Introduction
Cavendish banana and Dajiao (Musa sp) are the most popular fruits and vital staple food around the globe. Low temperature is one of the key environmental stresses which greatly affect the global banana production. Musa sp. exhibits a high degree of genetic variability for cold tolerance, with Cavendish banana (Musa sp. Cavendish, AAA Group) being more cold sensitive than Dajiao (Musa sp. Dajiao, ABB Group). In contrast to the Cavendish “dessert” banana, the Dajiao species, Dajiao has superior cold tolerance, enabling it to tolerate temperatures of 0-4°C, and has been proposed as a potential germplasm resource of cold tolerance in banana breeding.

Aim
To understand the molecular mechanism of the different cold tolerance between Cavendish banana and Dajiao.

Material and Methods
Plant Materials
Seedslings of the cold-tolerant Dajiao (Musa sp. Dajiao, ABB Group) and the cold-sensitive Cavendish banana (Musa sp. Cavendish, AAA Group) with a uniform growth stage were obtained from Institute of Fruit Tree Research, Guangdong Academy of Agricultural Sciences, Guangzhou, P. R. of China.

Transcriptomics
Transcriptomics Experimental Design and Cold Stress Treatment
10°C was used as cold treatment temperature and reduced exposure time at 0, 3 and 6 hours respectively. The comparative transcriptomic analysis was conducted for the cold stressed seedlings, followed by large scale identification and functional categorization of the differentially expressed, early responsive genes. Furthermore, quantitative real time-PCR was carried out to validate the early cold-responsive transcriptomic results and some late-responsive genes with the extended time of cold treatment for 24 and 48 hours. Seedlings were grown in a growth chamber at 30°C (daytime), a photon flux density of 240μmol m-2 s-1 throughout a 12 h photoperiod, and a relative humidity of 68-80%. Six-leaf stage seedlings were used in the experiment. Low temperature treatments were started at 12:00 AM on the first day by setting the temperature to 10°C, which was reached about 30 min later. The first young leaf was detached from the top of each of the 5 plants at each time point (10°C for 0, 3 and 6 h) for each biological replicate. The leaves from the 5 plants were cut into pieces (1.5 x 1.5 cm) and mixed well. Aliquots of the mixed tissues were frozen in liquid N2 and stored at -80°C until use.

Plant transformation
Product fusion construct 1301-MpMAPK5-GUS under the control of the Ub promoter. The double stranded RNA interference (dsRNA) construct was produced via a PCR-mediated approach and two products were cloned into pYL-RNAi under the control of the Ub promoter. The newly constructed 1301-MpMAPK5-GUS plant expression vector and RNAi vector were transferred into A. tumefaciens strain EHA105 by heat shock. The procedures of transformation is based on the method reported by Ibha, including Agrobacterium tumefaciens transformation, liquid medium screening, resistance genes screening, germination of resistant embryo and plant regeneration.

Phosphoproteomics
Leaves’ crude proteins were isolated and purified as described by Isaacsan (Isaacsan et al., 2006), with modifications. Further processing of the proteins was then performed according to Thermo Scientific’s TMT Mass Tagging Kit, and Kegg and Reagents protocol (http://www.perceoton.com/instructions/2152275.pdf) with a slight modification. The nano LC-MS/MS analysis was carried out using an Orbitrap Elite (Thermo Fisher Scientific, San Jose, CA) mass spectrometer equipped with a nano ion source according to Yang et al (Yang et al., 2012). The resulting MS and MS/MS data were processed using MaxQuant (Max Planck Institute of Biochemistry, Munich, Germany) with integrated Andromeda search engine (v.1.4.1.2) and Proteome Discoverer v. 2.0 software (Thermo Fisher Scientific, Bremen, Germany).

Results

differential expression genes
To thoroughly investigate the differences in gene expression between cold-sensitive Cavendish banana and cold-tolerant Dajiao in response to cold stress, we performed comparative transcriptomic analysis using the aligned reads (Table 1).

Table 1 Differential expressed genes (DEGs) in Cavendish and Dajiao during cold treatment for 3 and 6 hours.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cavendish</th>
<th>Dajiao</th>
<th>Unique in Cavendish</th>
<th>Unique in Dajiao</th>
<th>Common in both</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEGs at 3h</td>
<td>Total 60</td>
<td>33</td>
<td>10</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Up-regulated</td>
<td>35</td>
<td>26</td>
<td>2</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Down-regulated</td>
<td>7</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DEGs at 6h</td>
<td>Total 238</td>
<td>108</td>
<td>18</td>
<td>58</td>
<td>50</td>
</tr>
<tr>
<td>Up-regulated</td>
<td>138</td>
<td>94</td>
<td>54</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Down-regulated</td>
<td>40</td>
<td>14</td>
<td>10</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

MpICE1 interacts with MpMAPK5
In our recent experiment, MpICE1 interacts with MpMAPK5 (Figure 2). In order to select MAPK genes which response to cold stress, the expressions of all MAPKs in Musa genome were determined by RT-PCR under 3 hours cold stress. The results showed only the expression of MpMAPK5 was significantly up-regulated in Cavendish banana and Dajiao under cold stress. Besides, the expression of MpMAPK5 in Dajiao is almost 5 times of it in Cavendish banana. For the above reasons, MpMAPK5 (MpMAPK5 as follow) was chosen as the object of this study.

Cold resistance evaluation in transgenic Dajiao plants
Compared with wild type, RNAi transgenic plants showed dwarf phenotype under normal condition (Figure 6). After 5 days of cold treatment, wild type Dajiao showed normal phenotype, while the leaves of two RNAi transgenic lines became yellowing and water loss. After 7 days of cold treatment, minor injuries were found in wild type leaves, while the leaves of RNAi transgenic plants displayed severe necrosis and wilting symptoms (Figure 7). The expression of MAPK5 and cold resistance related genes (including MYB14, ICE1, MYB33, DREB1G, DREB1D, COR1 and SPC4) were verified by RT-PCR under cold stress.

Validation of the DEGs by RT-PCR analysis
10 DEGs of Dajiao identified in this study after 5 h cold treatment and 2 critical cold-response genes (ICE1 and MYB83) were selected for quantitative RT-PCR analysis. The considerably different expression profiles of the two important transcriptional factors with a remarkable time delayed response in Cavendish banana versus Dajiao suggest that the specific time course-based expression of ICE1 and MYB83 in Dajiao might be related to its cold tolerance.

MpzMAPK5 Bioinformaton
In the cells transformed with the MpzMAPK5-GFP fusion construct, green fluorescence was observed in both the nucleus and the cytoplasm of the cell (Figure 4b), and fluorescence was detected in both the nucleus and the cytoplasm of the cell transformed with the control vector (Figure 4a). The phylogenetic tree analysis revealed that MpzMAPK5 was clearly clustered with A subgroup and close to zMAPK5 of Arabidopsis and rice.

Phosphoproteomics
After analyzed by bioinformatics, we found that the abundance of phosphopeptides related to oxidoreductase activity showed remarkable difference between wild type and RNAi transgenic plants. In wild type plants, all 27 phosphopeptides related to oxidoreductase activity showed significant change after cold treatment. However, only 3 phosphopeptides showed significantly change in transgenic plants. The results indicated that suppression of MpzMAPK5 expression had a great effect on the oxidation reduction metabolism related proteins of Dajiao. To prove the results of above analysis, MDA contents and POD activity of wild type and transgenic plants were determined.

Discussion
1. The rapid activation and selective induction of ICE1 and MYB83 cold tolerance pathways in Dajiao, along with expression of other cold-specific genes, may be one of the main reasons that Dajiao has higher cold resistance than Cavendish banana.

2. MpMAPK5 is a positive regulator of cold signaling confers the cold tolerance of Dajiao by effecting ROS pathway.

Conclusion

Figure 1. Relative mRNA levels in Cavendish banana and Dajiao seedlings were determined by quantitative RT-PCR analysis.

Figure 2. The BIFC between MpICE1 and MpMAPK5.

Figure 3. The expression of MAPK family in Cavendish banana and Dajiao under 3 hour cold stress.

Figure 4. Salivary Gland Localization of MpMAPK5.

Figure 5. Phosphorylinic analysis of Arabidopsis amino acid sequences of MpMAPK5 with Rice and Arabidopsis MAPK amino acid sequences.

Figure 6. Phenotype of wild type and transgenic lines under normal condition.

Figure 7. Phenotype of WT and transgenic lines under cold stress.

Figure 8. Transcript levels of zMAPK5 and cold relative genes in wild type and transgenic lines under cold stress.

Figure 9. (a) MDA content (b) POD activity, (c) the ratio of POD activity, (d) DAR staining, (e) NRT staining in wild type and transgenic lines under cold stress.