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ptr-MIR169 is a posttranscriptional repressor of *PtrHAP2* during vegetative bud dormancy period of aspen (*Populus tremuloides*) trees

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ABSTRACT

Dormancy is a mechanism evolved in woody perennial plants to survive the winter freezing and dehydration stress via temporary suspension of growth. We have identified two aspen microRNAs (ptr-MIR169a and ptr-MIR169h) which were highly and specifically expressed in dormant floral and vegetative buds. ptr-MIR169a and its target gene *PtrHAP*2–5 showed inverse expression patterns during the dormancy period. ptr-MIR169a transcript steadily increased through the first half of the dormancy period and gradually declined with the approach of active growing season. *PtrHAP*2–5 abundance was higher in the beginning of the dormancy period but rapidly declined thereafter. The decline of *PtrHAP*2–5 correlated with the high levels of ptr-MIR169a accumulation, suggesting miR169-mediated attenuation of the target *PtrHAP*2–5 transcript. We experimentally verified the cleavage of *PtrHAP*2–5 at the predicted miR169a site at the time when *PtrHAP*2–5 transcript decline was observed. HAP2 is a subunit of a nuclear transcription factor Y (NF-Y) complex consisting of two other units, HAP3 and HAP5. Using digital expression profiling we show that poplar *HAP2* and *HAP5* are preferentially detected in dormant tissues. Our study shows that microRNAs play a significant and as of yet unknown and unstudied role in regulating the timing of bud dormancy in trees.

1. Introduction

Dormancy is a temporal suspension of any sign of visible growth of plant structure containing meristem [1], and is a mechanism that allows woody plants to endure freezing and dehydration stress during winter conditions. In most woody plants, including *Populus*, cessation of shoot growth and the induction of dormancy (also known as endodormancy) are either induced or accelerated by short days (SDs), and prevented or delayed by long days (LDs) [2]. Once plants become dormant, they need to meet a chilling requirement, lasting approximately 3 months under near-freezing temperatures, before active growth can resume [3]. Once chilling requirement is fulfilled, resumption of active growth is regulated by temperature [4]. The underpinning molecular regulation of these series of events is still poorly understood.

MicroRNAs (miRNAs[1]) play critical roles in regulation of plant development [5–7]. The functional roles of many miRNA families and their target genes in annual plant development have been elucidated, yet their role in woody perennial biology like for instance regulation of bud dormancy is still poorly understood and studied.

The genome sequence of the *Populus trichocarpa* tree [8] has enabled comparative analysis of miRNAs in annual and woody perennial plant lineages [5]. Among the 20 conserved miRNA gene families, only two (miR159 and miR169) had significantly expanded (doubled) in *Populus*, suggesting a functional diversification of some members related to unique aspects of tree biology [8]. miR169 was first isolated from an *Arabidopsis* small RNA library and was found to be expressed at very low levels compared to other miRNAs in several tissue types [9]. miR169 target genes encode HAP2 (Hem Activator Protein2), a subunit of a transcription factor complex known as NF-Y [5]. NF-Y consists of three subunits (HAP2, HAP3, and HAP5), binds to a CCAAT *cis*-element and assembly of all three is needed before the complex can bind to the DNA to regulate transcription [10,11]. In yeast and vertebrates, each of the HAP subunits is encoded by a single gene [12,13]. In plants, HAP genes have undergone substantial expansion and each subunit is represented by approximately 10 gene family members. This expansion suggests a functional diversification and specialization of HAP genes in plants [14–16].

HAP family members have been found to play roles in regulation of seed dormancy, another type of dormancy process. Mutations in two *Arabidopsis* HAP3 genes, Leafy Cotyledon1 (LEC1) and Leafy Cotyledon-Like1 (LCL1), condition various defects in seed dormancy [17–19]. A poplar HAP2 ortholog was specifically and highly expressed at the onset of cambium dormancy [20].

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[1] miRNA abbreviation will be used in reference to mature ~20 nt molecule while MIRNA will be used to indicate precursor transcript.

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Particularly intriguing is the role of HAP2 in regulation of flowering time in *Arabidopsis*. HAP2 has a protein structure similar to the CCT domain proteins like Constans (CO) [21]. CO can form a complex with HAP3 and HAP5 [21]. The CO/HAP3/HAP5 complex binds to the CCAAT box to activate the Flowering Time (FT) gene [21]. In contrast, upregulation of *HAP2* leads to FT repression and delayed flowering [21], suggesting that HAP2 may impair the formation of a functional CO/HAP3/HAP5 complex and thus repress FT expression and delay flowering. The FT/CO regulatory module in trees in addition to regulating reproductive onset, like in annuals, also provides regulation of bud dormancy [22]. FT promotes active growth, while its absence leads to dormancy [22]. Thus the relative levels of HAP2 and CO can determine the FT abundance and thus the dormancy/growth outcome. The role of poplar CO genes in regulation of dormancy is already established [22]. However the regulatory mechanism of HAP2 and if it plays any role in regulation of dormancy is still unknown.

We show that at least one poplar HAP2 ortholog, *PtrHAP2–5* is regulated during the dormancy period in aspen (*P. tremula*) and this regulation involves posttranscriptional repression mediated via its regulatory ptr-miR169a. Our findings demonstrate a novel regulatory mechanism involved in the control of bud dormancy in *Populus*, and perhaps other woody perennial plants.

2. Materials and methods

2.1. Sequence analyses

Arabidopsis miRNA sequences were obtained from the miRNA registry [23]. Publicly available *Populus* EST and genomic sequences were searched for conserved miRNAs using Blastn searches. Sequences with three or less mismatches were selected as putative miRNAs. All EST sequences were first subjected to CAP3 filtering [24] to remove any redundant sequences, and were then analyzed for the presence of open reading frames using combinations of Blastx searches [25] and GENSCAN predictions [26]. Sequences which showed the presence of protein coding sequence were discarded as putative miRNAs. The remaining sequences were inspected for the presence of secondary structures using MFOLD [27,28] (Supplementary Fig. 1). The predicted fold-back secondary structures were manually inspected for miRNA-like helicity, unpaired residue on both the sense and anti-sense strands (internal loops and bulges), G:U base pairing, miRNA base pairing and free energy for folding as previously described [29].

Primer design was performed using GCG (Accelrys, San Diego, CA). Poplar orthologs of *Arabidopsis* HAP2, 3, and 5 genes were identified via Blastp searches against the poplar genome sequence (phytozome.org). ESTs corresponding to each of the HAP2, 3, and 5 genes were identified using Blastn searches against the NCBI ESTdb database.

2.2. Plant material

Apex, young leaves, and stem tissues were collected from greenhouse-grown aspen (*P. tremuloides*) clone 271 plants. Root tissue was collected from tissue culture-grown plants of the same clone. Vegetative and floral dormant buds were collected from wild aspen plants growing in the vicinity of the Michigan Technological University campus. Collected tissues were frozen in liquid nitrogen and RNA immediately extracted, or the tissue was stored at −80 °C until further processed.

2.3. RNA extraction, cDNA synthesis, and RT-PCR

RNA was extracted from approximately 0.2 g of tissue using a Qiagen RNeasy Mini kit (Qiagen, Valencia, CA). Total RNA was treated with DNA-free™ DNase kit (Ambion, Inc. Austin, TX) for removal of any residual genomic DNA. One microgram of total RNA was used for cDNA synthesis using SuperScript III and oligodT primer (Invitrogen, Carlsbad, CA). RT-PCR was used to measure target gene and primary miRNAs expression. For measuring target gene expression primer pair flanking the putative miR169 target site were designed so that only uncleaved mRNA could be amplified. Gel pictures were obtained via UVP Gel Doc System (UVP, Upland, CA) and quantified using ImageJ software (NIH: http://rsb.info.nih.gov/ij/). All amplicons were sequence-verified prior to performing the analysis. Primers used for RT-PCR were designed using Accelrys GCG software package (Supplementary Table S1).

2.4. Small RNA Northern blots

Five micrograms of total RNA was resolved on a 17% polyacrylamide gel and blotted onto a nylon membrane (Bio-Rad Laboratories, Hercules, CA). DNA oligonucleotides complimentary to miRNA

Table 1
Identification and validation of miRNAs from aspen (*P. tremuloides*).

miRNA	miRNA sequence	Arm	Length	EST/GenomePosition[a]	Northern blot	Identity (%)
ptr-miR156a	UGACAGAAGAGAGUGAGCAC	5′	20	CK087760	+	96.89
ptr-miR159a	UUUGGAUUGAAGGGAGCUCUA	5′	21	BU888582	+	94.81
ptr-miR160a	UGCCUGGCUCCCUGUAUGCCA	5′	21	Chr10:20,626,079–20,626,680	+	95.45
ptr-miR162a	UCGAUAAACCUCUGCAUCCAG	5′	21	Not Amplified	+	–
ptr-miR164a	UGGAGAAGCAGGGCACGUGCA	5′	21	CK113235	+	95.00
ptr-miR166a	UCGGACCAGGCUUCAUUCCCC	3′	21	Chr07:12,408,931–12,409,551	+	97.80
ptr-miR167a	UGAAGCUGCCAGCAUGAUCUA	5′	21	BU886852	+	98.50
ptr-miR168a	UCGCUUGGUGCAGGUCGGGAA	5′	21	BU893331	+	98.20
ptr-miR169a	CAGCCAAGGAUGACUUGCCGA	5′	21	BU865420	+	96.45
ptr-miR169h	UAGCCAAGGAUGACUUGCCUG	5′	21	CK111070	+	92.98
ptr-miR171a	UGAUUGAGCCGCGCCAAUAUC	3′	21	Chr02:10,769,358–10,769,978	+	89.12
ptr-miR172a	AGAAUCUUGAUGAUGCUGCAU	3′	21	Not Amplified	+	–
ptr-miR319c	UUGGACUGAAGGGAGCUCCC	3′	20	Chr19:15,787,236–15,787,8	+	94.12
ptr-miR390a	AAGCUCAGGAGGGAUAGCGCC	3′	21	CK114877	+	97.16
ptr-miR393a	AUCCAAAGGGAUCGCAUUGAUCCU	5′	24	CF231897	+	98.85
ptr-miR395a	CUGAAGUGUUUGGGGGAACUC	5′	21	Chr6:7,613,013–7,613,407	+	87.50
ptr-miR397a	CAUCAUUGAGUGCAGCGUUGAUG	5′	23	BU827068	–	94.39
ptr-miR398a	UGUGUUCUCAGGUCACCCCUUUG	5′	23	AJ774602	–	97.11
ptr-miR399b	UGCCAAAGGAGAGUUGCCCU	3′	20	BU887031	–	97.07
ptr-miR403a	UUAGAUUCACGCACAAACUCG	3′	21	Chr02:15,447,814–15,448,4	+	98.39

[a] Indicates the genome sequence used to design primers for amplification from aspen tissue.

sequences (Supplementary Table S2) were end labeled with γ ^{32}P-ATP using Optikinase (Amersham, Piscataway, NJ). Hybridizations and washings were performed as previously described [30] and blots were exposed overnight at −20 °C.

2.5. 5′-RACE

Validation of target gene cleavage was performed using a modified 5′ RNA ligase-mediated rapid amplification of cDNA ends (5′-RACE) using GeneRacer kit (Invitrogen, Carlsbad, CA), and as previously described [31]. mRNA was purified with an Oligotex mRNA Midi kit (Qiagen, Carlsbad, CA) and directly ligated without further manipulations to GeneRacer RNA oligo. GeneRacer oligodT primer was used for cDNA synthesis. PCR and nested PCR were performed using the 5′, and nested 5′ GeneRacer primers supplied with the kit and gene specific reverse primers (Supplementary Table S1) designed approximately 150 bp downstream of the putative cleavage site. Amplified sequences were cloned in TOPO-TA vector and sequenced using M13 primer.

3. Results

3.1. Cloning and validation of aspen miRNAs

We amplified, cloned, sequenced and validated by Northern blot 20 putative miRNAs using RNA extracted from various aspen (*P. tremuloides*) tissues, organs and developmental stages (Table 1). We were able to amplify, clone and sequence 18 aspen (*P. tremuloides*) precursor miRNA sequences. Comparison to their cottonwood (*P. trichocarpa*) orthologs showed high sequence identity ranging from 87% to 98% (*average* = 96.16%) indicating high degree of sequence conservation at the primary transcript level between closely related lineages (e.g., both aspen and cottonwood belong to genus *Populus*) (Table 1).

Using Northern blots we detected the presence of 17 of the 20 studied miRNAs (Table 1). For all miRNAs we detected one band corresponding to their predicted size. For miR159 and miR319 we detected two bands (Supplementary Fig. 2). These two miRNAs have almost identical sequences with a difference of only three base-pairs. Therefore miR159 and miR319 probes can cross

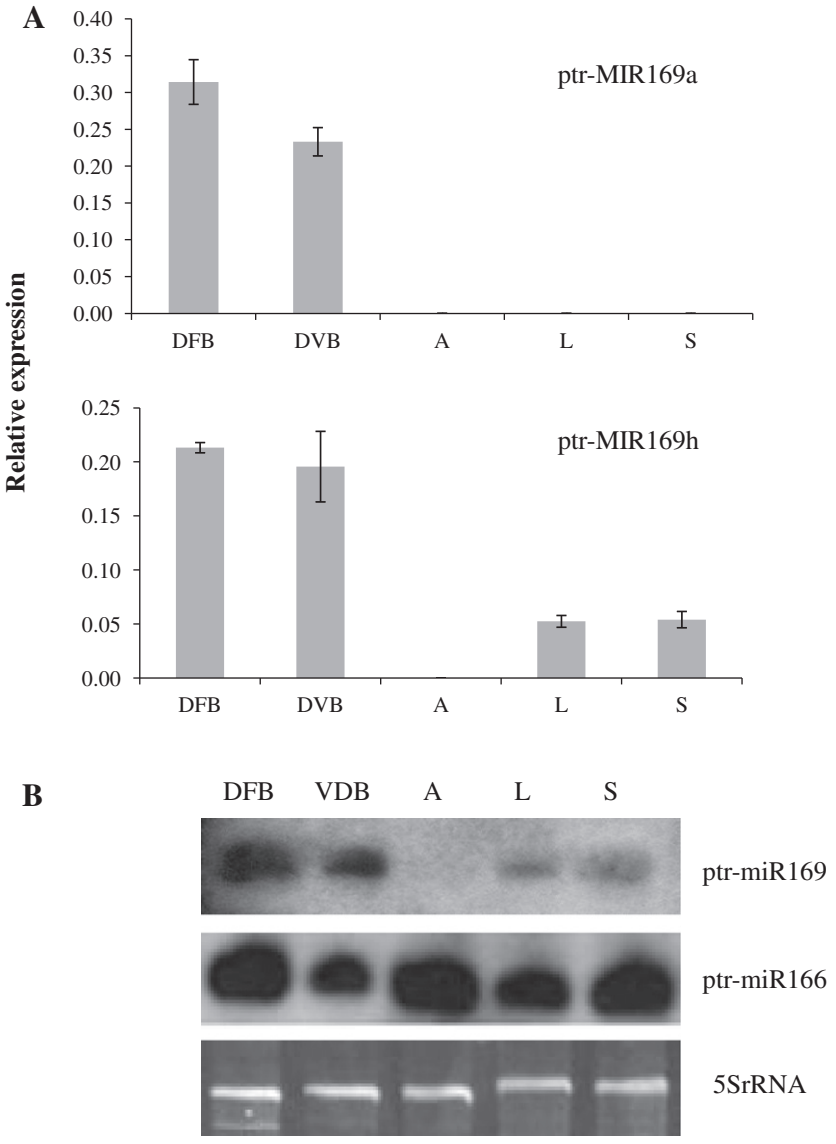

Fig. 1. Expression of precursor (A) and mature (B) ptr-miR169 in dormant and actively growing tissues. Expression estimates in (A) were normalized for loading differences using ubiquitin-like gene. Bars represent standard error of three biological replications. In (B) Northern blot of mature ptr-miR169. DFB: dormant floral buds, VDB: dormant vegetative buds, A: apex, L: leaves, S: stem.

Table 2
Genome position and model number of HAP2 genes with conserved miR169 target sequence in the version 2.2 (v.2.2) and 3 (v.3) of the *Populus* genome sequence.

Name	Gene identifier (v.2.2)	Gene identifier (v.3)	Position of miR169a site
PrtHAP2–1	POPTR_0009s06540	Potri.009G060600	Exon6
PtrHAP2–2	POPTR_0018s07220	Potri.018G064700	3′ UTR
PtrHAP2–3	POPTR_0001s27300	Potri.001G266000	3′ UTR
PtrHAP2–4	POPTR_0001s38100	Potri.001G372100	None
PtrHAP2–5	POPTR_0009s05760	Potri.009G052900	3′ UTR
PtrHAP2–6	POPTR_0006s14740	Potri.006G145100	3′ UTR
PtrHAP2–7	POPTR_0006s21640	Potri.006G201900	3′ UTR
PtrHAP2–8	POPTR_0016s06860	Potri.016G068200	3′ UTR
PtrHAP2–9	POPTR_0011s10080	Potri.011G101000	None
PtrHAP2–10	POPTR_0006s05210	Potri.006G053500	3′ UTR
PtrHAP2–11	POPTR_0001s26480	Potri.001G257600	3′ UTR

hybridize resulting in two bands on the Northern blots. In *Arabidopsis* high expression of miR159 and low expression of miR319 results in detection of only miR159 [32]. Similarly, on our blots *Arabidopsis* miR159 could be readily detected as a single band with higher intensity in wild type (WT) and lower in the *dcl* mutant deficient in the primary transcript processing because of the lesion in the DICER-LIKE enzyme [33]. However in *Populus* tissues both miR159 and miR319 can be readily detected suggesting equal abundance of both and producing two bands (Supplementary Fig. 2).

3.2. Two aspen miR169s differentially accumulate in dormant buds

Using combination of Northern blots and semi-quantitative RT-PCR we found the expression of the mature miR169 to be restricted to dormant buds (Fig. 1). The primary transcript of ptr-MIR169a was only detected in dormant buds (Fig. 1A). Although ptr-MIR169h was also detected in leaves and stems expression level in dormant buds was approximately threefold higher. Correspondingly mature miR169 was detected at high levels in both dormant floral and vegetative buds and low in stems and leaves (Fig. 1B). We did not find expression patters specific to dormant buds in any of the other miRNAs examined during this study.

3.3. miR169 target genes in poplar are upregulated during dormancy induction

Regulatory role of miRNAs is associated with negative regulation of their target genes. In *Arabidopsis* the target genes of miR169 encode HAP2 transcription factors [9]. Using homology searches in the *Populus* genome we found 11 putative HAP2 gene family members (Table 2). We identified the conserved miR169 target site in nine genes. Similar to *Arabidopsis*, eight of these genes had the miR169 target site in the 3′ UTR and only one in an exon region. We took advantage of a published microarray survey of expression changes occurring in poplar prior to entry in dormancy [34]. We identified 5 HAP2 genes whose expression was significantly (ANOVA, $p \leqslant 0.05$) changed during dormancy onset (Fig. 2). All of these five genes were severely downregulated under LD conditions (16 h/8 h light/dark) when the plants were actively growing. All five genes highly increased in expression during SD photoperiod (8 h/16 h light/dark), which is the primary dormancy induction signal, reaching highest levels during 6 weeks in SD. Of the five genes, four had the miR169a target site; only *PtrHAP*2–4 did not have the miR169 site.

3.4. Inverse expression patterns of PtrHAP2–5 is and ptr-MIR169a during dormancy period

We studied the transcript accumulation of both ptr-MIR169a and one of the four PtrHAP2 genes (*PtrHAP*2–5) that have

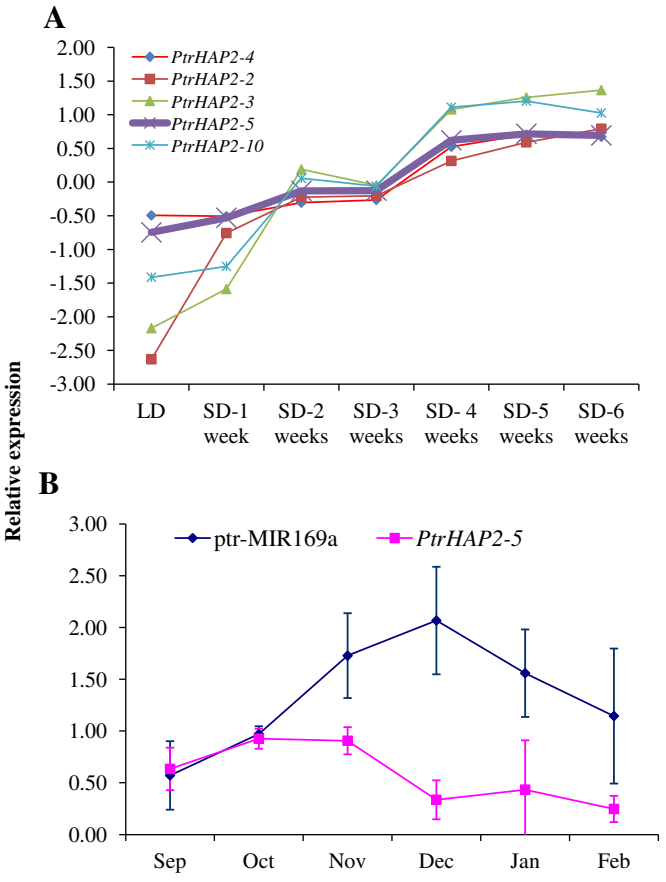

Fig. 2. Expression dynamics of ptr-MIR169 and its target PtrHAP2 genes during dormancy cycle. (A) Expression of 5 PtrHAP2 genes during SD-induced bud dormancy. (B) Expression of ptr-MIR169a and *PtrHAP*2–5 during 6 months of dormancy. Expression values were normalized for loading differences using ubiquitin-like gene. Bars show one standard error over three independent trees mean.

miR169a target site sequence in three wild growing aspen trees during 6 months of the dormancy period (Fig. 2B). The primers for amplification of the target genes were designed on each side of the miR169a target site so that only intact, uncleaved transcript would be amplified. The level of ptr-MIR169a transcript steadily increased through the first half of the dormancy period, reaching peak in December and gradually declining with the approach of active growing season. *PtrHAP*2–5 abundance was high in the beginning of the dormancy period but rapidly declined after November (approximately 3 months into dormancy). The

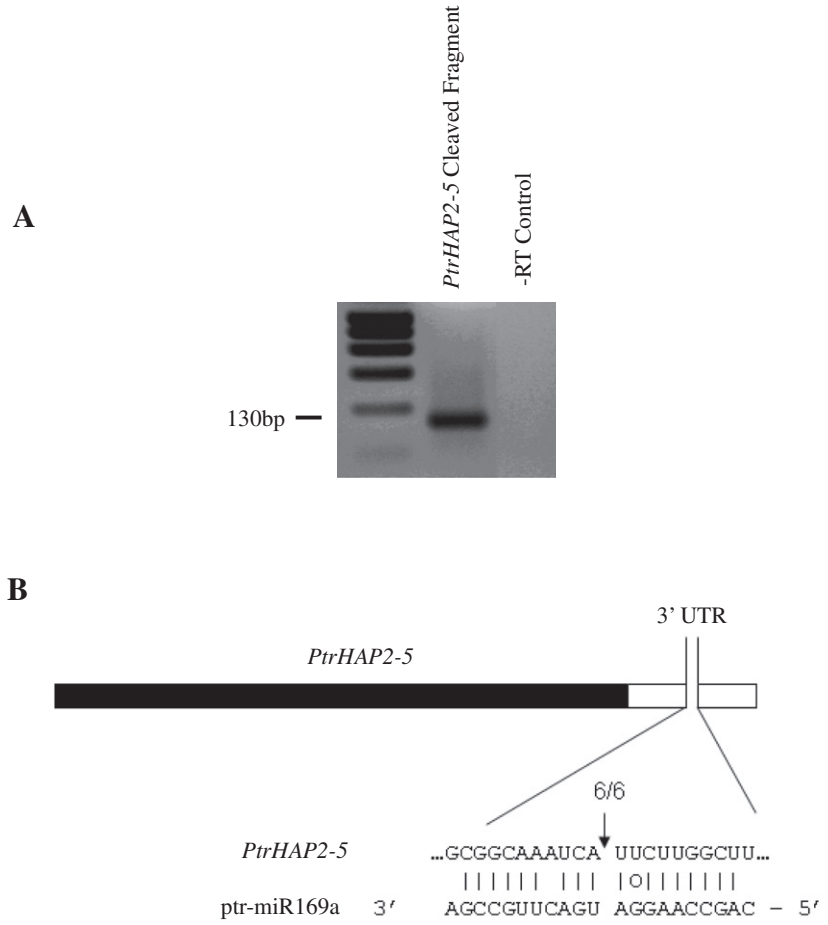

Fig. 3. Experimental validation of the miR169-directed cleavage of *PtrHAP*2–5 mRNA. (A) Amplified cleaved product. (B) Position and sequence of the cleavage. Arrow indicates the position of the cleavage and numbers the frequency of cleavage at this site. The solid black bar represents the coding and the white 3′ UTR region. Exact position of the miR169 shown above the solid bar. 6/6 indicates that 6 out of 6 sequences recovered via 5′-RACE mapped the cleavage product at the same position indicate by the arrow.

decline of *PtrHAP*2–5 correlated with the high levels of ptr-MIR169a accumulation in November and December, suggesting miR169-mediated attenuation of the target *PtrHAP*2–5 transcript.

3.5. ptr-miR169a-directed cleavage of PtrHAP2–5 in dormant buds

Although in plants translational repression has been reported the predominant miRNA mode of action appears to be RNA cleavage [9,35]. The miRNA-directed cleavage can be detected by using a modified form of 5′-RACE [31,36]. The *PtrHAP*2–5 gene carries the miR169a site. To verify that miR169a can initiate direct cleavage of the PtrHAP2–5 mRNA transcript *in vivo* during dormancy as predicted by our expression analysis, we isolated RNA from vegetative dormant buds collected in December and performed the 5′-RACE procedure using primers specific to *PtrHAP*2–5. A single amplification product of the predicted 130 bp size was obtained (Fig. 3A), cloned into TOPO-TA and the fragment sequenced in six different independent clones. In all six cases the 5′ cleaved end of *PtrHAP*2–5 mapped to the nucleotide that pairs with the 10th nucleotide of *ptr*-miR169a corresponding exactly to the predicted cleavage site (Fig. 3B). This indicates that the *PtrHAP*2–5 gene is indeed, at least in part, regulated *in vivo* during dormancy by miR169a-directed cleavage.

3.6. PtrHAP2, and PtrHAP5 EST abundance is associated with dormant tissues

Because HAP2, 3, and 5 bind together to form a regulatory complex, we reasoned that they must be coordinately regulated during dormancy. Using extensive poplar EST resources we searched for EST sequences corresponding to HAP2, HAP3 and HAP5 genes and scored their occurrence in various cDNA libraries (Fig. 4). HAP2 and HAP5 EST occurrence was found to be primarily associated with dormant tissues including dormant vegetative and floral buds as well as dormant cambium. Although we did identify HAP3 ESTs from dormant tissues, their presence in this tissue type was not as prevalent as HAP2 and HAP5.

4. Discussion

We present several lines of evidence indicating putative role(s) of two aspen ptr-MIR169 loci and at least one of their target genes (*PtrHAP*2–5) in regulation of bud dormancy. First, we demonstrated that the precursor ptr-MIR169a and ptr-MIR169h, as well as mature ptr-miR169, are predominantly or exclusively expressed in the dormant vegetative and floral buds. Second, we found that the expression dynamics of ptr-MIR169a and one of its target genes, *PtrHAP*2–5, were inversely correlated, particularly in the first half of the dormancy period when increase in ptr-MIR169a

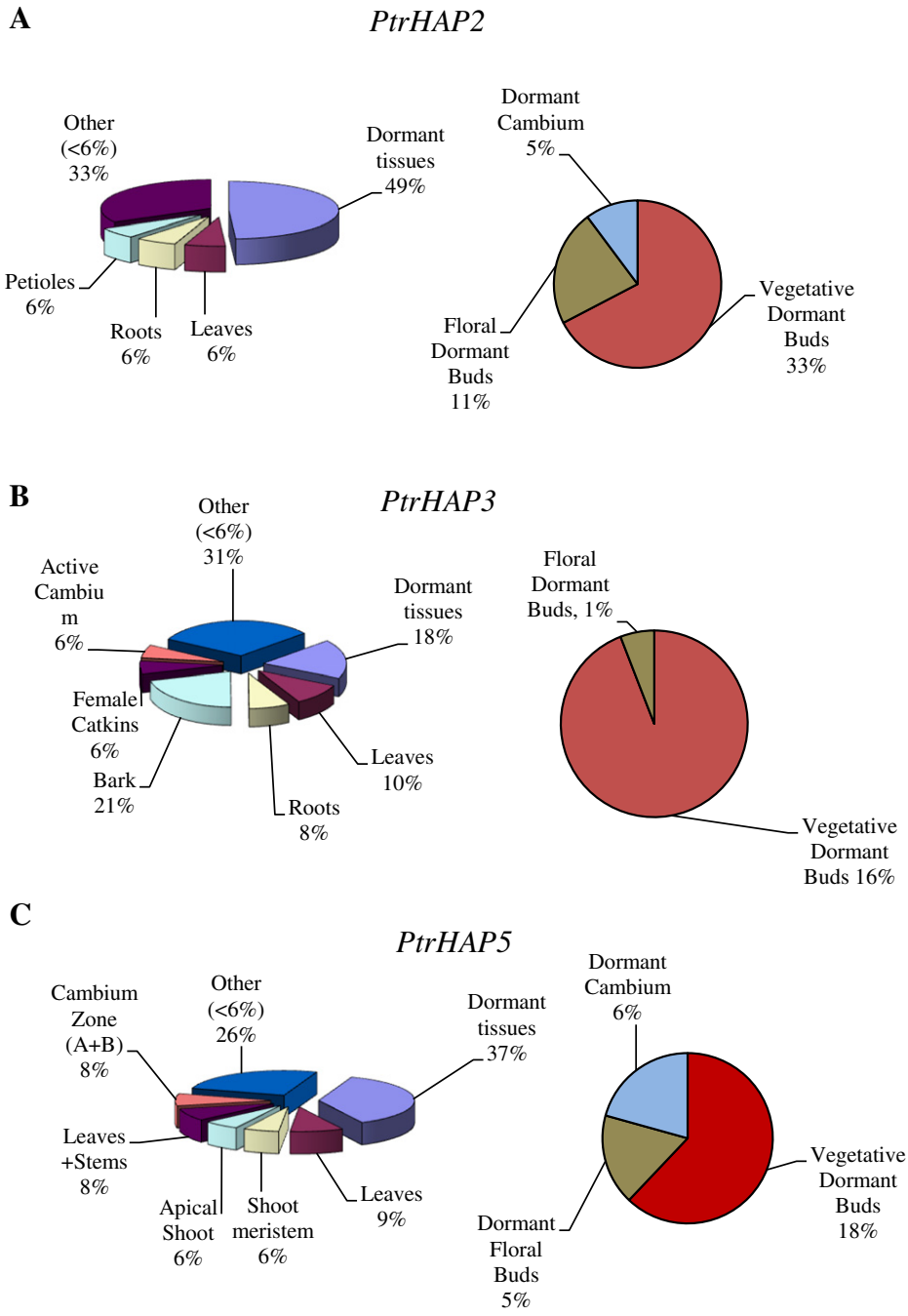

Fig. 4. Abundance of poplar HAP2 (A), HAP3 (B), and HAP5 (C) homologous sequences in poplar ESTs. Pie charts to the right indicate the breakdown of the dormant tissues shown in the left chart into different tissue types.

abundance correlated with decline in *PtrHAP*2–5 transcript level, suggesting of a miR169-mediated attenuation. Nearly half of the HAP2 gene family members (5/11) are significantly upregulated during dormancy induction. Third, using 5′-RACE we demonstrated *in vivo* that *PtrHAP*2–5 transcript is indeed cleaved at the predicted cleavage site. Finally, the HAP2 gene is part of a transcriptional regulatory complex, and we provide evidence that at least one of the complex partners, HAP5, is predominantly expressed in dormant tissues.

Our data suggest that high expression of PtrHAP2 genes, specifically *PtrHAP*2–5 at the onset of dormancy creates a cellular environment in vegetative buds promoting entry into dormancy. The molecular mechanism is still unclear but the most likely connection is through regulation of the FT gene(s). In aspen trees, the poplar ortholog of FT (*PtrFT*) induces first time flowering and prevents cessation of growth which precedes entry into dormancy [22]. *PtrFT* is activated by a poplar ortholog of CO (PtrCO) under LD. This activation promotes active growth and prevents entry into dormancy. Under SD, PtrCO circadian-rhythm-entrained repression leads to decline of *PtrFT* transcript, growth cessation, and subsequently dormancy. Recent evidence in *Arabidopsis* shows that CO can act as transcription factor by binding directly to the FT promoter and activating transcription [21,37]. Alternatively, CO can replace HAP2 in the HAP2/HAP3/HAP5 complex. HAP2 incorporation in the HAP complex has a negative effect on FT expression and flowering [21]. Overexpression of HAP2 leads to delay of

flowering and low *FT* levels [21]. Thus we believe that incorporation of CO and HAP2 in the HAP complex may have the opposite effect on FT transcription activity. Therefore in aspen, high level of PtrHAP2 and low levels of PtrCO transcription factors in the fall as shown in this study lead to PtrHAP2 predominant incorporation and repression of *PtrFT* genes which sets the stage for dormancy induction. The gradual quenching of PtrHAP2 transcript abundance via miR169-mediated degradation throughout the dormancy period leads to relief of *PtrFT* gene repression and return to growth. The temporal regulation of PtrHAP2 by ptr-miR169 is reminiscent of a molecular clock that measures the duration of winter. Similar temporal regulation of miRNA/target gene expression associated with respective regulation of timing of developmental events has been reported in both plants and animals [38–40].

Precise regulation of bud dormancy is critical for adaptation of wild and domesticated woody perennial species. Understanding the involved regulator mechanisms can therefore lead to improved methods for helping wild and cultivated perennial plants to cope with environmental variation, including climate change.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.027.

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