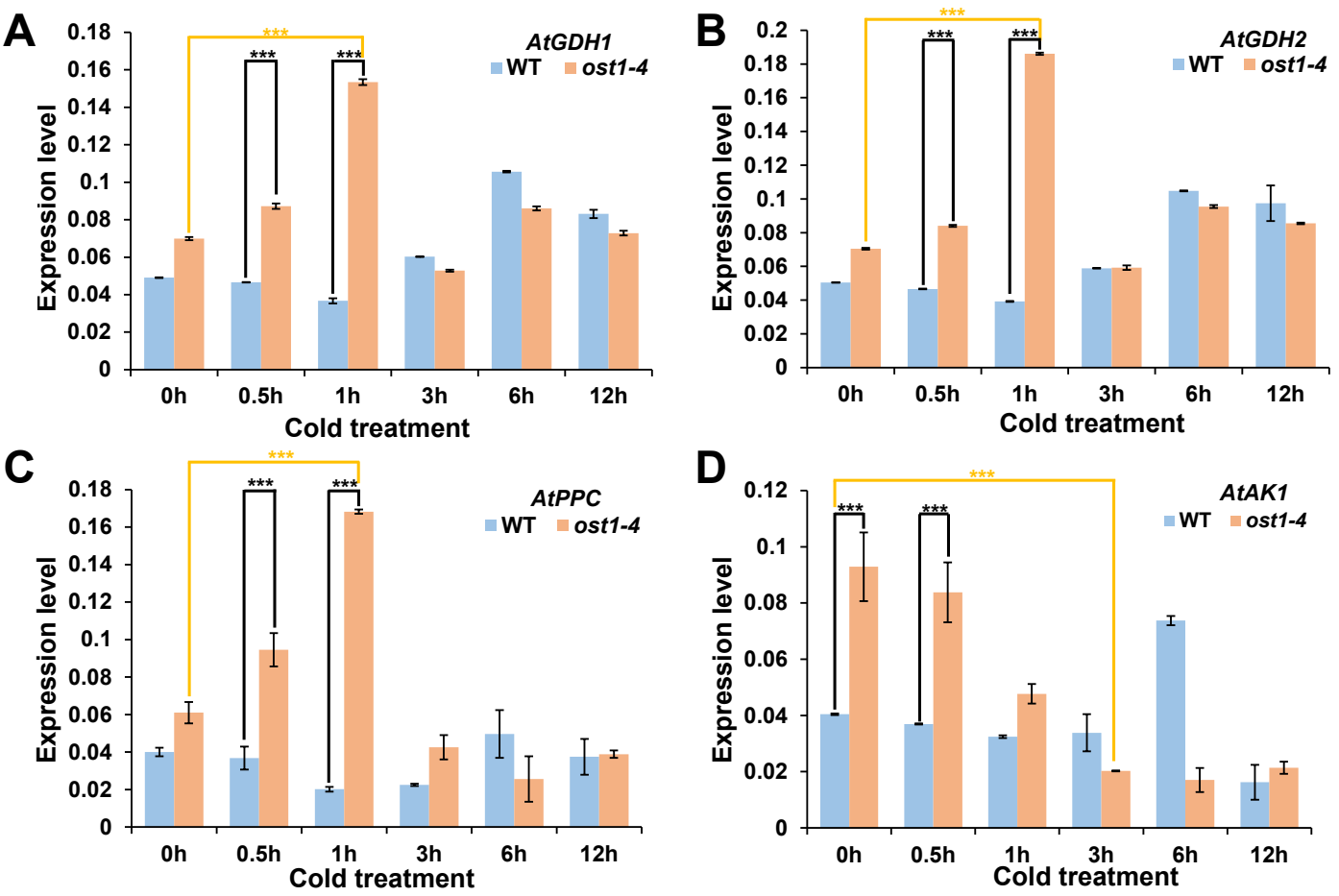

Figure 5. Gene expression in *ost1-4* mutant and WT plant after cold treatment. WT = wild type; *ost1-4* = *ost1-4* mutant line. *** = significant different ($p < 0.01$). (**A**) Relative expression of *AtGDH1*; (**B**) Relative expression of *AtGDH2*; (**C**) Relative expression of *AtPPC*; (**D**) Relative expression of *AtAK1*.

4. Discussion

Cold stress is one of the most important environmental stresses that plants face in their natural environment. To cope with the adverse impacts of stress conditions, plants evolved a series of complex response mechanisms. It has been proved that ABA signaling is a central signaling pathway that play a significant role in plants stress response [27]. The plant specific protein kinases SnRK2 played critical roles in ABA-dependent and independent signaling pathway [28,29]. Besides, as a member of SnRK2 family, OST1/SnRK2.6 especially expressed in stomata and mediated stomatal closure. Stomatal closure was a crucial in regulating stress response of the plant. Therefore, OST1 must play a very important role in stress response regulation. Recently, several lines of study had proved this hypothesis. For instance, overexpression of *Brassica oleracea* OST1 gene *BolOST1* had been reported to positively modulate drought response in transgenic Arabidopsis [30]. OST1 could also improve cold tolerance in Arabidopsis by enhancing ICE1 stability [16]. Despite several studies have been carried out to investigate the function of OST1 in plant stress response, the detailed metabolic profiles regulated by OST1, especially in response to cold stress was lacking. In this study, metabolic profiling of *ost1-4* mutant and WT plants under cold stress were investigated. Two most important stress indicators, such as MDA and EL were measured after cold treatment. The results showed that *ost1-4* mutant Arabidopsis exhibited significantly higher MDA content and relative EL after cold treatment, indicating that *ost1-4* mutant was more sensitive to cold stress than the WT. In support to the above results, the OJIP curve and F_V/F_M were declined remarkably in *ost1-4* mutant Arabidopsis after cold treatment, indicating a noticeable effect on photosynthesis. Taken together, physiological analysis showed that OST1 loss-of-function mutation could enhance cold stress sensitivity in plants. In support, a study revealed that OST1 plays a positive in cold stress response by phosphorylating BTF3 and BTF3L in Arabidopsis [14]. Moreover, it was also noticed that there no significant growth and physiological phenotype differences between *ost1-4* mutant and WT, this might be because of the functional redundancy of SnRK2 family and other proteins.

When plants exposed to abiotic stress, immediate responses could be induced at molecular, biochemical and physiological levels to establish a new homeostasis [31]. This new homeostasis, including change in metabolites could help plants to cope with damages caused by stresses. Therefore, understanding the metabolic profiles changed in response to abiotic stress is imperative significant to uncover complex tolerance mechanisms. Recently metabolomic analysis has been performed to uncover cold stress response in different plant species, including maize (*Zea mays*) [32], wheat (*Triticum aestivum*) [33] and Eucalyptus species [34]. Given the crucial role of OST1 in cold stress response and the important of metabolomics analysis, here we carried out metabolomics profiling of *ost1* mutant and WT plants under cold stress. As expected, cold stress induced several metabolic changes in both *ost1* mutant and WT Arabidopsis plants. Many SDMs were identified in comparison of $ost1\text{-}4_{CK}$vsWT_{CK}, and most of the SDMs were carbohydrates and organic acids. This suggested that the mutation of *OST1* induced the rearrangement of the metabolites in plant cell even under normal condition. When exposed to cold stress, although less SDMs were identified in comparison of $ost1\text{-}4_{cold}$vsWT_{cold} than that of $ost1\text{-}4_{CK}$vsWT_{CK}, larger percentage of the SDMs had higher content in *ost1-4* mutant than that in WT plant compare to that of normal condition. This result was counterintuitive because higher contents of soluble substances in cells usually implied better adaptability of the plant. And hundreds fold of upregulation of some metabolites were indeed detected in comparisons of WT_{cold}vsWT_{CK} and $ost1\text{-}4_{cold}$vs$ost1\text{-}4_{CK}$. However, the physiological investigation revealed that *ost1-4* mutant was sensitive to cold stress. These results were understandable when the trait of *ost1* mutant was considered. The typical phenotype of *ost1* mutant was stay-open stomata even under stress condition [35]. Cold stress could induce stomatal closure in WT plant, so that the plant could avoid water loss. But in *ost1-4* mutant, the stomata keep opening under cold stress condition, the relative high content of the metabolites might be play roles in keeping the water potential of the cells which induced stay opening of the stomata.

Resent study reported that OST1 regulated the stomatal movement via regulating the ion channels [36]. Considering that the stomatal movement was control by hydraulic and chemical signaling, so whether OST1 was involved in regulating the water potential was needed to study further.

The changes of massive metabolites in *ost1-4* mutant and WT plant before and after cold stress treatment were too complex. The KEGG analysis could be used as a useful tool to reveal the deep regulation mechanisms. With the KEGG analysis, several pathways that related to OST1 involved cold stress response were identified. Under normal temperature, pathways of alanine, aspartate and glutamate metabolism, citrate cycle (TCA cycle) and cyanoamino acid metabolism were found to be significantly changed in *ost1-4* mutant and WT plant which implied that these pathways were regulated by OST1. Alanine, aspartate and glutamate metabolism pathway was related to numbers of metabolism pathways; hence it was one of the core metabolism pathways to regulate the material and energy cycle in the cell. As the results showed, contents of two core metabolites including aspartate and glutamine were remarkably decreased in *ost1-4* mutant after cold treatment, which was distinguished from the change in WT plant. This indicated that aspartate metabolism was severely suppressed in *ost1-4* mutant when exposed to cold stress. Although the contents of glutamine increased after cold treatment in both genotypes, the difference of change fluctuations suggested the disruption by mutation of OST1 in Arabidopsis. Therefore, the integrate changes of the key metabolites content resulted in regulation of alanine, aspartate and glutamate metabolism pathway. Previous studies showed that alanine, aspartate and glutamate metabolism pathway was involved in the regulation of drought stress in *Salvadora persica* [37] and heat stress in *Sargassum fusiforme* [38]. which suggested the crucial roles of this pathway in stress response of plant. Besides, 3-cyanoalanine was exactly the upstream metabolite of aspartate and asparagine in cyanoamino acid metabolism pathway, and contents changes of these three metabolites were coincident. So, the pathway of cyanoamino acid metabolism should also be regulated by OST1. Citrate cycle was a crucial pathway for energy metabolism and carbon cycle in cells, and it also connected aspartate and glutamate metabolisms. Citrate cycle had been reported in cold stress response of strawberry [39]. In the present study the results showed that under normal condition, increase of citric acid content maybe induced by high content of pyruvic acid in *ost1-4* mutant under normal condition, which further induced increase of malic acid and α-ketoglutaric acid contents. Depside that the contents of metabolites in citrate cycle were downregulated by cold stress, they are still higher in *ost1-4* mutant than WT plant after cold treatment. High activity of citrate cycle could provide energy and osmosis substances which might be contributed to keep continuously stomatal opening. Furthermore, KEGG pathways of ascorbate and aldarate metabolism and arginine and proline metabolism were also enriched in the present study which was in accorded with the results that was found in pumpkin (*Cucurbita maxima*) [40].

Gene expression could reveal more information of metabolites regulation. Given that the pathways of alanine, aspartate and glutamate metabolism and citrate cycle (TCA cycle) were presumptive pathways which the most be related to OST1 regulated cold stress response. Several genes expressions related to these pathways were investigated further. The results showed that both *AtGDHs* were upregulated in *ost1-4*. *AtGDH* genes encoded glutamate dehydrogenase [41], and this enzyme catalyzed the deamination of l-glutamate to 2-oxoglutarate. This result suggested that the metabolic direction of glutamate was changed in *ost1-4* mutant, it preferred be catalyzed into glutamine other than 4-aminobutanoate. Maybe this could explain why the contents of α-ketoglutaric acid and glutamine were upregulated, but downregulation of 4-aminobutyric acid and succinic acid contents in *ost1-4* mutant compare to WT plant. AK1 was a kinase that catalyzed aspartate to aspartylphosphate [42], its activity was related to the content of aspartate. The results of this study also showed that under normal condition, the content of aspartate was higher in *ost1-4* mutant than that in WT plant. When the plants were exposed to cold stress, the content of aspartate increased in WT plant but decreased in *ost1-4* mutant. *AtPPC1*

encoded phosphoenolpyruvate carboxylase which catalyzed phosphoenolpyruvate and CO_2 to oxaloacetate [43]. The oxaloacetate could be catalyzed to citrate and malate. Hence, upregulation of *AtPPC1* expression could induce upregulation of citric acid and malic acid contents which results were detected by metabolic analysis.

5. Conclusions

Although OST1 was confined to expressed in guard cells and vascular system and regulated stomata closure, it played an important role in regulating cold stress response of the plants. Mutation of *OST1* could induce cold sensitivity of plant. Massive of SDMs were identified to be related to cold stress response of Arabidopsis. Most of the SDMs with high contents in *ost1-4* mutant after cold treatment were soluble carbohydrates and organic acids that might be contribute to keep continuous stomatal opening of the mutate plant under cold stress. The KEGG analysis revealed that pathways of alanine, aspartate and glutamate metabolism, citrate cycle (TCA cycle) and cyanoamino acid metabolism were most related to OST1 regulated cold stress response of Arabidopsis. The gene expression analysis of *AtGDH, AtPPC1* and *AtAK1* validated the presumption of metabolic analysis further. Overall, the present study provided some information on the metabolomic regulation of *OST1* related cold stress response in plant, but the mechanisms of OST1 related cold stress response was still largely unknown in plant, and it should to study further in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13102567/s1, Figure S1: Hierarchical clustering analysis for the SDMs in comparisons of $ost1\text{-}4_{cold}$ vs$ost1\text{-}4_{CK}$ and WT_{cold}vsWT_{CK}; Table S1: Primer sequences used in this study; Table S2: Metabolites identified in this study; Table S3: Classification of the metabolisms; Table S4: Data of hierarchical clustering analysis of the SDMs; Table S5: Pathway analysis of the SDMs.

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References

1. Eremina, M.; Rozhon, W.; Poppenberger, B. Hormonal control of cold stress responses in plants. *Cell. Mol. Life Sci.* **2016**, *73*, 797–810. [CrossRef]
2. Kazemi-Shahandashti, S.S.; Maali-Amiri, R. Global insights of protein responses to cold stress in plants: Signaling, defence, and degradation. *J. Plant Physiol.* **2018**, *226*, 123–135. [CrossRef]
3. Ding, Y.; Lv, J.; Shi, Y.; Gao, J.; Hua, J.; Song, C.; Gong, Z.; Yang, S. EGR2 phosphatase regulates OST1 kinase activity and freezing tolerance in *Arabidopsis*. *EMBO J.* **2019**, *38*, e99819. [CrossRef] [PubMed]
4. Wei, Y.; Chen, H.; Wang, L.; Zhao, Q.; Wang, D.; Zhang, T. Cold acclimation alleviates cold stress-induced PSII inhibition and oxidative damage in tobacco leaves. *Plant Signal. Behav.* **2022**, *17*, 2013638. [CrossRef]
5. Agurla, S.; Gahir, S.; Munemasa, S.; Murata, Y.; Raghavendra, A.S. Mechanism of stomatal closure in plants exposed to drought and cold stress. In *Survival Strategies in Extreme Cold and Desiccation. Advances in Experimental Medicine and Biology*; Iwaya-Inoue, M., Sakurai, M., Uemura, M., Eds.; Springer: Singapore, 2018; Volume 1081, pp. 215–232.
6. Lantzouni, O.; Alkofer, A.; Falter-Braun, P.; Schwechheimer, C. Growth-Regulating Factors interact with DELLAs and regulate growth in cold stress. *Plant Cell* **2020**, *32*, 1018–1034. [CrossRef]

7. Kidokoro, S.; Shinozaki, K.; Yamaguchi-Shinozaki, K. Transcriptional regulatory network of plant cold-stress responses. *Trends Plant Sci.* **2022**, *27*, 922–935. [CrossRef] [PubMed]
8. Guo, Q.; Li, X.; Niu, L.; Jameson, P.E.; Zhou, W. Transcription-associated metabolomic adjustments in maize occur during combined drought and cold stress. *Plant Physiol.* **2021**, *186*, 677–695. [CrossRef] [PubMed]
9. Bürger, M. Escaping the drought: The OST1-VOZ1 module regulates early flowering in tomato. *Plant Cell* **2022**, *34*, 1886–1887. [CrossRef]
10. Matrosova, A.; Bogireddi, H.; Mateo-Peñas, A.; Hashimoto-Sugimoto, M.; Iba, K.; Schroeder, J.I.; Israelsson-Nordström, M. The HT1 protein kinase is essential for red light-induced stomatal opening and genetically interacts with OST1 in red light and CO_2 -induced stomatal movement responses. *New Phytol.* **2015**, *208*, 1126–1137. [CrossRef]
11. Mustilli, A.C.; Merlot, S.; Vavasseur, A.; Fenzi, F.; Giraudat, J. Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* **2002**, *14*, 3089–3099. [CrossRef]
12. Liu, Z.; Hou, S.; Rodrigues, O.; Wang, P.; Luo, D.; Munemasa, S.; Lei, J.; Liu, J.; Ortiz-Morea, F.A.; Wang, X.; et al. Phytocytokine signalling reopens stomata in plant immunity and water loss. *Nature* **2022**, *605*, 332–339. [CrossRef] [PubMed]
13. Lang, Z.; Zhu, J. OST1 phosphorylates ICE1 to enhance plant cold tolerance. *Sci. China Life Sci.* **2015**, *58*, 317–318. [CrossRef] [PubMed]
14. Ding, Y.; Jia, Y.; Shi, Y.; Zhang, X.; Song, C.; Gong, Z.; Yang, S. OST1-mediated BTF3L phosphorylation positively regulates CBFs during plant cold responses. *EMBO J.* **2018**, *37*, e98228. [CrossRef] [PubMed]
15. Wang, D.Z.; Jin, Y.N.; Ding, X.H.; Wang, W.J.; Zhai, S.S.; Bai, L.P.; Guo, Z.F. Gene regulation and signal transduction in the ICE–CBF–COR signaling pathway during cold stress in plants. *Biochemistry* **2017**, *82*, 1103–1117. [CrossRef] [PubMed]
16. Ding, Y.; Li, H.; Zhang, X.; Xie, Q.; Gong, Z.; Yang, S. OST1 kinase modulates freezing tolerance by enhancing ICE1 stability in *Arabidopsis*. *Dev. Cell* **2015**, *32*, 278–289. [CrossRef]
17. Zhang, J.; Luo, W.; Zhao, Y.; Xu, Y.; Song, S.; Chong, K. Comparative metabolomic analysis reveals a reactive oxygen species-dominated dynamic model underlying chilling environment adaptation and tolerance in rice. *New Phytol.* **2016**, *211*, 1295–1310. [CrossRef]
18. Taïbi, K.; Campo, A.D.D.; Vilagrosa, A.; Bellés, J.M.; López-Gresa, M.P.; López-Nicolás, J.M.; Mulet, J.M. Distinctive physiological and molecular responses to cold stress among cold-tolerant and cold-sensitive *Pinus halepensis* seed sources. *BMC Plant Biol.* **2018**, *18*, 236. [CrossRef]
19. Kaplan, F.; Kopka, J.; Haskell, D.W.; Zhao, W.; Schiller, K.C.; Gatzke, N.; Sung, D.Y.; Guy, C.L. Exploring the temperature-stress metabolome of *Arabidopsis*. *Plant Physiol.* **2004**, *136*, 4159–4168. [CrossRef]
20. Angelcheva, L.; Mishra, Y.; Antti, H.; Kjellsen, T.D.; Funk, C.; Strimbeck, R.G.; Schröder, W.P. Metabolomic analysis of extreme freezing tolerance in Siberian spruce (*Picea obovata*). *New Phytol.* **2014**, *204*, 545–555. [CrossRef]
21. Rasmussen, S.; Parsons, A.J.; Newman, J.A. Metabolomics analysis of the *Lolium perenne–Neotyphodium lolii* symbiosis: More than just alkaloids? *Phytochem. Rev.* **2009**, *8*, 535–550. [CrossRef]
22. Catola, S.; Marino, G.; Emiliani, G.; Huseynova, T.; Musayev, M.; Akparov, Z.; Maserti, B.E. Physiological and metabolomic analysis of *Punica granatum* (L.) under drought stress. *Planta* **2016**, *243*, 441–449. [CrossRef] [PubMed]
23. Hu, L.; Zhang, P.; Jiang, Y.; Fu, J. Metabolomic analysis revealed differential adaptation to salinity and alkalinity stress in Kentucky bluegrass (*Poa pratensis*). *Plant Mol. Biol.* **2015**, *33*, 56–68. [CrossRef]
24. Fan, J.; Ren, J.; Zhu, W.; Amombo, E.; Fu, J.; Chen, L. Antioxidant responses and gene expression in bermudagrass under cold stress. *Am. Soc. Hort. Sci.* **2014**, *139*, 699–705. [CrossRef]
25. Fan, J.; Hu, Z.; Xie, Y.; Chan, Z.; Chen, K.; Amombo, E.; Chen, L.; Fu, J. Alleviation of cold damage to photosystem II and metabolisms by melatonin in Bermudagrass. *Front. Plant Sci.* **2015**, *6*, 925. [CrossRef]
26. Chen, L.; Ren, F.; Zhong, H.; Jiang, W.; Li, X. Identification and expression analysis of genes in response to high-salinity and drought stresses in *Brassica napus*. *Acta Biochim. Biophys. Sin.* **2010**, *42*, 154–164. [CrossRef]
27. Canales, F.J.; Rispail, N.; García-Tejera, O.; Arbona, V.; Pérez-De-Luque, A.; Prats, E. Drought resistance in oat involves ABA-mediated modulation of transpiration and root hydraulic conductivity. *Environ. Exp. Bot.* **2021**, *182*, 104333. [CrossRef]
28. Brandt, B.; Brodsky, D.E.; Xue, S.; Negi, J.; Iba, K.; Kangasjärvi, J.; Ghassemian, M.; Stephan, A.B.; Hu, H.; Schroeder, J.I. Reconstitution of abscisic acid activation of SLAC1 anion channel by CPK6 and OST1 kinases and branched ABI1 PP2C phosphatase action. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 10593–10598. [CrossRef]
29. Zhang, L.; Takahashi, Y.; Hsu, P.K.; Kollist, H.; Merilo, E.; Krysan, P.J.; Schroeder, J. FRET kinase sensor development reveals SnRK2/OST1 activation by ABA but not by MeJA and high CO_2 during stomatal closure. *eLife* **2020**, *9*, e56351. [CrossRef]
30. Wang, M.; Yuan, F.; Hao, H.; Zhang, Y.; Zhao, H.; Guo, A.; Hu, J.; Zhou, X.; Xie, C.G. BolOST1, an ortholog of Open Stomata 1 with alternative splicing products in Brassica oleracea, positively modulates drought responses in plants. *Biochem. Biophys. Res. Commun.* **2013**, *442*, 214–220. [CrossRef]
31. Fürtauer, L.; Weiszmann, J.; Weckwerth, W.; Nägele, T. Dynamics of plant metabolism during cold acclimation. *Int. J. Mol. Sci.* **2019**, *20*, 5411. [CrossRef] [PubMed]
32. Urrutia, M.; Blein-Nicolas, M.; Prigent, S.; Bernillon, S.; Deborde, C.; Balliau, T.; Maucourt, M.; Jacob, D.; Ballias, P.; Bénard, C.; et al. Maize metabolome and proteome responses to controlled cold stress partly mimic early-sowing effects in the field and differ from those of Arabidopsis. *Plant Cell Environ.* **2021**, *44*, 1504–1521. [CrossRef] [PubMed]

33. Zhao, Y.; Zhou, M.; Xu, K.; Li, J.; Li, S.; Zhang, S.; Yang, X. Integrated transcriptomics and metabolomics analyses provide insights into cold stress response in wheat. *Crop. J.* **2019**, *7*, 857–866. [CrossRef]
34. Oberschelp, G.P.J.; Guarnaschelli, A.B.; Teson, N.; Harrand, L.; Podestá, F.E.; Margarit, E. Cold acclimation and freezing tolerance in three Eucalyptus species: A metabolomic and proteomic approach. *Plant Physiol. Biochem.* **2020**, *154*, 316–327. [CrossRef]
35. Kimura, H.; Hashimoto-Sugimoto, M.; Iba, K.; Terashima, I.; Yamori, W. Improved stomatal opening enhances photosynthetic rate and biomass production in fluctuating light. *J. Exp. Bot.* **2020**, *71*, 2339–2350. [CrossRef] [PubMed]
36. Wu, Q.; Wang, M.; Shen, J.; Chen, D.; Zheng, Y.; Zhang, W. ZmOST1 mediates abscisic acid regulation of guard cell ion channels and drought stress responses. *J. Integr. Plant Biol.* **2019**, *61*, 478–491. [CrossRef] [PubMed]
37. Rangani, J.; Panda, A.; Parida, A.K. Metabolomic study reveals key metabolic adjustments in the xerohalophyte *Salvadora persica* L. during adaptation to water deficit and subsequent recovery conditions. *Plant Physiol. Biochem.* **2020**, *150*, 180–195. [CrossRef] [PubMed]
38. Liu, L.; Lin, L. Efect of heat stress on Sargassum fusiforme leaf metabolome. *J. Plant Biol.* **2020**, *63*, 229–241. [CrossRef]
39. Davik, J.; Koehler, G.; From, B.; Torp, T.; Rohloff, J.; Eidem, P.; Wilson, R.C.; Sønsteby, A.; Randall, S.K.; Alsheikh, M. Dehydrin, alcohol dehydrogenase, and central metabolite levels are associated with cold tolerance in diploid strawberry (*Fragaria* spp.). *Planta* **2013**, *237*, 265–277. [CrossRef]
40. Li, F.; Lu, X.; Duan, P.; Liang, Y.; Cui, J. Integrating transcriptome and metabolome analyses of the response to cold stress in pumpkin (*Cucurbita maxima*). *PLoS ONE* **2021**, *16*, e0249108. [CrossRef] [PubMed]
41. Pageau, K.; Reisdorf-Cren, M.; Morot-Gaudry, J.F.; Masclaux-Daubresse, C. The two senescence-related markers, *GS1* (cytosolic glutamine synthetase) and *GDH* (glutamate dehydrogenase), involved in nitrogen mobilization, are differentially regulated during pathogen attack and by stress hormones and reactive oxygen species in *Nicotiana tabacum* L. leaves. *J. Exp. Bot.* **2006**, *57*, 547–557.
42. Yoshioka, Y.; Kurei, S.; Machida, Y. Identification of a monofunctional aspartate kinase gene of Arabidopsis thaliana with spatially and temporally regulated expression. *Genes Genet. Syst.* **2001**, *76*, 189–198. [CrossRef] [PubMed]
43. Feria, A.B.; Ruíz-Ballesta, I.; Baena, G.; Ruíz-López, N.; Echevarría, C.; Vidal, J. Phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxylase kinase isoenzymes play an important role in the filling and quality of *Arabidopsis thaliana* seed. *Plant Physiol. Biochem.* **2022**, *190*, 70–80. [CrossRef] [PubMed]