SPL9 mediates freezing tolerance by directly regulating the expression of CBF2 in Arabidopsis thaliana

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Abstract

Background: Freezing stress inhibits plant development and causes significant damage to plants. Plants therefore have evolved a large amount of sophisticated mechanisms to counteract freezing stress by adjusting their growth and development correspondingly. Plant ontogenetic defense against drought, high salt, and heat stresses, has been extensively studied. However, whether the freezing tolerance is associated with ontogenetic development and how the freezing signals are delivered remain unclear.

Results: In this study, we found that the freezing tolerance was increased with plant age at the vegetative stage. The expressions of microRNA156 (miR156) and SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9 (SPL9), playing roles in regulation of ontogenetic development, were induced by cold stress. Overexpression of SPL9 (rSPL9) promoted the expression of C-REPEAT BINDING FACTOR 2 (CBF2) and hereafter enhanced the freezing tolerance. Genetic analysis indicated that the effect of rSPL9 on freezing tolerance is partially restored by cbf2 mutant. Further analysis confirmed that SPL9 directly binds to the promoter of CBF2 to activate the expression of CBF2, and thereafter increased the freezing tolerance.

Conclusions: Therefore, our study uncovers a new role of SPL9 in fine-tuning CBF2 expression and thus mediating freezing tolerance in plants, and implies a role of miR156-SPL pathway in balancing the vegetative development and freezing response in Arabidopsis.

Keywords: SPL9, CBF2, Freezing tolerance, Age, Arabidopsis

Key message

SPL9 directly binds to the promoter of CBF2 and activate CBF2 expression to balance the freezing tolerance and plant development.

Background

Plants frequently experience unexpected environmental changes, including drought and high or low temperatures, during their life cycle. The ability of plants to defend against these environmental changes is one of the major determinants of survival in natural populations. Plants therefore have evolved a plethora of sophisticated mechanisms to counteract the changing environment by adjusting their growth and development correspondingly; or plants select suitable regions to inhabit for reducing the freezing and other injuries [4]. It is well known that the interplay between ontogeny and abiotic stresses is of great importance in plants [6, 43]. Moreover, some stress responsive genes are highly upregulated in juvenile primordia in maize [37] for developmental priming and protecting against the damage by unexpected stresses. Evidences have shown that plants at the juvenile stage are more resistant to drought, heat stress and high salt stress.
respectively), tandemly clustered in an 8.7-kb region of freezing stress (< 0 °C), is one of the major environmental genes. Under cold condition by interacting with cold-responsive transcription factors play critical roles in cold tolerance. Some cold-repressed genes are enriched in the regulation of transcription, and response to hormone stimulus [16, 38, 41, 54]. In addition, some cold-induced genes are enriched in the responses to abiotic stresses; some cold-repressed genes are enriched in the regulation of transcription, and response to hormone stimulus [16, 38, 41, 54]. In Arabidopsis Hypocotyls and cotyledons, whereas CBF1 and CBF3 genes are mainly expressed in roots, hypocotyls and cotyledons, whereas CBF2 is expressed in hypocotyls, cotyledons and leaves under normal condition; when the plants are treated with cold, CBF1, CBF2 and CBF3 genes are all expressed in leaves, but CBF2 is also expressed in shoot stems [36]. Overexpression of CBFs leads to dwarf plants and enhanced cold tolerance [17, 23]. Since the three CBF proteins exhibit very high sequence similarity, they possibly have functional redundancy. cfb1 and cfb3 single mutants have slightly greater freezing tolerance than the wild type, whereas the mutation in CBF2 gene increases sensitivity to freezing with lower survival rate and greater ion leakage [54]. These results show that CBF2 is more important than CBF1 and CBF3 in the regulation of freezing tolerance.

Cold stress, including chilling stress (0 °C to 15 °C) and freezing stress (<0 °C), is one of the major environmental factors inhibiting plant growth and development, and even causing significant crop losses [40]. Under cold stress, the plasma membrane of plant is damaged with increased electrolyte leakage. Moreover, the number of stomata is associated with the cold-tolerance of varieties in plants: the sensitive cultivars and genotypes have the highest number of stomata [3]. Deciphering the mechanisms underlying the response to cold stress helps to accelerate the breeding of cold-tolerant varieties in plants. In recent years, many differently-expressed genes that play roles in the cold responsive network in plants have been identified by transcriptomic analysis. Of them, some cold-induced genes are enriched in the responses to abiotic stresses; some cold-repressed genes are enriched in the regulation of transcription, and response to hormone stimulus [16, 38, 41, 54]. In addition, some flowering genes, including FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1), are increased by cold to control flower development [21, 39], indicating that some developmental genes function in cold response to balance the developmental processes under cold condition by interacting with cold-responsive genes.

Some studies have indicated that the C-REPEAT BINDING FACTOR/DRE BINDING FACTOR1 (CBF/DREB1) transcription factors play critical roles in cold tolerance response in plants. CBFs are directly downstream of ICEs (INDUCER OF CBF EXPRESSION) and directly bind to the C-repeat (CRT)/dehydration-responsive element (DRE; G/ACCGAC) of the Cold-Regulated (COR) genes, known as the “CBF regulon”, to activate their expressions for further increasing the cold tolerance [5, 29, 46]. In Arabidopsis, three CBFs, including CBF1, CBF2, and CBF3 (also called DREB1b, DREB1c, and DREB1a, respectively), tandemly clustered in an 8.7-kb region of the fourth chromosome, are strongly and transiently up-regulated within 1 ~ 3 h after cold treatment, and thereafter their expressions are rapidly declined [18, 33, 34, 44, 47]. However, their expression patterns are different: CBF1 and CBF3 genes are mainly expressed in roots, hypocotyls and cotyledons, whereas CBF2 is expressed in hypocotyls, cotyledons and leaves under normal condition; when the plants are treated with cold, CBF1, CBF2 and CBF3 genes are all expressed in leaves, but CBF2 is also expressed in shoot stems [36]. Overexpression of CBFs leads to dwarf plants and enhanced cold tolerance [17, 23]. Since the three CBF proteins exhibit very high sequence similarity, they possibly have functional redundancy. cfb1 and cfb3 single mutants have slightly greater freezing tolerance than the wild type, whereas the mutation in CBF2 gene increases sensitivity to freezing with lower survival rate and greater ion leakage [54]. These results show that CBF2 is more important than CBF1 and CBF3 in the regulation of freezing tolerance.

The precise expression regulation of CBFs is of key importance for maintaining the balance between cold tolerance and plant growth. Therefore, how CBFs are regulated in response to cold tolerance and how CBFs regulate cold responsive genes expression are widely studied. A series of regulators, including INDUCER OF CBF EXPRESSION 1/2 (ICE1/2) [14, 29], MYB15 [2, 50], ZINC FINGER OF ARABIDOPSIS THALIANA 12 (ZAT12) [47], CALMODULIN-BINDING TRANSCRIPTION ACTIVATORS (CAMTA) [11], PHYTOCHROME-ASSOCIATED PROTEINS (PIFs) [26], CESTA (CES) [13], ETHYLENE-INSENSITIVE 3 (EIN3) [42] REVEILLE4/LHY-CCA1-Like 1 (RVE4/LCL1) and RVE8/LCL5 [27] that control the expression of CBF genes upon cold stress have been identified [25, 26]. ICE1 plays key roles in cold stress response by directly binding to the promoters of CBFs to control their expressions. The kinase OPEN STOMATA 1 (OST1) phosphorylates and stabilizes ICE1 to facilitate the expression of the CBF genes [9]; in addition, OST1 enhances the interaction between BASIC TRANSCRIPTION FACTOR 3 (BTF3s) and the CBFs for the stability of CBF proteins under cold stress [10]. Yet the protein kinase BRASSINOSTEROID-INSENSITIVE2 (BIN2) phosphorylates ICE1 for promoting ICE1 degradation by boosting the interaction between ICE1 and the E3 ubiquitin ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1), and thereby downregulating CBF gene expression [53]. BRASSINAZOLE-RESISTANT 1 (BZR1) and its closest homolog BRI1-EMS-SUPPRESSOR 1 (BES1), downstream of BIN2, positively regulate plant cold tolerance [32]. Further studies show that BZR1 directly binds to the promoters of CBF1 and CBF2 genes in vivo to activate their expressions in Arabidopsis [32].
CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) directly bind to the promoters of CBF1, CBF2, and CBF3, and have a direct role in their circadian regulation [12]. Moreover, CBF2 negatively regulates the expression of CBF1 and CBF3 [35]; and CBF1 and CBF3 also negatively affect the gene expression of CBF2 [54].

CBFs are the key factors in the complex cold-responsive network, and are also major players in determining the cold tolerance of plants. Moreover, the cbf triple mutants exhibit smaller rosette leaf number and size and lower fresh weight than the wild type [54], suggesting that CBFs function in the balance of plant development and cold-tolerance. To date, miR156-SPLs pathway also plays critical roles in plant development and cold response; and whether there is the interaction between CBFs and miR156-SPLs pathway in regulating cold tolerance is not reported. In this study, we provide evidence that the capacity of freezing tolerance in plants is increased with plants age; and SPL9 directly binds to the promoters of CBF2 to promote CBF2 expression for enhancing freezing tolerance. Overexpression of SPL9 (rSPL9) reduced the juvenile stage, and conferred enhanced freezing tolerance. SPL9 acts as a direct transcriptional activator to promote the expression of CBF2. Therefore, SPL9 constitutes a new hub to balance the development and cold tolerance in plants.

Results

Age-dependent freezing tolerance during vegetative development

To understand the mechanistic connection between freezing tolerance and vegetative development, we first confirmed the growth time point of vegetative developmental stages and examined the capacity of freezing tolerance in different vegetative development stages under long-day condition. The Col-0 plants produced abaxial trichomes on leaf 5.2 at about 11 days after transferring to green house, suggesting that the 11th-day was the developmental transition time (Fig. S1A, B) from juvenile phase to adult phase. Therefore, we chose the 9-day-old (as juvenile stage), 11-day-old (as transition stage) and 14-day-old (as adult stage) plants to perform the freezing-treatment experiment. After freezing-treatment, the bigger plants had more surviving ones (Fig. 1A). The statistical results showed that the survival rate of 9-day-old plants was 17.6%, which was significant lower than that of 11-day-old plants (31.6%); and the survival rate of 11-day-old plants was significant lower than that of 14-day-old plants (42.1%) (Fig. 1B). The result indicated that the capacity of freezing tolerance was increased with plant age, suggesting that the freezing-tolerance during vegetative stage was age-dependent.
Overexpression of SPL9 enhances the freezing tolerance

The conserved miR156-SPL pathway has been shown to regulate vegetative phase change and involve in cold response in plants [7, 51]. However, how miR156-SPL pathway responds to the low temperature remains unclear. We analyzed the expression of miR156-SPL pathway genes after low-temperature exposure and found that the levels of mature miR156 and its pri-mRNAs, miR156a and miR156c, were elevated under low temperature (Fig. 2). The expressions of SPL3 and SPL13, targets of miR156, were correspondingly reduced (Fig. S2), whereas the expression of another target, SPL9, was upregulated under low temperature (Fig. 2), suggesting that SPL9 might be positively involved in cold response. However, herein low temperatures induced the expression of both miR156 and SPL9, inconsistent with the normal miR156-SPL regulation. We detected the expression of SPL9 in Col-0, 156OE, MIM156 and rSPL9 plants under normal and cold conditions. The results showed that low temperature induced the expression of SPL9 in Col-0, 156OE, and MIM156 plants, whereas the level of SPL9 was lower in 156OE plants and higher in MIM156 plants than in Col-0, indicating that SPL9 was regulated by both miR156 and low temperature (Fig. S3). Interestingly, SPL9 expression was also elevated in rSPL9 plants, as miR156-insensitive SPL9-overexpressing transgenic plants, under low temperature condition (Fig. S3), showing that SPL9 might be partially regulated by low temperature via miR156-independent pathway.

To further examine the role of miR156-SPL9 pathway in freezing tolerance, we performed the freezing tolerance assay. The results showed that the 35S:miR156a plants and the spl9–4 mutant displayed reduced freezing tolerance based on the growth status, the survival rate and ion leakage as compared with Col-0 plants, whereas the UBI:MIM156 plants and the rSPL9 plants exhibited higher cold tolerance judged by their high growth status, the high survival rate and lower ion leakage than Col-0 plants (Fig. 3), as expected. This result indicated that overexpression of SPL9 enhanced the plant freezing tolerance.

SPL9 positively regulates the expression of CBF2

CBF2 is the master factor in regulation of cold tolerance [54]. Overexpression of CBF2 [54] and Overexpression of SPL9 both enhanced the freezing tolerance in Arabidopsis (Fig. 3). However, whether SPL9 relays the downstream signal CBF2 to mediate freezing tolerance is unknown. We first determined the expression of CBF2 in rSPL9 plants and Col-0, and the expression of SPL9 in cbf2 under normal condition using RT-qPCR. The expression of CBF2 was induced more than 6-fold in rSPL9 plants (Fig. 4A), but there was no significant change in the expression of SPL9 in cbf2 mutant (Fig. S4), indicating that SPL9 functions upstream of CBF2 to activate CBF2 expression. Under low temperature condition, CBF2 expression was induced in Col-0, spl9–4 and rSPL9 plants, whereas the expression of CBF2 in rSPL9 plants was relative lower than that in Col-0 (Fig. S5). Next, we asked if the expression of affected genes acting downstream of cold signaling in rSPL9 plants was also CBF2 dependent. We examined the expression of CBF2-regulated genes in Col-0 and rSPL9 plants. The expression of KINASE 1 (KIN1), RESPONSIVE TO DESICCATION 29A (RD29A), and COR47 was all significantly up-regulated in rSPL9 plants compared with Col-0 under normal conditions.
condition; while the expression of KIN1 was significantly up-regulated in rSPL9 plants compared with Col-0 under cold condition and the expression of RD29A and COR47 was down-regulated in rSPL9 plants compared with Col-0 under cold condition (Fig. S6). These findings do support the concept of developmental priming, which aims to protect developing rSPL9 seedlings against cold stress by elevating expression of CBF2.

To investigate the genetic interaction between SPL9 and CBF2 in regulating freezing response, we crossed cbf2 with rSPL9 to generate the rSPL9 cbf2 plants. The freezing-tolerance assay showed that cbf2 plants were more sensitive to cold with lower survival rate and higher ion leakage than Col-0; rSPL9 cbf2 plants exhibited lower survival rate and higher ion leakage than rSPL9, and exhibited greater survival rate and lower ion leakage than Col-0 and cbf2 (Fig. 4B, C, D), indicating SPL9 partially contributes to CBF2-mediated freezing tolerance in plants. Moreover, we introduced the rSPL9 to pCBF2:GUS plants to generate pCBF2:GUS rSPL9 plants. The GUS activity of pCBF2:GUS plant was high in the shoot meristem, but significantly lower than that of pCBF2:GUS rSPL9 plants (Fig. S7), demonstrating that SPL9 might enhance the activity of CBF2 promoter. These results suggested that SPL9 positively regulated the expression of CBF2 against freezing stress.

SPL9 promotes the transcription of CBF2 by directly binding to CBF2 promoter
To further investigate if CBF2 is a direct transcriptional target of SPL9, we used an inducible glucocorticoid receptor (GR) expression system to test this. The result showed that the expression of miR172B, a positive control, was elevated about 1.8-fold in DEX-treated
pSPL9:GR-rSPL9 samples compared with mock; and the expression of CBF2 was induced about 2.4-fold in DEX-treated pSPL9:GR-rSPL9 samples compared with mock, this result demonstrated that CBF2 is a direct transcriptional target of SPL9 (Fig. 5A). In addition, CBF1 and CBF3 are important to cold response in plants. Therefore, we determined whether SPL9 directly regulated the expression of CBF1 and CBF3 by GR system, whereas no significant changes of CBF1 and CBF3 expressions were found in mock and DEX-treated pSPL9:GR-rSPL9 samples (Fig. S8), indicating that SPL9 did not directly regulate the expression of CBF1 and CBF3 in Arabidopsis.

It has been reported that SPL9 binds to the motif “GTAC” of target genes to regulate their expressions. Consequently, we detected two sites with “GTAC” in the CBF2 promoter, and designed two pairs of primers surrounding those sites for ChIP-qPCR assay. The ChIP-qPCR analysis demonstrated that the DNA at the “Site1” and “Site2” was significantly enriched about 3.0-fold and 3.8-fold, respectively, compared with the negative control ACTIN2 (ACT2) (Fig. 5B), implying that SPL9 regulates
**Discussion**

Ontogenetic resistance against biotic and abiotic stresses has been extensively studied in plants [6, 15, 30, 43]. Abiotic stress tolerances, including salt, drought and heat stress tolerances are decreased with plant age, while we show here that the cold tolerance is increased with plant age. Cold stress has a profound impact on plant ontogenetic development, leading to growth repression and reduced yields. Overexpression of miR156 or loss-of-function of SPL9 delays the ontogenetic development, induces the drought, heat and salt tolerances, and decreases the cold tolerance in *Arabidopsis* and rice [6, 7, 43]. To conclude, it is convinced that miR156 served opposite roles in respond to cold stress and salt/drought/heat stresses. And it is still a mystery what mechanism drives the differential responses of miR156-SPL pathway. In this study, we found a molecular link between SPL9 and CBF2, the master transcription regulators in age- and cold-signal pathway, respectively. SPL9 directly binds to CBF2 promoter to activate CBF2 expression as developmental priming for the protection of shoot development under unexpected cold stress.
To date, the CBF-dependent cold signaling pathway has been studied widely. CBF1/2/3, APETALA2/ETHYLENE-RESPONSIVE (AP2/ERF1) family transcription factors, directly bind to the conserved CRT/DRE motifs of COR genes to activate their expression for cold response [18, 33, 34, 44, 47]. Overexpressing CBFs increases the expression of COR genes and enhances freezing tolerance in plants [46]. Consequently, some factors, partially or negatively regulating the CBFs expression, act critical roles in the cold response. ICE1 directly binds to CBF2 promoters to activate the expression of CBF genes for increasing freezing tolerance [29]. Mitogen-activated protein kinase 3 (MPK3) and MPK6 interact with and phosphorylate ICE1, hereafter reduces its stability and transcriptional activity, and finally represses the CBFs and CORs transcripts [31] and freezing tolerance. AtMYB15 and OsMYBS3 directly repress the expression of CBFs and negatively regulate chilling tolerance in Arabidopsis and rice, respectively [2, 8]. In this study, we found another transcription factor SPL9 directly bound to the “GTAC” motif of CBF2 promoter to activate CBF2 expression and thus enhanced the freezing tolerance against unexpected freezing stress.

It is of exceeding importance that the timing of the developmental stage transition determines the success of reproduction. SPL9 acts in regulation of vegetative phase change and floral transition by promoting the expression of miR172B and SOC1, FRUITFULL (FUL) genes [48, 51]. Low temperature inhibits the plant development leading to late flowering by elevating the expression of miR156 and some flowering-related genes [28]. Herein, we assured that SPL9 was a key factor in regulation of ontogenetic development and freezing response. Loss-of-function of SPL9 accelerates the leaf emergence, while rSPL9 plants reduce the initial rate of leaves [51]. The plants with the changes of CBFs levels have the similar phenomenon in the ontogenetic development as SPL9-related plants. The CBF-overexpressing plants display growth retardation and reduced plant biomass [1, 17, 23], whereas cbf triple mutants are larger than control under chilling stress [24]. Moreover, SPL9 and CBF2 are highly co-expressed in the shoot meristem. Therefore, SPL9 and CBF2 are considered as the important regulators for balancing plant growth and cold responses. The high levels of CBF2 and CBF2 targets, KIN1, RD29A and COR47, in rSPL9 plants under normal condition trigger the cold-defense priming. When the freezing stress occurs, the rSPL9 plants can reduce stress response for less deleterious effect, while the wild type plants dramatically elevated the CBF2 level to against cold injury by stopping development. Previous studies have shown that CBF2 is mainly involved in the regulation of cold acclimation [29, 36, 40, 54]. Herein, we found that the freezing-tolerance capability of rSPL9 was no significant difference compared with Col-0 (Fig. S10) after cold acclimation, even though CBF2 was activated by SPL9 in Arabidopsis. It’s possible that pre-cold treatment resulted in higher CBF2 expression of Col-0 and rSPL9 plants (Fig. S5), as well as reduced the difference of freezing tolerance between Col-0 and rSPL9 plants. These results support our conclusion that the high expression of CBF2 in the rSPL9 plants under normal condition is critical for triggering cold defense priming against unexpected freezing stress.

As important direct target of SPL9, the expression of CBF2 was significantly increased in rSPL9 plants. However, the genetic results showed that the cbf2 mutation only slightly affected the freezing phenotype of the rSPL9 plants, suggesting that SPL9 may also regulate other components to promote freezing tolerance. Recent study indicates that BZR1, positive regulator in freezing tolerance [32], physically interacts with SPL9 to regulate the vegetative phase change in Arabidopsis [49]. And BZR1 positively modulates plant freezing tolerance through CBF-dependent and CBF-independent pathways [32]. Herein, we found that SPL9 could regulate CBF target genes, KIN1, RD29A and COR47, and CBF-independent COR genes, WRKY6, SENESENCE-ASSOCIATED GENE 21 (SAG21) and SOC1 (Fig. S6), to respond to freezing stress. SOC1, as a direct target of SPL9 and BZR1 [32, 48], functions negatively in regulating plant responses to cold stress and positively in regulation of flowering [39]. WRKY6 directly regulated by BZR1 positively modulates freezing tolerance [32]. Thus, SPL9 might serve as a mediator of the crosstalk in cold and developmental signaling pathways. Our results indicate that SPL9, similar with BZR1, positively controls plant freezing tolerance via CBF-dependent and CBF-independent pathways.

In this study, we detected that miR156 was increased under low temperature treatment in Arabidopsis, the same as miR535 [45] and difference with OsmiR156k in rice [7]. Correspondingly, the expression of SPL3 and SPL13, target of miR156, were downregulated in Arabidopsis, whereas the transcript of SPL9 was elevated, indicating that SPL9 was mainly regulated by cold, and partially regulated by miR156. The expression of SPL9 in rSPL9 plants was induced by low temperature, suggesting that the expression of SPL9 may be independent miR156 under cold condition. This fine-tuned regulation by miR156-SPL indicates its function in balancing plant growth and cold tolerance. Previous studies have demonstrated the roles of miR156 superfamily including miR156, miR529, and miR535 in repressing the cold tolerance in rice. In rice, four SPL genes including OsSPL2, OsSPL14, OsSPL17 and OsSPL18 are co-targeted by OsmiR156/miR529/miR535; and six SPL genes,
including OsSPL4, OsSPL7, OsSPL11, OsSPL12, OsSPL16 and OsSPL19 are co-targeted by OsmiR156/miR535 [45]. Overexpression of OsmiR156k, which is down-regulated under cold stress, reduces tolerance to cold stress in rice by suppressing the expression of Os01g22249, OsP5CS (Oryza sativa DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE), OsSPL3, OsSPL14 and OsSPL17 [7]. However, the expression of OsmiR535 is rapidly induced by cold stress in rice. The OsmiR535-overpressing rice plants display significantly lower reactive oxygen species (ROS) scavenging enzyme activity and accumulate much more malondialdehyde (MDA) under cold stress than the wild type. Overexpression of OsmiR535 inhibits the expression of OsCBF1, OsCBF2, and OsCBF3 under normal and cold conditions and its targets, including OsSPL14, OsSPL11 and OsSPL4 [45]. But there is no evidence to certificate if SPL could directly bind to CBFs and regulate CBFs expression under cold stress in rice. These results indicate that Arabidopsis has different strategies to balance the cold stress and development compared with rice. In Arabidopsis, miR156 and SPL9 are both induced by cold; miR156 functions in reducing the growth rate by repressing SPL3 and SPL13, whereas its another target, SPL9, acts as a commander to direct the expression of CBF2 against the cold damage by triggering defense priming. Most importantly, SPL9 functions in regulation of flowering [48]. If only the expression of SPL9 is induced under cold condition, the plants will bloom earlier resulting in damage of floral organ caused by cold; while with the high level of miR156 under cold condition, the developmental transition of plants will be restrained to reduce the injury by low temperature. That’s a fine regulatory mechanism in balancing development and freezing stress in Arabidopsis. However, the rice miR156 and miR535 have adverse expression pattern under cold stress; the balance between miR156 and miR535 is critical for the interaction between cold and development. That’s to say different plants generate different mechanisms to respond to the freezing stress and maintain their lives by adjusting their growth conditions under harsh environments.

**Conclusions**

Based on our findings, we propose a model for the role of SPL9 in regulation of freezing tolerance during the vegetative stage (Fig. 6). According to this model, age and low

![Fig. 6 Model for SPL9-CBF2 complex in regulating freezing tolerance in Arabidopsis](image-url)
temperature induce the transcript of SPL9; the elevated
SPL9 promotes the binding of CBF2 promoter to facili-
ties CBF2 expression, and thus increases higher capacity
to tolerate freezing. miR156, as a master age-regulator, is
induced by cold, and then inhibits the expression of SPL3
and SPL13 to control the plant developmental transition.
Moreover, the capacity of freezing tolerance is increased
with plant age. Therefore, miR156-SPL pathway functions
as a balance sponge to maintain the plant development
and freezing tolerance. So far, crop improvement for
cold tolerance through conventional breeding approach
showed little success due to the complexity of stress tol-
erance traits. This work promotes this process, decipher
the mechanisms underlying the response to cold stress
and is of significant importance for boosting the breeding
of cold-tolerant plant varieties.

Materials and methods
Plant material and growth conditions
The plant materials, including 35S:miR156a (156OE,
miR156 overexpressing line), UBI:MIM156 (MIM156,
mir156 repression line by target mimic), spl9–4
(cs807258, null allele of SPL9), pEG302a-rSPL9 (rSPL9,
SPL9-promoter-driven miR156-insensitive SPL9-over-
expressing line), pSPL9:GR-rSPL9 (miR156-insensitive
SPL9 protein fused with Glucocorticoid Receptor (GR)
gene), and pSPL9:3 × FLAG-rSPL9 (miR156-insensitive
SPL9 protein tagged with 3 × FLAG), were in a Colum-
bia-0 (Col-0) background as reported previously [51].
The cbf2 (SALK_025195, Col-0 background) mutant
was a kind gift from Dr. Juan Lin (College of Life Sci-
fence, Fudan University, Shanghai, China). The rSPL9 cbf2
plant was generated by crossing rSPL9 with cbf2 mutant.
Seeds were sown in the media with soil and vermiculite
(1:1), treated at 4 °C for 2 days, and then transferred to the
long-day greenhouse (22 °C, 16-h light/8-h dark cycles,
with light intensity 130 μmmol•m−2•s−1). The plant age,
abaxial trichomes and leaf shapes were measured based
on our previous report [19, 20].

Freezing-treatment assay
All seeds used for freezing-treatment experiments were
collected at the same time and were naturally dried at
the room temperature. The 9-day-old (juvenile stage),
11-day-old (transition stage) and 14-day-old (adult stage)
Col-0 plants grown in soil under long day condition
were treated at −11 °C for 5 h to analyze the age-
dependent cold-tolerance. For freezing-treatment assay,
seeds of each genotype were washed twice with sterile
water, sterilized with 75% ethanol for 10 min, washed
more than five times with sterile water, and put on the
sterile paper until dried. The sterilized seeds were sown
one by one on 1/2MS medium plates (1% sucrose, 1.5%
agar, pH 5.8), treated at 4 °C for 2 days, and then trans-
ferred to the long-day growth chamber. Arabidopsis
seedlings were grown at 22 °C under long day condition
for 9 day, and then treated at −11 °C for 5 h. Thereaf-
ther, the seedlings were treated at 4 °C for 1 h and were
transferred to long day conditions for 5 days for recov-
er. The pictures were photographed, the surviving
seedlings were counted and the survival rates (surviving
seedling/total seedling × 100%) were measured. For the
electrolyte leakage assay [22], the cold-treated seedlings
were transferred carefully to tubes containing 10 mL of
deionized water and vacuumed for 1 min, and the con-
ductivity (S0) of the solution was measured. After 2-h
 treatment at room temperature, the conductivity (S1) of
the solution was measured again. Next, the tubes with
the samples were boiled for 5 min. After cooling down
to room temperature, the boiled conductivity (S2) of the
solution was measured finally. The percentage of elec-
trolyte leakage was calculated: ion leakage (%) = (S1-S0)
/ (S2-S0) × 100%. For the cold-acclimation assay, the
plants were treated at 4 °C for 3 days, and then treated
at −11 °C for 6 h and recovered for 5 days. After that
the survival rates were measured. The experiments had
been done more than three biological repeats under the
same conditions in this study.

RNA extraction and real-time quantitative PCR (RT-qPCR)
The 9-day-old seedlings were treated at 0 °C for 3 h, and
then were sampled. RNA extraction, first-strand cDNAs
synthesis, and RT-qPCR of mRNA and miRNA were car-
ried out as previous report [19]. The Arabidopsis TUBU-
LIN BETA CHAIN 2 (TUB2) gene was as internal control
for mRNA detection, and SNOR101 was as internal con-
trol for miRNA detection, respectively. The primers were
listed in Table S1. All experiments had been done more
than three times.

GR induction assay
The 9-day-old pSPL9:GR-rSPL9 seedlings were harvested
and immersed in 0.1% ethanol (as mock), or 10 mM
Dexamethasone (DEX) in 0.1% ethanol in 50-mL tubes.
Thereafter, the treated samples were put into the long-
day growth chamber to incubate for 8 h, and then were
used to extract total RNAs for the expression analysis of
CBF2, CBF1, CBF3 and miR172B (as positive control) by
RT-qPCR.

Chromatin immunoprecipitation quantitative PCR
(ChiP-qPCR) analysis
The SPL9 binding site, “GTAC” sequence, in the pro-
motor of CBF2 was analyzed. The ChiP-qPCR prim-
ers (Table S1) were designed based on the predicted
SPL9-binding sites. About three grams of 9-day-old
pSPL9:3 × FLAG-rSPL9 and rSPL9 (as a negative control) seedlings under long-day condition were harvested. ChIP-qPCR was performed as previous report [19]. This experiment had more than three biological replicates.

**Transient activation assay**

The about 1-kb promoter of CBF2 genes were amplified and fused into the pCAMBIA3301 vector to generate pCBF2:GUS vector. The CBF2 cis-regulatory sequences (GTAC) possibly bound to by SPL9 were mutated to “tgca” using overlapping PCR to generate pΔCBF2:GUS vector. The control vector (pSY06, Vec), UBI:rSPL9 (rSPL9), pCBF2:GUS and pΔCBF2:GUS were transformed into Agrobacterium tumefaciens strain GV3101, and then they were grouped into four combinations (Vec+pCBF2:GUS, Vec+pΔCBF2:GUS, rSPL9+pCBF2:GUS, and rSPL9+pΔCBF2:GUS) by pairwise mixing (1:1). Transient activation assay was performed as previous report [19]. The pCBF2:GUS plants were crossed with rSPL9 plants to generate pCBF2:GUS rSPL9 plants. Moreover, the injected tobacco leaves were stained with GUS staining solution, decolorized with 75% alcohol, and photographed. The pCBF2:GUS transgenic Arabidopsis lines and pCBF2:GUS rSPL9 Arabidopsis plants were examined for GUS activity by histochemical staining using previous experimental procedure [19].

**Statistical analysis**

Statistically significant differences were analyzed using the SPSS software by t-test or one-way ANOVA with post hoc Least Significant Difference (LSD) multiple comparison test.

**Abbreviations**

miR156: microRNA156; SPL9: SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9; CBF2: C-REPEAT BINDING FACTOR 2; DFR: DIHYDROFLAVONOL-4-REDUCTASE; IPA1: IDEAL PLANT ARCHITECTURE 1; FT: FLOWERING LOCUS T; SOC1: SUPPRESSOR OF OVEREXPRESSION OF CO 1; COR: C-REPEAT BINDING FACTOR/DRE BINDING FACTOR 1; CBF/DREB1: C-REPEAT BINDING FACTOR/DRE BINDING FACTOR 1; WRKY6, WRKY7, SAG1, and SOC1, genes in Col-0 and rSPL9 plants under 22 °C and 0 °C by RT-qPCR. The numbers indicate the fold change of relative expression level. Figure S5. The expression of CBF2 in Col-0, spl9–4 and rSPL9 plants under 22 °C and 0 °C by RT-qPCR. The numbers indicate the fold change of relative expression level.

**Additional file 1: Figure S1.** Leaf shape, abaxial trichome phenotypes, and leaf initiation rate of Col-0. (A) Twenty-one-day-old Col-0 plant grown in long days, and its leaf shape and abaxial trichome phenotypes. Numbers indicate the first leaf with abaxial trichomes. (B) Leaf initiation rate of Col-0 in long days. Leaf numbers were scored at 7, 9, 11, 13, and 15 days (d) after transferring to greenhouse. The intersection point of two red lines represents the time and the leaf position when the vegetative phase transition is occurred. Figure S2. The expression of SPL3 and SPL13 in Col-0 under 22 °C and 0 °C by RT-qPCR. Asterisks indicate significant difference from 22 °C using Student’s t-test (**P < 0.01). Figure S3. The expression of SPL9 in Col-0, 156OE, MIM156 and rSPL9 plants under 22 °C and 0 °C by RT-qPCR. Asterisks indicate significant difference from 22 °C using Student’s t-test (**P < 0.01). Figure S4. The expression of SPL9 by RT-qPCR in Col-0 and cb2 plants. ns, not significant. Figure S5. The expression of CBF2 in Col-0, spl9–4 and rSPL9 plants under 22 °C and 0 °C by RT-qPCR. The expression levels of the genes in Col-0 at 22 °C were set as 1.0. Data are mean ± SD of two biological replicates. Figure S6. GUS activity by histochemical staining in pSPL9:3 plants in Col-0 and pCBF2:GUS plants. Figure S7. RT-qPCR analysis of CBF1 and CBF3 expression, respectively, after SPL9 activation in the presence of DEX. ns, not significant. Figure S9. GUS activity by histochemical staining in leaves of N benthamiana infiltrated with Agrobacterium with different combinations of constructs, including Vec + pCBF2:GUS, Vec + pΔCBF2:GUS, rSPL9 + pCBF2:GUS, and rSPL9 + pΔCBF2:GUS. Figure S10. Freezing phenotypes and survival rates of Col-0 and rSPL9 plants after cold-acclimation treatment. ns, not significant. Table S1. Primers used in this study.

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**Authors’ contributions**

C.G. and J.Y. conceived of the study; J.Z., M.S. and C.G. performed experiments and data analysis; J.Y. assisted in performing part of the experiments; C.G. wrote and revised the manuscript. All authors read and approved the manuscript.

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**Availability of data and materials**

The data and materials in the current study are available from the corresponding author on reasonable request.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.
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