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Overexpression of *OsmiR156k* leads to reduced tolerance to cold stress in rice (*Oryza Sativa*)

Na Cui · Xiaoli Sun · Mingzhe Sun · Bowei Jia · Huizi Duanmu · Dekang Lv · Xu Duan · Yanming Zhu

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Abstract The microRNA156 (miR156) family 'has' been well demonstrated to regulate plant growth and development. However, no reports focused on the roles of miR156s in environmental stress responses. In previous studies, we identified 18 cold stress responsive microRNAs in rice by using microarray analysis. Here, in the present study, we focused on the biological function of one of these cold responsive microRNAs, *OsmiR156k*. We generated the transgenic rice overexpressing *OsmiR156k* under the control of CaMV35S promoter, and verified the presence of *OsmiR156k* by using Southern blot analysis. We found that overexpression of *OsmiR156k* inhibited the seedling growth at the very early seedling stage under cold stress. Furthermore, *OsmiR156k* overexpression decreased plant cold tolerance at the young seedling growth stage, as evidenced by lower survival rates, chlorophyll contents and proline contents. As expected, we also suggested the down-regulated expression of the cold stress responsive genes, *01g22249* and *OsP5CS,* and *OsmiR156k*-tagated SPL genes, SPL3, SPL14 and SPL17, in the *OsmiR156k* transgenic lines. Taken together, our findings suggest that overexpression of *OsmiR156k* decreased the tolerance to cold stress in rice.

Keywords *OsmiR156k* · Rice · Cold tolerance · *SPLs*

Introduction

Rice (*Oryza sativa* L.), as an important worldwide gramineous economical crop, is grown over a wide range of climatic conditions. However, rice yield is severely restricted by adverse environmental stresses, especially cold stress (Li et al. 2007; Shi et al. 2012). During the early growth stages, cold stress severely inhibits rice seedling establishment and growth (Iba 2002; Tian et al. 2011; Wang et al. 2013a). Reproductive development of rice, from meiosis to seed set, is also highly vulnerable to cold stress (Xu et al. 2008; Zhang et al. 2011; Shi et al. 2012). It has been reported that plant height, panicle length, spike neck length, full grain, seed setting rate, anther length and anther volume were all negatively influenced by cold stress (Suh et al. 2010; Waterer et al. 2010; Zhou et al. 2012). Hence, it is of fundamental importance to explore cold-tolerant rice through rational breeding and/or genetic engineering strategies.

Na Cui and Xiaoli Sun have contributed equally to this work.

N. Cui · X. Sun · M. Sun · B. Jia · H. Duanmu · D. Lv · X. Duan · Y. Zhu (✉)
Key Laboratory of Agricultural Biological Functional Genes, Northeast Agricultural University, Harbin 150030, People's Republic of China
e-mail: ymzhu@neau.edu.cn

X. Sun
Agronomy College, Heilongjiang Bayi Agricultural University, Daqing 163319, People's Republic of China

MicroRNAs (miRNAs) are small single-stranded non-coding RNAs of just 21–30 nt in length, which regulate biological processes at the post-transcriptional level through mediating translational inhibition and/or cleavage of target transcripts (Bartel 2004; Kim 2005; Jones-Rhoades et al. 2006; Kim and Nam 2006; Ganie and Mondal 2015). Several studies have reported the involvement of miRNAs and their target genes in plant responses to cold stress (Jones-Rhoades and Bartel 2004; Phillips et al. 2007; Eldem et al. 2013). For example, bioinformatics analysis revealed that 36 conserved and 5 novel miRNAs in *Tnifoliate orange* (Zhang et al. 2014b) and 15 miRNAs in rice inflorescences were regulated by cold stress (Barrera-Figueroa et al. 2012). Under cold stress, variations in miRNAs expression (either up- or down-regulation) modify the transcript abundance of their target genes (Jeong and Green 2013; Zhang et al. 2014b; Nigam et al. 2015). Although expression analysis has suggested the involvement of miRNAs in cold stress responses, their precise biological function and molecular basis under cold stress have not been completely elucidated.

The highly conserved microRNA156 (miR156) family has been well suggested to play important roles in the regulation of plant growth and development by regulating their targeted SPL genes (Jones-Rhoades et al. 2006; Kim and Nam 2006). For example, in potato, miR156 is identified as a phloem-mobile signal regulating tuber development (Bhogale et al. 2014). Recent researches demonstrated that two *SPL* genes from *Gossypium hirsutum* were involved in the regulation of the development of leaves and second shoots, and also played an integral role in promoting flowering (Zhang et al. 2015).

In rice, a total of twelve miR156 precursors (*OsmiR156a-l*) were discovered, and among the 19 SPL family genes, 11 were suggested to be targets of *miR156*. Among the 12 Os*miR156* precursors, 10 members (*OsmiR156a-j*) produce the same mature miRNA sequence, indicating that a complex regulation network exists between *OsSPL* and *OsmiR156* family. For now, miR156s-SPLs were well demonstrated to regulate plant development and growth. For example, overexpression of *OsmiR156b* and *OsmiR156h* lead to significant dwarfism, maturity postponed, and small panicles by regulating the *SPL* genes (Xie et al. 2006). Furthermore, *miR156* was found to be involved in juvenile stage development in rice (Wu et al. 2009), and similar roles of *miR156* were also reported in Arabidopsis (Jung et al. 2011) and maize (Chuck et al. 2007). In addition, *OsmiR156e* overexpression in rice produced a bushy mutant, and appropriate down-regulation of *OsmiR156* expression resulted in the improvement of plant architecture(Chen et al. 2015). Therefore, miR156s and its target *SPLs* played multiple roles in regulating plant development. To date, although functions of *miR156* have been studied deeply, little is known about the role of *miR156s* in response to unfavorable environmental stimuli. Only a few researches reported the roles of *miR156s* in plant stress responses (Wang et al. 2013b). For example, in cotton (*Gossypium hirsutum*), *miR156* and *SPL2* showed significant regulation relationship under salinity stress (Wang et al. 2013b). However, until now, there is still no research illustrating the involvement and biological function of *miR156s* in cold stress responses.

In previous studies, we identified a total of 18 cold stress responsive miRNAs in rice by using the microarray data, containing 12 significantly down-regulated miRNAs corresponding to 10 families and 6 up-regulated miRNAs from 5 families (Lv et al. 2010). We then selected eight of them for further studies, including miR156k, miR319a/b (Wang et al. 2014), miR1868, miR1320, miR1850, and miR535. In this study, we focused on the biological function of OsmiR156k in response to cold stress. By using the overexpression transgenic rice lines, we gave the genetic evidence for the biological function of *OsmiR156k* in cold stress responses. We demonstrated that *OsmiR156k* overexpression decreased plant cold tolerance at both the very early seedling stage and the young seedling stage, and altered the expression of some cold responsive genes and its target SPL genes.

Materials and methods

Plant material, growth conditions and stress treatments

Seeds of rice (*Oryza Sativa* Kongyu 131) were surface-sterilized in 5 % sodium hypochlorite (NaClO) for 30–40 min, washed in distilled water for five times, placed on water-saturated Whatman filter paper, and kept at room temperature for 2 days and at 37 °C for another day to promote germination.

The seedlings were grown in Yoshida culture solution in a greenhouse under controlled conditions (28 °C/22 °C, 16 h light/8 h dark, and 80 % relative humidity)(Counce et al. 2000). To analyze the expression of cold responsive marker genes and *OsmiR156k* target SPL genes, the there-leaf seedlings were exposed to cold stress by being placed in a 4 °C freezer. Equal amounts of leaves were harvested at certain time points. After frozen in liquid nitrogen, the samples were stored at −80 °C for RNA extraction.

Transformation of *OsmiR156k* in rice

In order to overexpress *OsmiR156k* in rice, the *Pre-OsmiR156k* sequence were inserted into the pCAMBIA330035Su vector by USER^{TM} cloning technique (Nour-Eldin et al. 2006), under the control of CaMV35S promoter. Rice transformation was performed by the *Agrobacterium tumefaciens*-mediated co-cultivation approach as described previously (Upadhyaya et al. 2000). Transformed calli were selected on glufosinate-containing media, to produce *OsmiR156k* overexpression (OX) transgenic lines. The positive transformants were transplanted into Yoshida solution and grown in the greenhouse. The T_1 seeds were harvested from individual plants and sown again to generate T_2 plants. The T_2 lines, which showed no segregation for glufosinate resistance, were considered to be homozygote and carried forward to T_3 generation.

Presence of the *OsmiR156k* gene in transgenic rice was confirmed by PCR using the CaMV35S promoter specific forward primer and the *Bar* gene specific reverse primer. The PCR-positive seedlings were further confirmed by southern blot analysis as described previously (Sun et al. 2014).

Phenotypic analysis of transgenic rice under cold stress

The T_3 generation of homozygous transgenic lines was used for all phenotypic validation. To determine the cold tolerance at the early seedling growth stage, WT and OX rice seedlings with 5-mm buds were exposed to 4 °C (by placing the whole seedlings in a 4 °C freezer) for 7 days, and then recovered under controlled conditions for 4 days. On the 5th day, pictures were taken to show the growth performance of each line, and the root length and shoot height were measured and recorded. Thirty seeds from each line were used for each experiment and the experiments were repeated for three times.

For the cold tolerance test at the young seedling stage, 2-week-old WT and OX seedlings were exposed to 4 °C for 2 days, and then allowed for growth under normal conditions. The numbers of rolling leaves (Yang et al. 2012; Zhang et al. 2014a) and the content of free proline (Bates et al. 1973; Xiang et al. 2013), the chlorophyll content (Arnon 1949; Amin et al. 2012; Sun et al. 2013) and superoxide dismutase (SOD) activity (Asada et al. 1973; Lin and Lai 2013) were measured after cold treatment (before recovery). After recovery for 7 days, the survival rate was recorded. Pictures were taken before stress treatment, after cold treatment for 2 days, and after recovery for 7 and 14 days, respectively.

Quantitative real-time PCR assay

Total RNA was extracted by using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), and then subjected to reverse transcription with oligo d(T)18 reverse primer according to the manufacturer's instruction of Superscript III Kit (Invitrogen, Carlsbad, CA, USA). Prior to the qRT-PCR assays, the cDNA quality was assessed by PCR using *OsEf1-α* specific primers to exclude genomic DNA contamination. Quantitative real-time PCR was carried out on an ABI 7500 sequence detection system (Applied Biosystems, Carlsbad, CA, USA) by using the SYBR Premix Ex-Taq^{TM} II Mix (Takara Shiga, Japan). One microliter of each synthesized cDNA was used as template. Amplification of *OsEf1-α* was used as internal references. Relative intensities were calculated and normalized as described previously (Willems et al. 2008). To enable statistical analysis, three fully independent biological replicates were obtained and subjected to real-time PCR in triplicate. Primers for quantitative real-time PCR were listed in Table 1.

Statistical analyses

All numerical data were subjected to statistical analyses using EXCEL 2007 and analyzed by Student's *T* test. All experiments were repeated at least three times, and results from one representative experiment are shown.

Table 1 Primers of genes used for quantitative real-time PCR validation

Gene	Prime sequence
SPL3	Forward: 5′-CTTAAAGTCATCGTTGCGGGTCT
	Reverse: 5′-GGTTGTGGCTTCCGTCTGC
SPL14	Forward: 5′-AAACCCCTTTGGCATCACG
	Reverse: 5′-CCTTACGCTGCTTGGAACCCT
SPL17	Forward: 5′-GGCTTGCTGCATCCTTTCAT
	Reverse: 5′-CCCCTTGCCACTGGATTGAA
OsP5CS	Forward: 5′-CTCAAATCAAGGCGTCAACTAAGA-3′
	Reverse: 5′-TTTGTCAATATATACGTGGCATATACCA-3′
Os01g22249	Forward: 5′-AACGGAGTGGAAGCAGCGT-3′
	Reverse: 5′-CAGCACCTCTATGTTGCCCA-3′

Results

Generation of *OsmiR156k* transgenic *Oryza sativa* plants

In previous studies, we identified a total of 18 cold stress responsive miRNAs by using microarray data (Lv et al. 2010). Among them, miR156k was down-regulated under cold stress, which was further confirmed by quantitative real-time PCR analysis (Lv et al. 2010). In the current study, we focused on the biological function of *OsmiR156k* in response to cold stress. To this end, we constructed the *OsmiR156k* overexpression vector (Fig. 1a), and generated the transgenic *Oryza sativa* plants. Three transgenic lines (line #1, #2, #3) were verified by southern blot analysis (Fig. 1b), and two of them were single-copy T-DNA insertion, with a clear single band in southern blot analysis. The homozygous T_3 generation of line 1 and 3 were used in the following experiments.

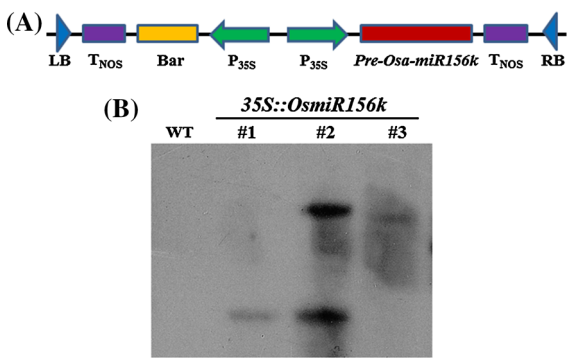

Fig. 1 Characterization of the *OsmiR156k* transgenic *Oryza sativa* plants. **a** Schematic representation of *OsmiR156k* OX construct used for *A. tumefaciens*-mediated transformation. **b** Southern blot analysis showing the presence of *OsmiR156k* in the transgenic lines (#1, #2 and #3). WT plants were used as negative controls

Overexpression of *OsmiR156k* resulted in reduced plant tolerance to cold stress

To test whether *OsmiR156k* overexpression affects plant tolerance to cold stress, we compared the growth performance of WT and OX seedlings. It was reported that miR156s influenced plant growth in juvenile-to-adult phase, for example the tiller number and panicle morphology. So in this study, we used the rice seedlings at the very early development stage (seedlings with 5 mm buds) and at the young seedling stage (2-week-old seedlings), which the seedlings did not start tilling. At the very early development stage, OX plants grew much more slowly (or even died), and appeared significantly shorter shoots (Fig. 2b) and roots (Fig. 2c) than WT after cold treatment for 7 days. These results demonstrated that overexpression of *OsmiR156k* inhibited seedling growth under cold stress at the early development stage.

Consistently, *OsmiR156k* overexpression also reduced plant cold tolerance at the young seedling stage (Fig. 3a). Following 4 °C incubation for 48 h, OX plants became severely wilted, whereas WT appeared relatively healthy (Fig. 3a, b). Previous studies reported that rice seedlings exhibited leaf rolling under cold stress treatment (Yang et al. 2012; Zhang et al. 2014a). As shown in Fig. 3c, OX seedlings exhibited more rolling leaves than WT under cold stress (Fig. 3c). After recovery for 7 days, the growth of most OX plants was severely ceased, and some OX seedlings even dead (Fig. 3a). After recovery for 14 days, 83 % WT seedlings survived, while

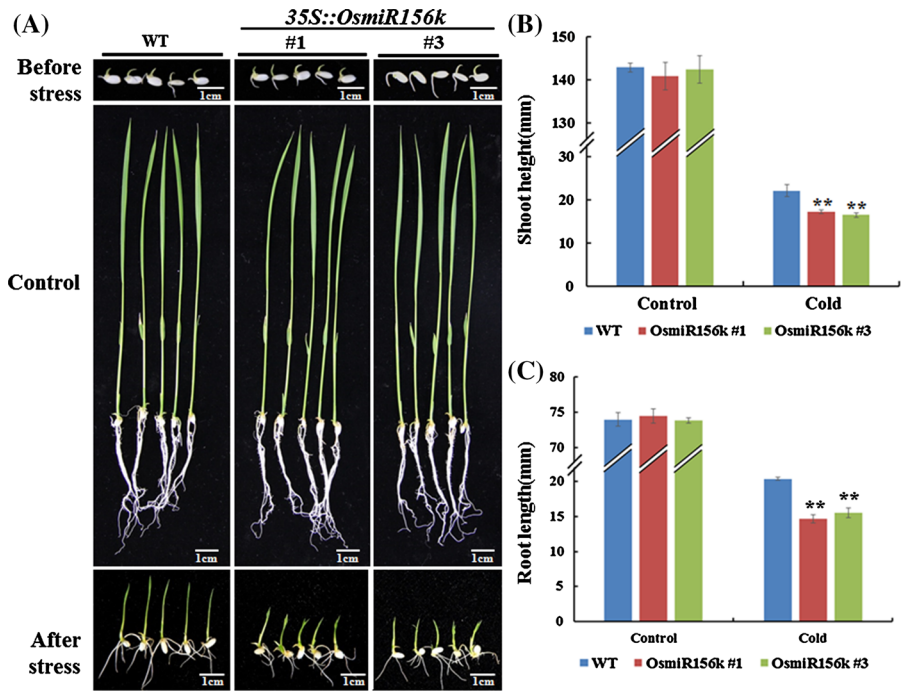

Fig. 2 Effect of cold stress on the growth of WT and *OsmiR156k* OX lines at the very early seedling stage. **a** Phenotypes of WT and OX transgenic plants in response to cold stress. For the cold stress tolerance test at the very early seedling stage, hydroponic-cultured seedlings with 5 mm bud burst were treated at 4 °C for 7 days, and then recovered under controlled conditions for 4 days. On the 5th day, pictures were taken to show the growth performance of each line. Seedlings of the WT and transgenic plants grown for 7 days at control conditions were used for control. **b** Root length of seedlings under normal and cold stress. **c** Shoot height of seedlings under normal and cold stress. Data are means (±SE) of three biological replicates. $^{**}P < 0.01$ by Student's *t* test

the survival rates of transgenic lines were only 40 % for line 1 and 30 % for line 3 (Fig. 3d). We also observed that after cold stress treatment, WT plants showed significantly higher levels of total chlorophyll content than the *OsmiR156k* OX lines (Fig. 3e), and the increased chlorophyll content might be helpful for photosynthesis and growth of WT seedlings under cold stress. These results confirmed that the cold tolerance of WT seedlings was greater than that of OX lines under cold stress.

Overproduction of free proline to facilitate osmoregulation is a common adaptive mechanism for plant responses to cold stress. To test whether the reduced cold tolerance of *OsmiR156k* OX plants is related to the capacity of proline accumulation, the free proline contents of WT and OX plants were investigated. There was no significant difference in proline contents between WT and OX plants under control conditions (Fig. 3f). An obvious increase of proline content was observed upon exposure to cold stress in both WT and OX plants. However, the increase degree in WT plants was significantly higher than that in OX lines (Fig. 3f). These results indicated that *OsmiR156k* negatively regulated plant cold tolerance, perhaps through decreasing the accumulation of free proline under cold treatment.

It has been well suggested that cold stress enhances the production of reactive oxygen species (ROS), which results in secondary oxidative damage and exaggerates the negative effects of cold stress on plant metabolism (Blokhina et al. 2003; Gill and Tuteja 2010). Superoxide dismutase (SOD) plays a central role in the ROS scavenging and plant defense against oxidative stress. In this study, we showed that under normal growth conditions, WT and OX plants displayed similar levels of SOD activity. As expected, after cold stress, the SOD activity of both OX and WT plants increased greatly; however, the SOD activity of OX lines was significantly lower than that of WT plants (Fig. 3g). These results implied that the decreased cold tolerance of *OsmiR156k* OX lines was partially due to enhanced

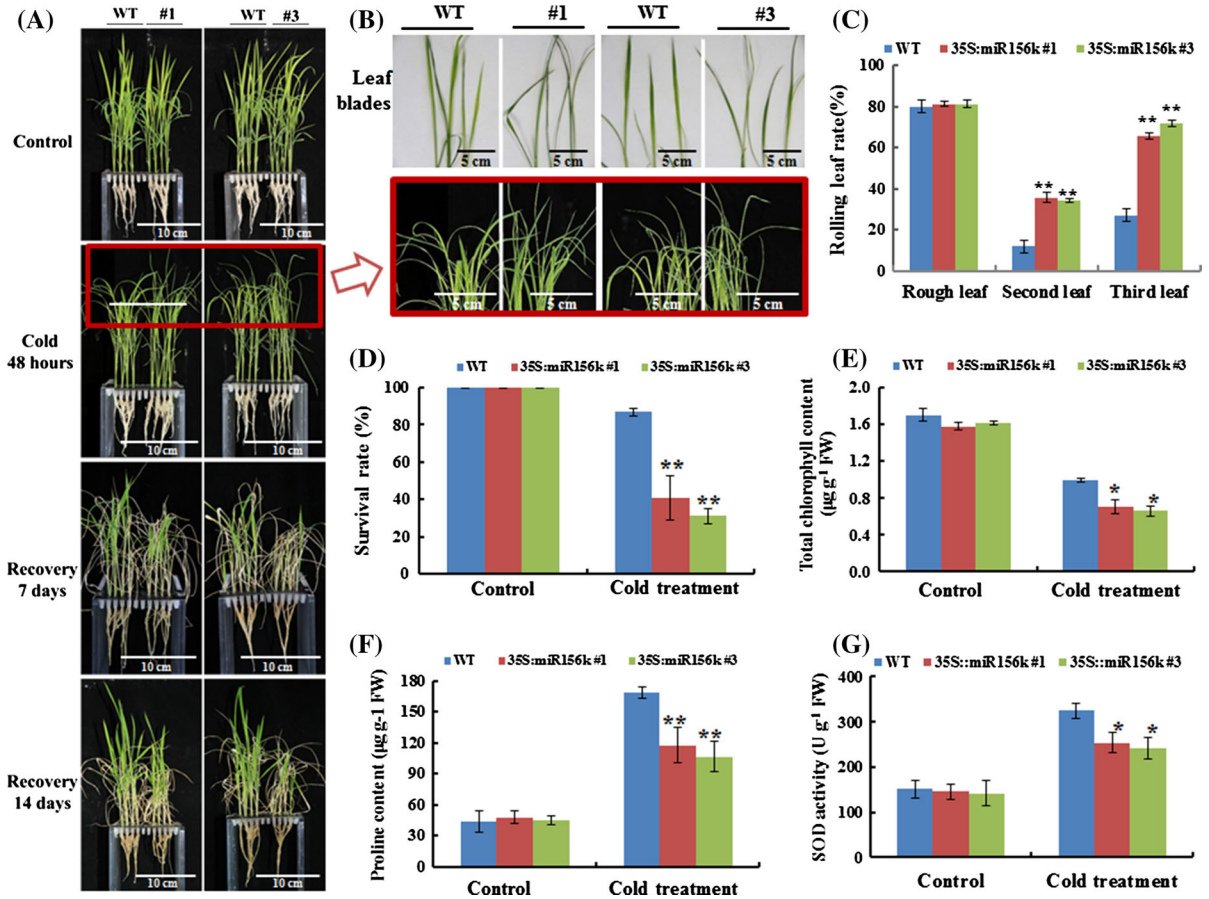

Fig. 3 Reduced cold tolerance of *OsmiR156k* transgenic plants to cold stress at young seedling stage. **a** Phenotypes of hydroponic-cultured WT and OX plants in response to cold stress. For the cold tolerance test at the young seedling stage, 2-week-old hydroponic-cultured seedlings were submerged in standard Yoshida solution at 4 °C for 2 days, and then recovered for 2 weeks. Twenty-four plants of each *line* were grown in one plate, and three plates were used. Photos were taken on the 2nd, 7th and 14th day after initial cold treatment. **b** Leaf blade after cold treatment for 2 days. **c** Leaf blade rates after cold treatment for 2 days. **d** The survival rates of WT and transgenic lines after cold stress. **e** The free proline content of WT and transgenic lines after cold stress. **f** The total chlorophyll content of WT and transgenic lines after cold stress. **g** The SOD activities of WT and transgenic lines after cold stress. Data are means (±SE) of three biological replicates. $*P < 0.05$, $**P < 0.01$ by Student's *t* test

oxidative damage caused by the reduced ROS-scavenging activity.

Expression levels of cold responsive genes and *OsmiR156k* target SPL genes were down regulated in transgenic lines

Above results indicated a potential role of OsmiR156k in proline accumulation and ROS scavenging. To gain more insight into the role of *OsmiR156k* in response to cold stress, we further examined the expression patterns of cold signaling-related genes in both WT and OX plants, including Os01g22249, a ROS scavenging gene (Chen et al. 2009) and OsP5CS, a proline synthase gene (Maruyama et al. 2014). Our real-time PCR data showed that in both WT and OX plants, *Os01g22249* and *OsP5CS* were up-regulated under cold stress and displayed similar expression profile expression, with the highest expression level at 9 h after treatment. However, the increased folds (9 h after treatment) in OX lines were smaller than that in WT, and at the earlier time points (1, 3 and 6 h), no obvious difference was observed between OX and WT (Fig. 4a, b).

We also investigated the expression patterns of *OsmiR156k* targeted SPL genes predicted previously (Klein et al. 1996; Xie et al. 2006). We firstly analyzed their expression changes in response to cold stress, based on the rice mRNA microarray data carried out our lab. As shown in Fig. 4c, several SPL genes were differentially expressed in response to cold stress. To identify if the SPL genes were regulated by *OsmiR156k* under cold stress, we selected three of them (*SPL3*, *SPL14* and *SPL17*), and investigated their expression in WT and OX plants. Our real-time PCR results further confirmed that expression levels of these three SPL genes were indeed induced by cold treatment, and higher transcript levels were found in WT plants than in OX lines. In details, the expression levels differ between OX and WT at 9 h post cold treatment, and stay different until 12 h (Fig. 4d–f). Taken together, these results suggested that *OsmiR156k* overexpression altered the expression of cold stress responsive genes and targeted SPL genes.

Discussion

It has been well demonstrated that miR156 and its target SPL genes could regulate flowering time (Cardon et al. 1997), juvenile-to-adult and floral phase transitions (Cardon et al. 1997; Wu and Poethig 2006; Wang et al. 2009; Wu et al. 2009), leaf and plastocron development (Moreno et al. 1997; Willems et al. 2008), plant architecture (Jiao et al. 2010), seed germination and seedling development (Martin et al.

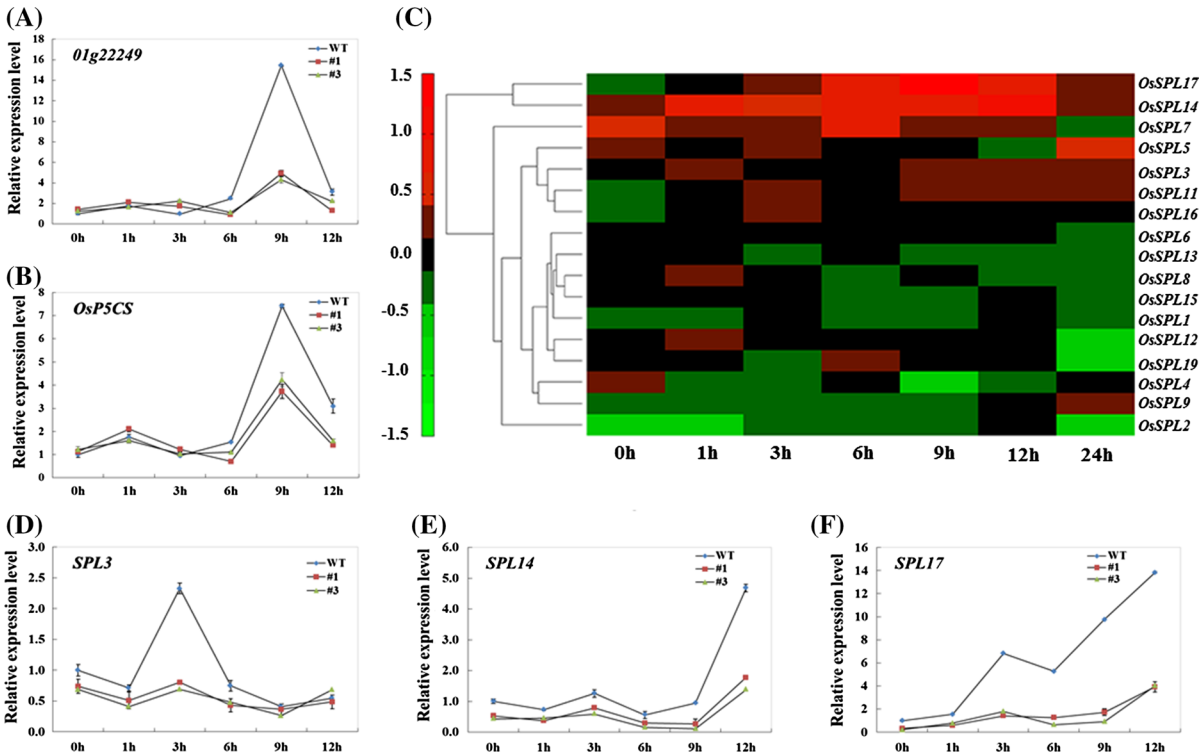

Fig. 4 *OsmiR156k* overexpression altered expression patterns of a set of cold stress responsive genes and SPLs genes targeted by *OsmiR156k*. **a** Expression patterns of *Os01g22249*, a ROS scavenging gene. **b** Expression patterns of *OsP5CS*, a proline synthase gene. **c** Seventeen genes targeted by miR156k from rice were differentially regulated in response to cold stress. Three criteria were used to determine the differential regulation of miRNAs: (1) miRNAs should be present at each time point; (2) fold changes should be less than 0.67 (down-regulated) or greater than 1.5 (up-regulated); (3) differences were significant at $P < 0.05$. The color saturation reflects the magnitude of the expression ratio for each transcript. *Red color* indicates higher transcript levels than observed in the controls, whereas green means lower transcript levels. **d–f** Expression patterns of target SPLs genes regulated by *OsmiR156k* under cold stress. Relative transcript levels were determined by quantitative real-time PCR using *OsEf1-α* as an internal control, and were normalized to WT plants at 0 h of treatment. Values represent the means of three fully independent biological replicates, and three technology replicates for each. (Color figure online)

2010), and sterility (Xing et al. 2010). Very recently, studies showed that miR156 isoforms were highly induced by heat stress (HS) and were functionally important for HS memory (Stief et al. 2014). Up to now, little is known about miR156 function in response to cold stress.

In our previous study, *OsmiR156k* was initially identified as a cold stress responsive gene from the large scale rice microarray dataset, and the cold induced expression was further verified by quantitative real-time PCR (Lv et al. 2010). In this study, we focused the biological function of *OsmiR156k* in cold stress responses, and characterized the cold tolerance of *OsmiR156k* transgenic rice during the seed germination and young seedling development stages. The period of imbibition during seed germination is most sensitive to low temperature (Thompson et al. 1977). After germination, water uptake increases and storage reserves are utilized to support seedling growth, then the seedling begins photosynthesis (Cheng et al. 2013), which process is also severely affected by low temperature (Smallwood and Bowles 2002). Therefore, cold tolerance of germination and seedling development play an important role in plant growth. By comparing the growth performance between WT and OX rice, we showed that *OsmiR156k* overexpression reduced plant cold tolerance at the very early seedling stage and the young seedling stage (Figs. 2, 3).

Accumulation of free proline could facilitate osmoregulation and protect plants from cold stress by reducing the water potential of plant cells (Liu and Zhu 1997; Armengaud et al. 2004). As expected, we also found that accumulation of free proline in *OsmiR156k* transgenic lines was significantly lower than WT plants, which may partially account for the decreased tolerance to cold stress (Fig. 3e). In addition, environmental stimuli, including cold stress, enhance the production of ROS (Blokhina et al. 2003; Skopelitis et al. 2006; Suzuki et al. 2012), which will decrease cell viability and even cause cell death (Vaidyanathan et al. 2003). SOD is an important part of the ROS-scavenging mechanisms (Mittler 2002; Blokhina et al. 2003; Vaidyanathan et al. 2003; Gill and Tuteja 2010). In this study, we found that the SOD content of OX lines was significantly lower than that of WT plants under cold stress (Fig. 3g). Notably, the down-regulated expression of *OsP5CS*, a proline synthase gene (Fig. 4a), and *Os01g22249*, a ROS scavenging gene (Fig. 4b), may help for explaining the lower levels of free proline content and SOD activity in *OsmiR156k* transgenic plants under cold stress.

SPLs genes comprise a family of transcription factors that contain the SBP domain, a highly conserved DNA-binding domain first identified in a protein that binds to the promoter region of the *SQUAMOSA (SQUA)* gene in *Antirrhinum majus* (Klein et al. 1996; Cardon et al. 1997, 1999). SPLs are widely identified as miR156 targets, and *SPL3, SPL4, SPL9, SPL15* are involved in the heteroblastic regulation of abaxial trichome production (Wu and Poethig 2006; Schwarz et al. 2008). The *SPL14* knockout mutant plant exhibited a truncated juvenile vegetative phase, and expression of a mutated *SPL14*, which cannot be regulated by miR156, showed higher grain productivity (Stone et al. 2005; Jiao et al. 2010; Luo et al. 2012). *SPL8* and the miR156-targeted *SPL* genes regulate gynoecium apical-basal patterning redundantly (Xing et al. 2013). In this study, we showed that SPL expression also responded to cold stress, and *OsmiR156k* altered *SPL* gene expression under cold stress (Fig. 4). These findings indicated a potential role of OsmiR156k-SPLs in cold stress responses.

In summary, *OsmiR156k* is suggested to be a negative regulator of plant tolerance to cold stress, and our findings broaden our knowledge of the role of miRNAs in environmental challenge. Our future studies will focus on the following parts: Firstly, we will explore the application of *OsmiR156k* in facilitating molecular breeding of crop with enhanced cold tolerance, for example through RNA interference or short tandem target mimic (STTM) technology (Tang et al. 2012). Secondly, it will be interesting to identify the biological function of *SPLs* in cold stress responses. Thirdly, we will further verify whether the cold stress responses mediated by *OsmiR156k* depends on *OsmiR156k-SPLs* pathway. Lastly, studies in different plant species have shown that *miR156* expression levels changed not only in response to cold stress but also under other stresses such as heat, salt, and drought. Hence, further detailed analysis of the stress responses mediated by *miR156* is still an important research field.

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