Cross-talk between freezing response and signaling for regulatory transcriptions of MIR475b and its targets by miR475b promoter in Populus suaveolens

Jun Niu*, Jia Wang*, Huiwen Hu, Yinlei Chen, Jiyong An, Jian Cai, Runze Sun, Zhongting Sheng, Xieping Liu & Shanzhi Lin

MicroRNAs (miRNAs) are small, non-coding RNAs that play important roles in post-transcriptional regulation of their target genes, yet the transcriptional regulation of plant miRNAs by promoter is poorly understood. Here, we firstly clone pri-miR475b cDNA and its native promoter from P. suaveolens, and characterize Psu-MIR475b as class-II gene transcribed by RNA polymerase II. By 5′ deletion analysis of Psu-miR475b promoter in a series of promoter-GUS chimeric vectors, we functionally identify three positive regulatory regions and multiple cis-acting elements responsible for Psu-miR475b promoter activity in response to freezing stress and exogenous hormone treatment. Moreover, the Psu-miR475b promoter activity displays a tissue-specific manner, negatively regulated by freezing stress and positively by MeJA, SA or GA treatment. Importantly, we comparatively analyze the time-course transcriptional profiles of Psu-miR475b and its targets in Psu-miR475b over-expression transgenic plants controlled by Psu-miR475b-specific promoter or CaMV 35S constitutive promoter, and explore the regulatory mechanism of Psu-miR475b promoter controlling transcriptional expressions of Psu-MIR475b and its targets in response to freezing stress and exogenous hormone treatment. Our results reveal that Psu-miR475b promoter-mediated transcriptions of Psu-MIR475b and its targets in response to freezing stress may be involved in a cross-talk between freezing response and stress signaling process.

Low temperature, especially freezing (< 0 °C), is one of the major environmental stresses that seriously influence the growth, development, distribution and productivity of plants1,2. Freezing tolerance and cold acclimation are highly complex process involved in physiological and metabolic modifications for cold response and a multiple gene expression network controlling plant tolerance to cold stress3–4. However, the regulatory networks of overall response of plants to low temperature stress still remains unclear. MicroRNAs (miRNAs) are a highly conserved class of endogenous single-stranded small non-coding RNAs that have been clearly shown to serve as negative regulators to modulate plant gene expression at post-transcriptional level by transcript cleavage or translational repression of target genes10–12. In recent years, the significant alterations in transcript levels of some miRNAs have been identified in response to cold stress in several plants such as Arabidopsis5,13–15, rice16, wheat17, Setaria italica18, Phaseolus vulgaris19, Brachypodium distachyon20, trifoliate orange21, celery22, Camellia sinensis23 and Populus24–27. Moreover, many predicted and experimentally confirmed targets of cold-responsive miRNAs encode a variety of transcription factors or other regulatory proteins implicated in low temperature response20–22,24,26,28. Also, genetic transformation of miRNAs and their targets has been recently demonstrated to alter cold stress tolerance capacity in plants26,29. All of these findings have shown a crucial role of miRNAs in the regulation of gene

College of Biological Sciences and Biotechnology, National Engineering Laboratory for Tree Breeding, Key Laboratory of Genetics and Breeding in Forest Trees and Ornamental Plants, Ministry of Education, Beijing Forestry University, Beijing 10083, China. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to S.L. (email: szlin@bjfu.edu.cn)
expression in response of plants to low temperature. Thus, an importance for understanding the initiation and regulation of miRNA gene transcription under low temperature stress.

The biogenesis of miRNAs is complex. Most plant miRNA genes (MIR), located in intergenic regions, are transcribed as independent transcriptional units by RNA polymerase II (Pol II) to produce primary transcripts (pri-miRNA) and then processed into stem-loop structured miRNA precursors (pre-miRNAs) by Dicer-like enzyme 1 (DCL1) in the nucleus. The mature miRNAs, transported to the cytoplasm, are incorporated into the RNA-induced silencing complex (RISC), and then lead to post-transcriptional gene silencing via transcript cleavage or translational repression of their target mRNAs by recognizing the base-pairing and interaction with their cognate targets. Although much effort has been focused on elucidating the regulatory function of plant miRNAs, little is known about how MIRED genes themselves are regulated. Recently, some studies have shown that plant miRNAs have the class II promoters and may be regulated by a similar mechanism as established for protein-coding genes. The promoters of miRNAs have been predicted in rice by bioinformatic analysis and A. thaliana by 5′ RACE, respectively, indicating the promoter as a crucial control region for the transcription initiation of miRNAs. However, direct evidence for transcriptional regulation of MIR genes by its native promoter is very little to date. Thus, the nature of miRNA promoter remains one of the most interesting open problems in the study of miRNA biogenesis.

*Populus suaveolens*, a typical freezing-resistant arbor tree of poplar species, can survive under a freezing temperature of approximately −43.5 °C in winter in the distribution of eastern Siberia regions and Great Xing'an Mountain, Northeast of China, and has emerged as a novel ideal model plant to study the freezing resistance mechanism in woody plants. Previously, we identified miR475b with a significant down-regulation in *P. suaveolens* under freezing stress (°C), and revealed that miR475b plays an important role in freezing resistance of *P. suaveolens*. In this continued study, we report the clone and analysis of miR475b gene and its native promoter from *P. suaveolens*, and explore the tissue-specific expression pattern of Psu-miR475b promoter. Also, we produce a series of 5′ promoter deletion-GUS reporter constructs, and perform a combination of the histochemical and fluorometric GUS assay and qRT-PCR to functionally characterize a set of regulatory regions and cis-acting elements responsible for the transcriptional activity of Psu-miR475b promoter. Importantly, we comparatively analyze the time-course transcriptional expression profiles of Psu-miR475b and its target genes in Psu-miR475b over-expression transgenic plants controlled by Psu-miR475b-specific promoter or cauliflower mosaic virus 35S (CaMV 35S) constitutive promoter, and investigate the regulatory mechanism of Psu-miR475b promoter for the transcripts of P-MIR475b and its targets in the transgenic plants subjected to freezing stress and exogenous hormone treatment. To our knowledge, this is the first report of functional identification and regulatory mechanism of Psu-miR475b promoter governing the transcriptional expressions of P-MIR475b and its targets in response to freezing stress.

**Results**

**Cloning and analysis of freezing-responsive Psu-miR475b and its promoter.** To elucidate the regulatory mechanism of miR475b transcription in response of *P. suaveolens* to freezing stress, the 1011-bp full-length freezing-responsive pri-miRNA475b with a putative 5′-cap structure and 3′-poly(A) tail (designated as Psu-MIR475b, Accession No. JX262380) was first cloned by 5′ and 3′ RACE from *P. suaveolens* cDNA (Fig. 1a,b). In order to gain insights into miR475b transcription, the secondary structure of RNA sequences generated from pri-miR475b cDNA was analyzed. We found that miR475b precursor has folding back free energy of −50.00 kcal/mol to form a stable stem-loop structure, and its mature sequence with 21nt length (Fig. 1c). Importantly, based on 5′ end sequence of Psu-miR475b gene, we used inversion-PCR (IPCR) to obtain full-length (939bp) Psu-miR475b promoter (Accession No. KM288552) from *P. suaveolens* genomic DNA (Fig. 1a,b).

Promoter is a crucial control region for transcription initiation of miRNAs. To understand the mechanism of the activation of Psu-MIR475b gene, it is required to locate cis-acting elements within its promoter region. By online programs, we characterized one core promoter element TATA box-like sequence (TAAAATAA, −32/−25), five CAAT-boxes as common cis-acting elements (−149/−146, −591/−588, −666/−663, −757/−754, −786/−783), five light responsive elements [CG motif (CCATGGGG, −57/−50), Box 4 (ATTAAT, −360/−355, −620/−615), GT1-motif (GTTTAAT, −550/−544), 1-box (−635/−629) and GAG-motif (AGAGATG, −817/−811), four stress-related elements [CGTCA-motif (CGTCA, −75/−71), TCA-element (AAGAAAAAGA, −297/−288), GARE-motif (AAACAGA, −452/−446) and TC-rich repeat (AAACAGA, −687/−678)], two cis-acting regulatory elements [5′UTR Py-rich stretch (TTCTTCTTCT, −838/−829) and ARE (TGGTTT, −342/−339)], and one endosperm-specific regulatory AACA-motif (AATCTAATT, −590/−581) within Psu-miR475b promoter (Fig. 1b and Table S1). Thus, an enrichment of diverse regulatory cis-acting elements in Psu-miR475b promoter.

**Tissue-specific activity for Psu-miR475b promoter in plants.** To explore whether Psu-miR475b promoter is similar to those of protein-coding genes, 939 bp full-length Psu-miR475b promoter (PromiR475) and CaMV 35S promoter (Pro35S) were respectively fused to GUS reporter gene and transferred into tobacco. We compared GUS activity in different tissues of transgenic PromiR475-GUS and Pro35S-GUS tobacco subjected to histochemical GUS staining. The PromiR475-GUS plants exhibited GUS expression in the stems and leaves, but no GUS staining was detected in the roots (Fig. 2a). In stark contrast with Pro35S-GUS plants, all tested tissues of PromiR475-GUS lines displayed a significant higher GUS expression (Fig. 2a). These results indicate that Psu-miR475b promoter is able to direct GUS gene expression, but differs from CaMV 35S constitutive promoter that served as positive control, directing a stronger expression of GUS.

Previously, we identified one preferential tissue-specific transcript of Psu-miR475b in the leaves and stems of *P. suaveolens*. Thus, it is useful to illustrate the tissue-specificity of Psu-miR475b promoter in *P. suaveolens*...
to address the regulatory mechanism of Psu-miR475b promoter controlling the transcription of Psu-miR475b. Here, the constructed promoter-GUS chimeric vectors (ProMIR475b:GUS and Pro35S:GUS) were transferred into *P. suaveolens* for the transcript level assay of GUS gene in different tissues by RT-PCR and qRT-PCR (Fig. 2b). As expected, Psu-miR475b promoter-driving GUS gene was transcribed in the leaves and stems of transgenic *P. suaveolens*, but no transcript observed for the roots, which was comparable with those observations in transgenic ProMIR475b:GUS tobacco (Fig. 2a). In addition, transgenic *P. suaveolens* driven by 35S promoter greatly increased GUS activity in all tissues examined (Fig. 2b). Thus, our findings reveal a typical tissue-specific expression pattern for Psu-miR475b promoter in plants.

Characterization of multiple cis-regulatory elements in Psu-miR475b promoter. To understand the regulatory mechanism controlling *Pse-MIR475b* gene expression by its native promoter, we first sought to determine the functionality of our predicted regulatory regions responsible for the Psu-miR475b promoter activity. Hence, we produced a series of 5′ promoter deletion-GUS constructs, covering different regions from −939 to −1, −569 to −1, −412 to −1, −351 to −1, −260 to −1, −95 to −1, −50 to −1 and −20 to −1 (Figure S1). The multiple transgenic tobacco plants (>12 independent lines) were obtained (Figure S2), and their stems were used as one predominant tissues (Fig. 2a) for the GUS expression analysis. The fluorometric GUS assay clearly demonstrated that compared with full-length promoter (939 bp), the deletion from −939 (relative to TSS) to −570, −569 to −413, −351 to −261, and −95 to −51 caused a significant reduction (about 1.0-, 1.8-, 1.6-, and 1.8-fold, respectively) in GUS activity in transgenic tobacco stems, whereas only 0.2-fold decrease was observed for the deletion from −412 to −352, and −261 to −96 (Fig. 2c). Intriguingly, GUS expression from −50 to −1 was significantly lower than others, while further deletion to −20 abolished the GUS expression (Fig. 2c). These investigations were entirely consistent with our results of histochemical GUS staining in the stems of transgenic tobacco plants (Fig. 2d). Thus, our data indicate that three positive regulatory regions (−939 to −413, −351 to −261 and −95 to −51) are responsible for the basal activity of Psu-miR475b promoter, and one region (−50 to −21) required for transcriptional initiation.

As we were surprised by a cluster of stress-related cis-elements within Psu-miR475b promoter (Fig. 1b and Table S1), we attempted to establish whether or not the basal activity of Psu-miR475b promoter could be affected by the treatments of stress-related stimuli. To achieve this, a series of 5′ promoter deletion-GUS transgenic
tobacco was subjected to the treatments of MeJA, SA, ABA and GA, respectively. By using fluorometric GUS assay, an obvious induction of GUS activity was observed in the stems of ProMIR475b-939/GUS and ProMIR475b-569/GUS plants upon treatment by SA, MeJA and GA, but a lower inducible ratio for ProMIR475b-569/GUS plants. In contrast, the GA-inducible expression of GUS gene seemed to disappear in ProMIR475b-412/GUS and ProMIR475b-351/GUS lines, which responded to SA and MeJA treatments. Also, ProMIR475b-260/GUS and ProMIR475b-95/GUS plants exhibited a higher GUS activity in response to MeJA, but not GA and SA. Notably, no inducible expression was shown for ProMIR475b-50/GUS and ProMIR475b-20/GUS plants (Fig. 2e). The present results indicate that the regions from −569 to −413, −351 to −261, and −95 to −51 are respectively required for GA-, SA- and MeJA-inducible activity of Psu-miR475b promoter. However, no response of GUS activity was observed with or without ABA treatment. It is also interesting to note that Pro35S/GUS lines showed no significant inducible expression under these imposed conditions (Fig. 2e).

**Cross-talk between freezing response and stress signaling for ProMIR475b activity regulation.** Recently, we identified the significant down-regulation of Psu-miR475b in response of *P. suaveolens* to 0°C stress[25], which allowed us to explore whether the activity of freezing-responsive Psu-miR475b promoter...
was specifically regulated by low temperature. Thus, we generated ProMIR475b-GUS and Pro35S-GUS transgenic *P. suaveolens* (Figure S2), and subjected them to the time-course analysis of GUS transcript level by qRT-PCR after 0 °C treatment for 0–48 h. We observed that during freezing-stress treatment, ProMIR475b-driving GUS expression was significantly decreased from 6 to 48 h, while no expression change was directed by CaMV 35S promoter in transgenic plants (Fig. 3a), indicating that Psu-miR475b promoter activity was specifically down-regulated by freezing stress. In addition, to investigate whether the signaling pathway was involved in the activity regulation of Psu-miR475b promoter, our analysis focuses on identifying the regulatory patterns of Psu-miR475b promoter by MeJA, SA and GA. In the case of treatment with exogenous MeJA, SA or GA for 0–48 h, we found that the Psu-miR475b promoter activity in response to freezing stress. Impressively, in those regions, MeJA-responsive CGTCA motif (−75/)−71), SA-responsive TCA element (−297/)−288) and GA-responsive GARE motif (−452/)−446) were identified (Fig. 2e). Also, the Psu-miR475b promoter activity was induced by the GA, SA, and MeJA treatments (Fig. 3b). Thus, it could be concluded that a cross-talk between freezing-stress response and hormone signaling may involve in the activity regulation of Psu-miR475b promoter.

**ProMIR475b**-mediated transcriptions of Psu-miR475b and its targets in transgenic *P. suaveolens*. To address the regulatory mechanism of Psu-miR475b-specific promoter controlling *Pst*-MIR475b expression, we analyzed the time-course transcription pattern of Psu-miR475b by qRT-PCR in Psu-miR475b-overexpressing *P. suaveolens* under the control of Psu-miR475b promoter or CaMV 35S promoter (Figure S4). Compared with the wild-type (WT) controls, the most striking difference between two types of transgenic lines was observed in ProMIR475b-MIR475b plants with about 6.1-fold increase of Psu-miR475b transcript, which is considerably higher than that (0.3-fold) in Pro35S-MIR475b lines (Fig. 4a), confirming an important contribution of Psu-miR475b promoter to regulate its native gene (*Pst*-MIR475b) transcription. Recently, we experimentally characterized 12 putative pentatricopeptide repeat protein (PPR) genes (XM_002319013.1, XM_002326743.1, XM_002336177.1, XM_002329199.1, DB891579, XM_002309526.1, XM_002301639.1, XM_002326793.1, XM_006377350.1, XM_002310640.2, XM_006389244.1, XM_006389560.1) as the targets of Psu-miR475b. To understand how Psu-miR475b regulates the expressions of its targets, and to examine whether Psu-miR475b promoter was involved specifically in the regulation of miR475b-mediated expression, we further checked the transcript levels of 12 miR475b-targeted PPR genes in transgenic plants. Compared to the WT controls, the transcripts of 12 PPR genes were all markedly reduced (4–9 fold) in ProMIR475b-MIR475b plants, but no significant alteration was observed for Pro35S-MIR475b lines (Fig. 4b). This indicates that the expressions of miR475b-targeted genes in Psu-miR475b-overexpressing transgenic plants are specifically regulated by Psu-miR475b promoter. Also, the fact of an inverse correlation between Psu-miR475b (up-regulation) and its targets (down-regulation) (Fig. 4a, b) in transgenic plants showed that the miR475b-targeted transcripts may be cleaved directly by Psu-miR475b, which was confirmed by our previous 5′ RLM- RACE.

**ProMIR475b**-mediated transcriptional regulation involved in freezing response and hormone signaling. Considering the fact that freezing stress has shown to confer a negative effect on the Psu-miR475b promoter-directed GUS expression (Fig. 3a), the obtained Psu-miR475b-overexpressing *P. suaveolens* were also exposed to 0 °C treatment for 0–48 h, and the temporal transcript profiles of Psu-miR475b and its targets were...
analyzed to reveal the regulatory mechanism of Psu-miR475b promoter for the transcriptional expressions of Psu-MIR475b and its targets in response to freezing stress. We found that the transcript level of Psu-miR475b in ProMIR475b:MIR475b plants was lower after 6 h and greatly decreased with longer stress, while a small decline was detected in Pro35S:MIR475b lines with minimum valve at 24 h, similar to that of the WT controls (Fig. 4c). This finding reveals that Psu-miR475b transcription is altered in response to freezing stress, and a significant down-regulation is specifically driven by its native promoter. Also, the strongly induced expression of all 12 miR475b-targeted genes by freezing was identified in ProMIR475b:MIR475b plants than in both Pro35S:MIR475b plants and WT controls (Fig. 4d), implying that the significant up-regulation of miR475b-targeted genes driven by Psu-miR475b promoter may be involved in freezing-stress response process.

Given that Psu-miR475b promoter activity is induced by MeJA, SA and GA (Fig. 3b), we attempted to address whether the application of these stimuli could trigger the transcriptions of Psu-miR475b and its targets. To this end, the transgenic P. suaveolens of ProMIR475b:MIR475b and Pro35S:MIR475b were respectively treated by MeJA, SA or GA for 0–48 h. Here, Psu-miR475b transcript was up-regulated approximately 17.3-, 9.6- and 5.1-fold in ProMIR475b:MIR475b plants by MeJA, SA and GA treatments for 48 h, peaked at 36, 24 and 24 h respectively, whereas a small induced expression for Psu-MIR475b was detected in both Pro35S:MIR475b lines and WT controls by these treatments (Fig. 4e). These results indicate that an obvious inducible transcription of Psu-miR475b by the MeJA, SA and GA treatments, which is tightly correlated with the stronger response of Psu-miR475b promoter to MeJA, SA and GA. The significantly down-regulated transcription of all 12 targets by freezing stress. (e) The significantly induced transcription of Psu-miR475b by the MeJA, SA and GA treatments, which is tightly correlated with the stronger response of Psu-miR475b promoter to MeJA, SA and GA. (f) The significantly down-regulated transcription of all 12 targets by the MeJA, SA and GA treatments. The finding indicates an important regulatory role of Psu-miR475b promoter for Psu-miR475b-mediated transcriptional repression of its targets responsive to MeJA, SA and GA.

Discussion
In recent years, bioinformatic analysis has been applied to predict the promoters of miRNAs in Arabidopsis and rice53,51,52,43–45, but the experimental cloning and functional identification of MIR promoter was only reported for...
miR171a, miR172a and miR390a/b in Arabidopsis to data48–50. This study presents for the first time a regulatory mechanism of Psu-miR475b promoter governing the transcription of Psu-miR475b and its targets in response of *P. suaveolens* to freezing stress, where pri-miR475b CDNA and its promoter were firstly cloned from *P. suaveolens* (Fig. 1a,b). A lower folding back free energy was predicted for Psu-miR475b precursor (Fig. 1c), as reported in *Triticum aestivum*26, *B. distachyon*28 and *P. tomentosa*29. Intriguingly, the primary transcript of Psu-miR475b was capped at the 5′ end and polyadenylated at the 3′ end (Fig. 1b), similar to the unique properties of class-II gene transcripts, which has been characterized in *A. thaliana* MIR genes31,43,45. Moreover, Psu-miR475b has one 21 nt-length mature sequence located in 3′ arm of stem-loop structure, and has uridine (U) as first nucleotide at 5′ end (Fig. 1c,b), which is entirely consistent with *P. trichocarpa* miR475b/U26, also identified as one characteristic feature of miRNAs in plants20,24,31. Together, all our findings reveal that Psu-MIR475b gene is transcribed as a single transcript unit by the RNA pol II mechanism.

The promoter contain essential components for the transcription regulation of MIR gene31,32. TATA-box, as a well-known core motif in the promoters of eukaryotic class-II genes, has been identified in most miRNA promoters of *A. thaliana* and *O. sativa*31,32,43,45, suggesting that most of plant MIR genes may present the same promoters as the protein-coding genes transcribed by RNA pol II. In this study, we identified one 8-nt TATA box-like sequence of Psu-miR475b promoter within −32 to −25 (Fig. 1c and Table S1) was compatible with those located in protein-coding genes, also correspond to authentic TATA box sequence within the core promoters of plant MIR genes31,32,44. Importantly, the up-regulated GUS was detected in both ProMIR475b-GUS and Pro35S:GUS transgenic tobacco, but the relative lower GUS activity was observed for ProMIR475b-GUS lines (Fig. 2c,d). All our results show indeed that Psu-miR475b promoter may be as pol II promoter, but exhibits a specificity for Psu-miR475b, which support the hypothesis of the differentiation between MIR genes and protein-coding genes45.

Earlier studies in four model species (*Caenorhabditis elegans*, *Drosophila melanogaster*, *A. thaliana* and *O. sativa*) have revealed many essential conserved motifs in the promoters of MIR genes52. Recently, 11 over-represented cis-elements (AtMYC2, ARF, SORLREP3, G-box, SORLIP1, RY-repeat, LTRE, Evening element, TELO-box, DRE-like and AtMYB2), and 9 under-represented cis-elements (GATA box, LHY motif, T-box, GCC-box, RAV1-B, Bellringer BS3, CArG, HSEs and CCA1) were identified in the promoters of Arabidopsis MIR genes by position weight matrices (PWM)45. In this work, we performed the PWM method to identify a total of 13 cis-acting elements in Psu-miR475b promoter (Fig. 1b and Table S1), among which 11 elements (Box 4, GT1-motif, CGTCA-motif, GAG-motif, CG-motif, TCA-motif, GARE-motif, 5′ UTR Py-rich stretch, TC-rich repeat, ARE and ACA motif) were not previously reported. Our data indicate that these putative cis-regulatory elements may be specific to Psu-miR475b promoter, probably owing to MIR gene promoter with specific cis-acting elements for governing unique transcription of miRNA53. In addition, we present the first 5′ promoter progressive deletion analysis to elucidate the functionality of these potential regulatory cis-elements for the Psu-miR475b promoter activity. The histochemical and fluorometric GUS assay (Fig. 2c,d), combined with our characteristics of cis-acting elements in Psu-miR475b promoter (Fig. 1b and Table S1), suggests that CGTCA motif (involved in MaJA responsiveness), GAR-motif (GA responsiveness), TCA-element (SA responsiveness), TC-rich repeat (stress responsiveness) and 5′ UTR Py-rich stretch (conferring high transcription level) may play the key role in the activity regulation of Psu-miR475b promoter, and TATA-box (as core promoter element) function as initiator for initiation of Psu-miR475b transcription. Importantly, the regions from −569 to −413, −351 to −261, and −95 to −51 were respectively required for GA-, SA-, and MeJA-inducible activity of Psu-miR475b promoter (Fig. 2e), also noted in the promoters of some cold-responsive miRNAs (such as miR167/393/408) in *A. thaliana*44. However, no response of GUS activity to ABA-treatment in transgenic plants may be correlated with the lack of ABA-responsive ABRE motif in the Psu-miR475b promoter (Table S1), as reported in the promoters of some *A. thaliana* miRNAs14, implying that the activity regulation of Psu-miR475b promoter may be ABA-independent pathway. Together, our deletion analysis suggest a complex regulatory mechanism for the activity of Psu-miR475b promoter controlled by its internal cis-acting elements.

Previously, miR475b has been shown to be down-regulated in response of *P. suaveolens* to freezing stress27. Our findings on the significant down-regulation of GUS gene by freezing stress in transgenic ProMIR475b-GUS plants (Fig. 3a) and the obvious induced expression by the exogenous SA, MeJA and GA (Fig. 3b) reveal that Psu-miR475b promoter is one low temperature-responsive promoter, and its activity is negatively regulated by freezing stress and positively by the applications of exogenous hormones. This also prompt us to consider that Psu-miR475b promoter is one low temperature-responsive promoter, and its activity is negatively regulated by freezing stress27. These results, combined with the finding that the promoter activity of Psu-miR475b is negatively regulated by freezing stress, confirm an involvement of Psu-miR475b promoter in the transcription regulation of Psu-MIR475b and its targets under freezing stress.

Many miRNAs were differentially regulated by exogenous application of plant hormones such as JA, SA, GA and ABA13,54. In this work, compared with Pro35S:MI475b plants, the significant differential expressions of Psu-miR475b (up-regulation) and its targets (down-regulation) were identified in transgenic Pro35S:MI475b plants by the MeJA, SA and GA treatments (Fig. 4e,f), indicating that the exogenous hormones (MeJA, SA and GA) could regulate the transcripts of Psu-miR475b and its targets, likely though mediating the Psu-miR475b promoter activity. This could be supported by the fact of a higher inducible activity of Psu-miR475b promoter by the treatments of MeJA, SA and GA (Fig. 3b). Thus, we conclude that Psu-miR475b promoter-triggered...
transcriptions of Psu-MIR475b and its targets may be involved in the complex signaling pathways, mediated by MeJA, SA and GA.

Taken together, our findings that freezing stress-responsive Psu-miR475b and its targets as well as the Psu-miR475b promoter activity are prone to being affected by the treatments of freezing stress and exogenous MeJA, SA or GA, reveal the existence of a great cross-talk between freezing response and stress signaling process. It was suggested that the PPRs may provide a signaling link between mitochondrial electron transport and regulation of stress and hormonal responses in A. thaliana. Microarray analysis of transcript expression has shown that many genes involved in the biosynthesis and signaling of plant endogenous hormone (such as JA, SA and GA) are down-regulated by cold stress in A. thaliana. The previously characterized 12 PPRs as the targets for psu-miR475b, integrated with all our investigations, suggest that the lower activity of Psu-miR475b promoter could be expected mainly due to the smaller biosynthesis capacity of endogenous hormone (MeJA, SA and GA) caused by freezing stress. Thus, the transcriptional expressions of Psu-miR475b and its targets by its native promoter may be involved in a complex signaling pathway and freezing-stressed response. Our findings also evidenced that Psu-miR475b-specific promoter is important determinant for the transcriptional regulation for Psu-miR475b and its targets in response of P. suaveolens to freezing stress.

Methods

Plant materials and growth conditions. P. suaveolens, obtained from Great Xing’an Mountain, Northeast of China, was used as the source material for this study. The plants were propagated by cutting and raised in pots within a controlled environment chamber (photoperiod: 16/8 h light/dark, minimum illumination: 0.2 mM s⁻¹ m⁻², day temperature: 20–30°C) at Beijing Forestry University. Tissue-cultured P. suaveolens plants were raised and synchronized on modified MS medium as our previously described. The fully developed leaves harvested from the tissue-cultured plantlets were subjected to genetic transformation. Tissue-culture tobacco (Nicotiana tabacum cv. W38) plants were performed on modified MS medium, and the fully developed tobacco leaves were then used for genetic transformation experiments.

Freezing stress treatment of P. suaveolens plantlets. The 2-month-old plantlets with identical growth status were exposed to 0°C, and the leaves were then harvested at time points 0, 6, 12, 24, 36 and 48 h post-treatment as described previously. All materials were collected from three individual plants and immediately frozen in liquid nitrogen and stored at −80°C.

Cloning of pri-miR475b and its promoter and transformation of P. suaveolens. Total RNA was isolated from the tested samples using SV Total RNA Isolation System (Promega, Madison, WI, USA). The synthesis of first strand cDNA, and the 3'- and 5'-RACE of pri-miR475b were performed according to SMARTer RACE cDNA kit (Clontech, USA) illustrate, and then the purified amplification products were sequenced to assemble the full-length sequence of pri-miR475b. 3'- and 5'-RACE outer/inner primer could be seen in Supplementary Table S2. The full-length cDNA sequence (pri-miR475b) was amplified from P. suaveolens genomic DNA by PCR using two gene-specific primers 5'-AGGTAGTCAAGCACCATCACAAA-3' (prime) and 5'-AACCTACAGCATGACCTAGAGGC-3' (reverse primer), and then cloned into pGM-T Vector, giving Psu-miR475b. The sequence of the amplified DNA fragment was veriﬁed by sequencing. A XbaI-Sacl fragment from pT-MIR475b containing the MIR475b sequence was then subcloned into the XbaI and SacI sites of vector pBI121 between the CaMV 35S promoter and the NOS 3'-poly (A) signal to generate the 35S:MIR475b construct (named as ProPS-MIR475b). For full-length Psu-miR475b promoter from P. suaveolens by IPCR, forward primers were (5'-AAATGTCAAGGGTGAACTAATTCTA-3' (F-1) and 5'-ATAAGTAGAAGTCTACCGGGA-3'(F-2), and reverse primers were 5'-AGGTAGTCAAGCACCATCACAAA-3' (R-1) and 5'-AACCTACAGCATGACCTAGAGGC-3' (R-2). To construct the Psu-miR475b-overexpression vector driven by Psu-miR475b promoter, the amplified Psu-MIR475b gene and its promoter were digested with XbaI and SacI, and then cloned into pGEM-T Vector, giving Psu-miR475b. The sequence of the amplified DNA fragment was veriﬁed by sequencing. A XbaI-Sacl fragment from pT-MIR475b containing the MIR475b sequence was then subcloned into the XbaI and SacI sites of vector pBI121 between the CaMV 35S promoter and the NOS 3'-poly (A) signal to generate the 35S:MIR475b construct (named as ProPS-MIR475b). For a negative control, empty effector plasmid 35S/Em was constructed by the replacement of GUS gene with a native sequence 5'-TCTAGAGGCTCAAGCGCGGCTAGCTACGC-3' in pBI121. These constructs were first introduced into Agrobacterium tumefaciens strain GV3101 via the freezing-thaw method, and then transferred into P. suaveolens by the leaf disc transformation method.

Promoter-GUS chimeric vector construction and tobacco transformation. A series of 5' progressive deletions of Psu-miR475b promoter, covering different regions from −939 to −1, −569 to −1, −412 to −1, −351 to −1, −260 to −1, −95 to −1, −50 to −1 and −20 to −1, were respectively generated by PCR using a 5' sequence of Psu-MIR475b as a template with forward primers containing Hind III restriction site (underlined): 5'-CCCCAGCTTTTTTTTCTGTTTGGTTGA-3'(f1), 5'-CCCCAAGCTTTTTTCTAGTTTGGTTGA-3'(f2), 5'-CCCCAAGCTTTTTTCTAGTTTGGTTGA-3'(f3), 5'-CCCCAAGCTTTTTTCTAGTTTGGTTGA-3'(f4), 5'-CCCCAAGCTTTTTTCTAGTTTGGTTGA-3'(f5), 5'-CCCCAAGCTTTTTTCTAGTTTGGTTGA-3'(f6), and a common reverse primer containing a Hind III site immediately downstream of the 35S promoter (underlined): 5'-CTCAGAGGCTCAAGCGCGGCTAGCTACGC-3'(r01). Each of the PCR amplified fragments was digested with XbaI and Hind III (Promega, USA) and purified with TIAN-quick Midi Purification Kit (TIANGEN, Beijing, China). They were then fused to the GUS reporter gene of the modified pBI121 vector (Clontech, USA) harboring an XbaI site immediately downstream of the Hind III site, which was previously digested with XbaI and Hind III to release 35S promoter. The resulting vectors, confirmed by DNA sequencing,
were respectively named as Pro\textsubscript{MIR475b-939/GUS}, Pro\textsubscript{MIR475b-569/GUS}, Pro\textsubscript{MIR475b-112/GUS}, Pro\textsubscript{MIR475b-351/GUS}, Pro\textsubscript{MIR475b-260/GUS}, Pro\textsubscript{MIR475b-95/GUS}, Pro\textsubscript{MIR475b-50/GUS} and Pro\textsubscript{MIR475b-20/GUS}.

The chimeric vectors for tobacco transformation were performed by the same method of \textit{P. suaveolens}. The putative transgenic plantlets resistant to kanamycin were further confirmed by PCR, as well as preliminary GUS staining. The verified transgenic tobaccos were then propagated and synchronized from primary transformants in MS medium. One month-old \textit{in vitro}-grown plantlets were used for subsequent experiments.

**Plant treatment.** For inducible expression analysis of GUS activity, the \textit{in vitro} transgenic tobacco plants were sprayed with 200 \( \mu \)M MeJA, 200 \( \mu \)M GA, 5 \( \mu \)M SA, 100 \( \mu \)M ABA, and then incubated at 23 °C for 24 h. To test the effects of the different defense-related stimuli on the activity of Psu-miR475b promoter, the aerial parts between the second and fourth leaves of the \textit{in vitro} transgenic \textit{P. suaveolens} plants were sprayed with 200 \( \mu \)M MeJA, 200 \( \mu \)M GA, 5 \( \mu \)M SA or 100 \( \mu \)M ABA solutions, and the poplar samples were then harvested at time points 0, 6, 12, 24, 36 and 48 h post-treatment. All these treatments were described by our previous studies\textsuperscript{56}, and untreated plantlets and plantlets treated with distilled water were used as the controls.

**Histochemical and fluorometric GUS assay.** For histochemical staining of GUS, fresh tissue samples were dissected from tobacco plants and immediately subjected to the X-Gluc solution\textsuperscript{56}. After overnight incubation at 37 °C, stained samples were bleached with 70% (v/v) ethanol and observed with OLYMPUS BX61 and SZX12 microscopes.

A fluorometric GUS assay was performed as previously described\textsuperscript{25} and the tobacco stem tissues were ground in liquid nitrogen and homogenized in freshly prepared GUS extraction buffer (50 mM NaH\textsubscript{2}PO\textsubscript{4}, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% (v/v) sodium laurylsarcosine, 10 mM 3-mercaptopropanol). After centrifuging for 15 min at 12,000 rpm at 4 °C, the GUS activity of the supernatant was determined using 4-methylumbelliferyl glucuronide (4-MUG) as a substrate. The fluorescence of the GUS-catalyzed hydrolysis reaction product, 4-methylumbelliferone (4-MU), was measured with the TECAN GENios system. Protein concentration in supernatant was assessed by the Bradford method\textsuperscript{54}, using bovine serum albumin as a standard. GUS activity was normalized to the protein concentration of each supernatant extract and calculated as pmol of 4-MU per milligram of soluble protein per minute.

**Gene expression analysis.** Genomic DNA was extracted from the mature leaves of \textit{P. suaveolens} with a Plant Genomic DNA Kit (TIANGEN, Beijing, China). Total RNA was isolated from the tested samples using SV Total RNA Isolation System (Promega, Madison, WI, USA), and treated with RNase-free DNase I to eliminate the residual genomic DNA, according to the manufacturer’s instructions (Promega, Madison, WI, USA).

Relative quantification of the expressions for Psu-miR475b and its targets, and GUS gene by quantitative real-time PCR (qRT-PCR) were performed on 7500 Real-Time PCR System, by using MiRcute miRNA SYBR Green Kit (TianGen, Beijing, China) and SuperScript\textsuperscript{™} III Platinum® Two-Step qRT-PCR Kit with SYBR® Green (Invitrogen, Carlsbad, CA, USA), respectively. According to the previous reports\textsuperscript{59,60} and our generated Solexa sequencing (data not shown), miR167e and miR168a-3p were used as inner references for Psu-miR475b, and poplar ACTIN gene as endogenous reference gene for miR475b-targets\textsuperscript{27}. Data were from at least three quantitative PCR replicates per sample and three biological replicates. The specific primers of Psu-miR475b and its target genes, GUS gene and reference gene are shown in Supplementary Table S2.

**Prediction of cis-acting elements within Psu-miR475b promoter.** The putative cis-elements of Psu-miR475b promoter were identified by online programs including Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), PLACE (http://www.dna.afrc.go.jp/PLACE/signalup.html), TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess) and PWM (http://users.soe.ucsc.edu/~kent/improbizer/motifMatcher.html).

**References**

1. Thomashow, M. F. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. \textit{Annu Rev Plant Biol} 50, 571–599 (1999).
2. Chinnusamy, V., Zhu, J. & Zhu, J. K. Cold stress regulation of gene expression in plants. \textit{Trends Plant Sci} 12, 444–451 (2007).
3. Fowler, S. & Thomashow, M. F. Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CRT cold response pathway. \textit{Plant Cell} 14, 1675–1690 (2002).
4. Hannah, M. A., Heyer, A. G. & Hincha, D. K. A global survey of gene regulation during cold acclimation in \textit{Arabidopsis thaliana}. \textit{PLoS Genet} 1, e26 (2005).
5. Lee, B.-h., Henderson, D. A. & Zhu, J. K. The \textit{Arabidopsis} cold-responsive transcriptome and its regulation by CBF. \textit{Plant Cell} 17, 3155–3175 (2005).
6. Benedict, C., Geisler, M., Trygg, I., Huner, N. & Hurry, V. Consensus by democracy. Using meta-analyses of microarray and genomic data to model the cold acclimation signaling pathway in \textit{Arabidopsis}. \textit{Plant Physiol} 141, 1219–1232 (2006).
7. Yamaguchi-Shinozaki, K. & Shinozaki, K. Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. \textit{Annu Rev Plant Biol} 57, 781–803 (2006).
8. Chinnusamy, V., Zhu, J. K. & Sunkar, R. Gene Regulation During Cold Stress Acclimation in Plants in \textit{Plant Stress Tolerance}, Vol. 639 (ed. Ramanjulu, S.) 39–55 (Springer, 2010).
9. Park, S. et al. Regulation of the \textit{Arabidopsis} CBF regulon by a complex low-temperature regulatory network. \textit{Plant J} 82, 193–207 (2015).
10. Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. \textit{Cell} 116, 281–297 (2004).
11. Jones-Rhoades, M. W., Bartel, D. P. & Bartel, B. MicroRNAs and their regulatory roles in plants. \textit{Annu Rev Plant Biol} 57, 19–53 (2006).
12. Voinnet, O. Origin, biogenesis, and activity of plant microRNAs. \textit{Cell} 136, 669–687 (2009).
13. Sunkar, R. & Zhu, J. K. Novel and stress-regulated microRNAs and other small RNAs from \textit{Arabidopsis}. \textit{Plant Cell} 16, 2001–2019 (2004).
14. Liu, H. H., Tian, X., Li, Y. J., Wu, C. A & Zheng, C. C. Microarray-based analysis of stress-regulated microRNAs in Arabidopsis thaliana. RNA 14, 836–843 (2008).
15. Zhou, X. F., Wang, G. D., Sutoh, K., Zhu, J. K & Zhang, W. X. Identification of cold-inducible microRNAs in plants by transcriptome analysis. BBA–Gene Regul Mech 1779, 780–788 (2008).
16. Lv, D. K. et al. Profiling of cold-stress-responsive miRNAs in rice by microarrays. Gene 459, 39–47 (2010).
17. Yang, Z. H. et al. Uncovering small RNA-mediated responses to cold stress in a wheat thermo-sensitive genic male-sterile line by deep sequencing. Plant Physiol 159, 721–738 (2012).
18. Khan, Y. et al. Comprehensive genome-wide identification and expression profiling of fotilax miRNAs in response to abiotic stress and development of miRNA database. Plant Cell Tiss Org 118, 279–292 (2014).
19. Arenas-Huertero, C. et al. Conserved and novel miRNAs in the legume Phaseolus vulgaris in response to stress. Plant Mol Biol 70, 385–401 (2009).
20. Zhao, J. Y., Xu, Y. Y., Huan, Q. & Chang, K. Deep sequencing of Brachypodium small RNAs at the global genome level identifies microRNAs involved in cold stress response. BMC Genomics 10, 449 (2009).
21. Zhang, X. N., Li, X. & Liu, J. H. Identification of conserved and novel cold-responsive microRNAs in trifoliate orange (Poncirus trifoliata (L.) Raf.) using high-throughput sequencing. Plant Mol Biol Rep 32, 328–341 (2014).
22. Jiang, Q. et al. High-throughput analysis of small RNAs and characterization of novel microRNAs affected by abiotic stress in a local celery cultivar. Sci Hort 169, 36–43 (2014).
23. Zhang, Y. et al. Identification and characterization of cold-responsive microRNAs in tea plants (Camellia sinensis) and their targets using high-throughput sequencing and degradome analysis. BMC Plant Biol 14, 271 (2014).
24. Liu, S., Sun, Y. H. & Chiang, V. L. Stress-responsive microRNAs in Populus. Plant J 55, 131–151 (2008).
25. Sun, R. Z. et al. Cloning and analysis of the low temperature stress-responsive microRNAs from Populus traeceolens. Genomics Appl Biol 30, 204–211 (2011).
26. Chen, L. et al. Genome-wide identification of cold-responsive and new microRNAs in Populus tomentosa by high-throughput sequencing. Biochem Biophys Res Commun 417, 892–896 (2012).
27. Hu, H. W. et al. Prediction and expressing analysis of the cold stress-responsive miR475 target genes from Populus traeceolens. Genomics Appl Biol 33, 1–6 (2014).
28. Hu, H. H. et al. Characterization of transcription factor gene SNAC2 conferring cold and salt tolerance in rice. Plant Mol Biol 67, 169–181 (2008).
29. Yang, C. H. et al. Overexpression of microRNA319 impacts leaf morphogenesis and leads to enhanced cold tolerance in rice (Oryza sativa L.). Plant Cell Environ 36, 2207–2218 (2013).
30. Lee, Y. et al. MicroRNA genes are transcribed by RNA polymerase II. EMBO J 23, 4051–4060 (2004).
31. Xie, Z. X. et al. Expression of Arabidopsis miRNA genes. Plant Physiol 138, 2145–2154 (2005).
32. Zhou, X. F., Ruan, J. H., Wang, G. D. & Zhang, W. X. Characterization and identification of microRNA core promoters in four model species. PLoS Comput Biol 3, e37 (2007).
33. Nozawa, M., Miura, S. & Nei, M. Origins and evolution of microRNA genes in plant species. Genome Biol Evol 4, 230–239 (2012).
34. Yang, X. Z., Zhang, H. Y. & Li, L. Alternative mRNA processing increases the complexity of microRNA-based gene regulation in Arabidopsis. Plant J 70, 421–431 (2012).
35. Call, C., Xie, Z. X., Kasschau, K. D. & Carrington, J. C. Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science 297, 2053–2056 (2002).
36. Vaucheret, H., Vazquez, F., Créte, P. & Bartel, D. P. The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. Gene Dev 18, 1187–1197 (2004).
37. Brodersen, P. et al. Widespread translational inhibition by plant miRNAs and sRNAs. Science 320, 1185–1190 (2008).
38. Zhu, J. K. Reconstituting plant miRNA biogenesis. P Natl Acad Sci USA 105, 9851–9852 (2008).
39. Beaucournu, L., Yu, A. & Bouché, N. microRNA-directed cleavage and translational repression of the copper chaperone for superoxide dismutase mRNA in Arabidopsis. Plant J 62, 454–462 (2010).
40. Bivakawa, H.-O. & Tomari, Y. Molecular insights into microRNA-mediated translational repression in plants. Mol cell 52, 591–601 (2013).
41. Liu, Q. K., Wang, F. & Axtell, M. J. Analysis of complementarity requirements for plant microRNA targeting using a Nicotiana benthamiana quantitative transient assay. Plant Cell 26, 741–753 (2014).
42. Wang, B. et al. HOS1 regulates Argonaut1 by promoting transcription of the microRNA gene MR168b in Arabidopsis. Plant J 81, 861–870 (2015).
43. Megraw, M. et al. MicroRNA promoter element discovery in Arabidopsis. RNA 12, 1612–1619 (2006).
44. Cui, X., Xu, S. M., Mu, D. S. & Yang, Z. M. Genomic analysis of rice microRNA promoters and clusters. Gene 433, 61–66 (2009).
45. Zhao, X., Zhang, H. Y. & Li, L. Identification and analysis of the proximal promoters of microRNA genes in Arabidopsis. Genomics 101, 187–194 (2013).
46. Lin, S. Z., Zhang, Z. Y. & Lin, Y. Z. Comparison of G6PDH activity and LT50 between Populus tomentosa and P. traeceolens during freezing acclimation. Forest Stud China 40 (2003).
47. Lin, S. Z. & Zhang, Z. Y. Studies on molecular biology and freezing-tolerance of poplar 146–150 (Environmental Science Press, Beijing, 2004).
48. Xue, Y. et al. Interaction between two timing microRNAs controls trichome distribution in Arabidopsis. PLoS Genet 10, e1004266 (2014).
49. Cho, H. J. et al. SHORT VEGETATIVE PHASE (SVP) protein negatively regulates miR172 transcription via direct binding to the pri-miR172a promoter in Arabidopsis. FEBS Letters 586, 2332–2337 (2012).
50. Montgomery, T. A. et al. Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. Cell 133, 128–141 (2008).
51. Yao, Y. et al. Cloning and characterization of microRNAs from wheat (Triticum aestivum L.). Genome Biol 8, R96 (2007).
52. Lu, S. F. et al. Novel and mechanical stress-responsive microRNAs in Populus trichocarpa that are absent from Arabidopsis. Plant Cell 17, 2286–2293 (2005).
53. Naqvi, A., Choudhury, N. & Haq, Q. Small RNA-mediated defensive and adaptive responses in plants. Sust Agr Rev 7, 129–160 (2011).
54. Pandey, R., Joshi, G., Bhardwaj, A. R., Agarwal, M. & Katiyar-Agarwal, S. A comprehensive genome-wide study on tissue-specific and abiotic stress-specific miRNAs in Triticum aestivum. PLoS One 9, e95800 (2014).
55. Lin, Y. Z., Lin, S. Z. & Zhang, Z. Y. Tissue Culture and Rapid Propagation of Populus suaveolens. Plant Physiol Commun 40, 463 (2004).
56. Lei, Y. et al. Cloning and expression of three pathogenesis related protein genes from Populus tomentosa. J Northeast Forest Univ 6, 67–75 (2012).
57. Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6, 3901–3907 (1987).
58. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248–254 (1976).
59. Lin, Y. L. & Lai, Z. X. Evaluation of suitable reference genes for normalization of microRNA expression by real-time reverse transcription PCR analysis during longan somatic embryogenesis. *Plant Physiol Bioch* 66, 20–25 (2013).

60. Feng, H. *et al.* Selection of suitable inner reference genes for relative quantification expression of microRNA in wheat. *Plant Physiol Bioch* 51, 116–122 (2012).

**Acknowledgements**

This research was supported by the National Natural Sciences Foundation of China (No. 31270698).

**Author Contributions**

Experiments were designed by J.N., J.W. and S.L. H.H., Y.C. and R.S. cloned and analyzed freezing-responsive Psu-miR475b and its promoter. J.A. and J.C. participated in transgenic plants. Z.S. and X.L. conducted some sequencing analysis and cis-acting regulatory elements characterization. J.N. and J.W. prepared Figures 1–4 and Figures S1–S4. JN wrote the manuscript with editorial assistance from S.L. All authors have read and approved the manuscript.

**Additional Information**

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Niu, J. *et al.* Cross-talk between freezing response and signaling for regulatory transcriptions of MIR475b and its targets by miR475b promoter in *Populus suaveolens*. *Sci. Rep.* 6, 20648; doi: 10.1038/srep20648 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/