

Expression patterns of conserved microRNAs in the male gametophyte of loblolly pine (*Pinus taeda*)

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Abstract MicroRNAs (miRNAs) are small RNAs that regulate genes involved in various aspects of plant development, but their presence and expression patterns in the male gametophytes of gymnosperms have not yet been established. Therefore, this study identified and compared the expression patterns of conserved miRNAs from two stages of the male gametophyte of loblolly pine (*Pinus taeda*), which are the mature (ungerminated) and germinated pollen. Microarray was used to identify conserved miRNAs that varied in expression between these two stages of the loblolly pine male gametophyte. Forty-seven conserved miRNAs showed significantly different expression levels between mature and germinated loblolly pine pollen. In particular, miRNAs representing 14 and 8 families were up- and down-regulated in germinated loblolly pine pollen, respectively. qRT-PCR was used to validate their expression patterns using representative miRNAs. Target genes and proteins were identified using psRNATarget program. Predicted targets of the 22 miRNA families belong mostly to classes of genes involved in defense/stress response, metabolism, regulation, and signaling. qRT-PCR was also used to validate the expression patterns of representative target genes. This study shows that conserved miRNAs are expressed in mature and germinated loblolly pine pollen.

Many of these miRNAs are differentially expressed, which indicates that the two stages of the male gametophyte examined are regulated at the miRNA level. This study also expands our knowledge of the male gametophytes of seed plants by providing insights on some similarities and differences in the types and expression patterns of conserved miRNAs between loblolly pine with those of rice and Arabidopsis.

Keywords Germination · Conifers · Germinated pollen · Pollen tube · Gymnosperms · Phase change

Introduction

MicroRNAs (miRNAs) make up a minute portion of the genome, but play important roles in post-transcriptional gene regulation through cleavage of target mRNAs or translational repression (Brodersen et al. 2008; Carthew and Sontheimer 2009). Plant miRNAs have a remarkable propensity to target genes involved in developmental processes, phase transitions, metabolism, and defense and stress responses (Jones-Rhoades et al. 2006; Mallory and Vaucheret 2006; Chen 2009; Lelandais-Briere et al. 2010). Many miRNAs are deeply conserved in land plants (Floyd and Bowman 2004; Axtell and Bartel 2005; Kidner and Martienssen 2005), but many have been found to be taxa, tissue, or stage specific (Arazi et al. 2005; Lu et al. 2005; Zhang et al. 2006a, b, 2010; Zhan and Lukens 2010). All these support the notion that the regulation of conserved miRNA activity at various stages of growth and in specific cell types is of central importance for normal plant development. In seed plants, our knowledge of miRNAs is mostly from reports on structures representing the sporophyte (diploid) phase of the life cycle (e.g., embryos,

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seedlings, leaves, stems, wood, etc.) (Floyd and Bowman 2004; Axtell and Bartel 2005; Jones-Rhoades et al. 2006; Lu et al. 2007; Chen 2009; Zhao et al. 2010; Zhang et al. 2012). Several studies have also demonstrated miRNA expression from the male gametophyte (haploid) phase in various species of angiosperms (Fujioka et al. 2008; Chamber and Shuang 2009; Grant-Downton et al. 2009; Grant-Downton 2010; Le Trionnaire and Twell 2010; Borges et al. 2011; Le Trionnaire et al. 2011; Wei et al. 2011; Peng et al. 2012), but none from any gymnosperm.

In seed plants, the male gametophyte phase produces the mature pollen grain which is typically formed in the microsporangia in the pollen cones of gymnosperms and anthers in the flowers of angiosperms. It becomes dispersed and typically ends up in the ovule (in gymnosperms) or stigma (in angiosperms) where it germinates and forms a pollen tube that generally functions to deliver the sperm to the egg. In conifers, mature pollen grains are dispersed at one- to five-cell stage, except in Podocarpaceae where the prothallial cells may proliferate and so their pollen grains may be shed with as many as 40 nuclei (Fernando et al. 2010). Pine pollen grains are typically composed of five cells at the time that they are released from the pollen cones. Three of these cells (two prothallial cells and a sterile cell) have no known function in post-pollination processes, whereas the vegetative and generative cells are involved in pollen tube growth and gamete formation, respectively. Pine pollen germinates on the nucellus of the ovule within 5 days from pollination, and the pollen tube grows to about half of the thickness of the nucellus and then ceases to elongate. The pollen tube remains dormant for about a year inside the ovule with the generative cell remaining undivided. Therefore, much of the post-pollination processes in pines and conifers in general are involved in pollen tube growth. There are many other major morphological and developmental differences between the male gametophytes of gymnosperms and angiosperms (Singh 1978; Gifford and Foster 1989; Fernando et al. 2010; Williams 2012), but it remains to be seen whether these are accompanied by major differences at the molecular level. Nevertheless, proper regulation of gene expression is necessary in the transition of the male gametophyte from the mature (ungerminated) to germinated phase, two stages that are spatially and temporally separated in vivo and represent pre-pollination and post-pollination stages, respectively. As such, many transcripts and proteins have been shown to be differentially expressed between mature and germinated pollen in gymnosperms (Fernando 2005; Chen et al. 2006) and angiosperms (Dai et al. 2007; Zou et al. 2009; Zhu et al. 2011) and likely include the miRNAs that are involved in modulating these two stages. In angiosperms, many miRNAs show dynamic and complex changes related to the development of

microspores to mature pollen with expressions that are distinct from those of the sporophytic tissues (Fujioka et al. 2008; Chambers and Shuai 2009; Grant-Downton et al. 2009; Borges et al. 2011; Le Trionnaire et al. 2011; Wei et al. 2011; Peng et al. 2012). MiRNAs that are associated with bicellular and tricellular pollen of rice have also been described, and many differences between their miRNA profiles have been found (Peng et al. 2012). Thus far, our knowledge of spatiotemporal difference in miRNA expression during male gametophyte development in seed plants is confined to pre-pollination stages. Therefore, much remains to be discovered regarding the miRNAs in the male gametophyte phase, particularly those that are related to the post-pollination stages such as the transition from mature to germinated phase.

There is limited information about miRNA analysis in gymnosperms and almost entirely based on sporophytic tissues from conifers. Lu et al. (2007) identified miRNAs from loblolly pine (*Pinus taeda*) stem xylem and examined their expressions in relation to fusiform gall development. Qiu et al. (2009) examined the miRNAs that are up-regulated in a *Taxus chinensis* somatic cell line upon exposure to methyl jasmonate. Using Norway spruce (*Picea abies*) seedlings, novel and conserved miRNAs were identified, but most of which targeted unknown genes (Yakovlev et al. 2010). In Japanese larch (*Larix leptolepis*), four miRNA families are differentially expressed between embryonic and non-embryonic callus (Zhang et al. 2010). Stage-specific expression of nine representative miRNAs and their targets demonstrated their potential in modulating somatic embryogenesis in Japanese larch (Zhang et al. 2012). A remarkable finding regarding large-scale comparison of miRNAs in gymnosperms (including *Cycas*, *Ginkgo*, and *Ephedra* but mostly conifers) versus angiosperms is the prevalence of 21nt long small RNAs, which has been considered as a gymnosperm-unique silencing signature and is believed to be related to specific Dicer-like family genes in gymnosperms (Dolgosheina et al. 2008; Morin et al. 2008; Yakovlev et al. 2010). However, recent reports indicate that this size bias is associated only with vegetative organs and tissues since reproductive structures show appreciable amounts of 24nt long small RNAs (Wan et al. 2012a; Nystedt et al. 2013; Zhang et al. 2013). The only report on gametophytic tissue expression in gymnosperm is that of Oh et al. (2008), which compared the expression of five conserved miRNAs between the female gametophyte and zygotic embryo of loblolly pine. To fill the gap in our knowledge of miRNA expression in the male gametophytes in gymnosperms, this study aimed to identify conserved miRNAs from mature and germinated pollen of loblolly pine through microarray analysis, validate their expression patterns through qRT-PCR, and predict their target genes and functions using web-based computational approaches.

Materials and methods

Collection and in vitro germination of pollen grains

Pollen cones were collected from several loblolly pines (*P. taeda*) growing in the campus of North Carolina State University, Raleigh, NC, 1–2 days before pollen grains were released from the microsporangia. The pollen grains at this stage are five-celled and are in the process of dehydration in preparation for dormancy and dispersal. The pollen cones per individual tree were labeled and separately processed for surface sterilization following Fernando et al. (1997). Prior to storage of the sterile pollen grains at -80°C , average pollen germination was determined to be 98 %. Total RNA was isolated from the frozen pollen grains to represent the mature pollen used in the study. For germinated pollen, stored pollen grains were evenly dispensed on modified Brewbaker and Kwack (1963) medium (pH 5.8) composed of $0.1\text{ mg ml}^{-1}\text{ H}_3\text{BO}_3$, $0.3\text{ mg ml}^{-1}\text{ Ca}_2\text{NO}_3$, $0.2\text{ mg ml}^{-1}\text{ MgSO}_4$, and $0.1\text{ mg ml}^{-1}\text{ KNO}_3$, supplemented with 145 mM sucrose and 0.4 % phytigel (Fernando et al. 1997). A 25-mm nitrocellulose membrane (Millipore, Billerica, MA) was placed on the surface of the solidified germination medium, and 30 mg pollen grains were dispensed onto the membrane by gently shaking a sterile spatula containing pollen grains over it. The culture plates were incubated at 25°C in the dark for 2 days (48 h) to allow pollen grains to germinate. Pollen grains were considered germinated if the pollen tube was at least as long as the diameter of the pollen grain. Average pollen germination was 98 %. DAPI staining indicates that both mature and germinated pollen grains are composed of five nuclei.

RNA preparation, miRNA array, and data analysis

Using pollen grains from three different loblolly pine individuals as biological replicates, total RNA was extracted from mature (directly from frozen and stored pollen grains) and germinated (2-day-old pollen cultures) pollen using TRIzol Reagent (Invitrogen, Carlsbad, CA). The three biological samples from mature pollen were designated as U1, U2, and U3 and for germinated pollen as G1, G2, and G3. Total RNAs from these six samples were sent to LC Sciences Inc. (Houston, TX) for the miRNA array procedure. Briefly, this involved the use of a custom $\mu\text{paraflo}^{\text{TM}}$ microfluidic array containing MiRBase (Release 10.1) plant miRNA probes. The quality of the total RNA was verified, and the samples were size fractionated to isolate the small RNAs ($<300\text{nt}$), to which 3' poly (A) tails were added. An oligonucleotide tag was ligated to the poly (A) tail for use in fluorescent dye staining. The 20mer RNA controls, PUC2PM-20B and

PUC2MM-20B, were spiked into the small RNA samples for use in quality control during the miRNA array assay. The small RNAs were then labeled with Cy3 or Cy5 fluorescent dyes and hybridized to a dual-channel $\mu\text{paraflo}^{\text{TM}}$ microfluidics chip. The chip contained probes of chemically modified nucleotide-coding segments complementary to the 653 unique plant miRNA sequences from MiRBase (Release 10.1), representing miRNAs from 17 plant species. For quality control, the chip also included probes for maize 5S rRNA, an inner positive control, and PUC2-20B (an artificial non-homologous nucleic acid), an external positive control. Blank and non-homologous nucleic acids were also used as negative controls. Following hybridization, images from the chip were collected with a laser scanner and digitized using Array-Pro image analysis software (Media Cybernetics, Silver Springs, MD). The signal values from the miRNA array were corrected by subtracting the background using a local regression method. To remove any system-related signal variations, the data were also normalized to the statistical mean of all detectable transcripts using a cyclic locally weighted regression (LOWESS) filter following background subtraction. The log-transformed ratios of the detected signals for mature and germinated samples were calculated along with the p values of the t test. Only miRNAs with log ratios of one or more and negative one or less (which were equal to fold changes of two or greater) and p values of equal to or less than 0.01 were considered as differentially expressed.

qRT-PCR and data analysis

The expressions of 12 representative differentially expressed conserved miRNAs representing 12 different families were validated through qRT-PCR using the NCode SYBR GreenER miRNA qRT-PCR kit (Invitrogen, Carlsbad, CA). The specific miRNAs used to represent the family were chosen generally, based on those that showed the highest and lowest expression levels from the microarray analysis. For each miRNA and the internal reference (Supplement 1), three biological replicates were performed for both mature (U1, U2, and U3) and germinated (G1, G2, and G3) loblolly pine pollen. Sequence-specific primers were used (Supplement 1). Primer specific for *P. taeda* 5S rRNA was used as the reference for internal normalization for comparative C_T analyses. All qRT-PCRs were performed in a MiniOpticon Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Results were viewed using the CFX Manager Software (Bio-Rad, Hercules, CA) and exported to Microsoft Excel to calculate the mean C_T , ΔC_T , $\Delta\Delta C_T$, and fold change. The fold change was calculated as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_T$ germinated pollen $-\Delta C_T$ mature pollen and $\Delta C_T = C_T$ target miRNA $-C_T$ of

reference RNA. A fold change ≥ 1 indicated an increased expression of the miRNA in germinated pollen as compared to mature pollen. For fold change values < 1 , the negative inverse was calculated to indicate the fold change reduction in expression in germinated pollen. A fold change of -1.0 or lower indicated decreased miRNA expression in germinated pollen as compared to mature pollen.

Target prediction for conserved miRNAs

Mature miRNAs sequences from each of the representative miRNAs were retrieved from miRBase (<http://www.sanger.ac.uk/Software/Rfam/mirna/>). Their potential targets were determined by searching for complementary hits using the psRNATarget program (<http://plantgrn.noble.org/psRNATarget/>) with default parameters (Dai and Zhao 2011) using the *Pinus* (Pine) DFCI Gene Index version 9 as the source of sequence information. The psRNATarget program used the scoring scheme of miRU by Zhang (2005) where the “maximum expectation” was considered as the threshold of the score. The miRNA and target pair were discarded if the score was greater than the threshold, which was set at a default value of 3.0. More stringent cutoff threshold was 0–2.0 for lower false-positive prediction, and although not used in this study, more relaxed cutoff threshold was 4.0–5.0 for higher prediction coverage according to Zhang (2005). The psRNATarget program also provided accession numbers of target genes and identity of the target proteins based on complementary matches with other species. Identities of target proteins presented in Tables 3 and 4 were limited to land plant species when available.

Validation of potential target genes by qRT-PCR

Total RNA was isolated from mature and germinated loblolly pine pollen and from three different individuals, as described above. cDNA synthesis was done using QuantiTect Reverse Transcription Kit following the manufacturer’s protocol (Qiagen, Valencia, CA). Primers were designed for the potential targets of pta-miR166 and pta-miR951 based specifically on sequences derived from the Plant Gene Index (<http://compbio.dfci.harvard.edu/tgi/tgipage.html>), which were TC189536 and DR054135, respectively (Tables 3, 4). Primers for TC189536 are 5′-GATGCTAGCCCCGCTGG A-3′ and 5′-TGCTGGAGGCACATTCTGTAA-3′; primers for DR054135 are 5′-ACAATTGGCCTTTCATCTGC-3′ and 5′-TAAGTTCTGCCCTCCTCCT-3′. The 18s rRNA was used as the reference gene (using 5′-CATGGCCGTT CTTAGTTGGT-3′ and 5′-GAGTTGATGACACGCGCT TA-3′ as left and right primers, respectively). qRT-PCR was done in triplicate for each combination of primers and cDNA

Table 1 Differentially expressed conserved miRNAs from loblolly pine male gametophyte based on microarray analysis

miRNA	Log2	Fold change	miRNA	Log2	Fold change
Up-regulated in germinated pollen			osa-miR166g	1.03	2.06
tae-miR1125	3.15	6.3	ath-miR166a	1.01	2.02
ath-miR395b	3.11	6.22	ath-miR162a	1.00	2.0
mtr-miR395g	2.7	5.4	bna-miR169g	1.00	2.0
osa-miR395	2.65	5.3	Down-regulated in germinated pollen		
mtr-miR395a	2.57	5.14	pta-miR946a	-1.0	-2.0
vvi-miR396b	2.49	4.98	ath-miR844	-1.2	-2.4
mtr-miR395h	2.39	4.78	ptc-miR474b	-1.34	-2.68
vvi-miR396a	2.22	4.44	ptc-miR482	-1.35	-2.7
ath-miR390a	1.91	3.82	ptc-miR474a	-1.36	-2.72
zma-miR171a	1.84	3.68	ptc-miR474c	-1.37	-2.74
ptc-miR397b	1.70	3.4	pta-miR950a	-1.41	-2.82
ath-miR397a	1.65	3.3	ptc-miR156 k	-1.43	-2.86
ptc-miR169z	1.63	3.26	sbi-miR156e	-1.44	-2.88
ath-miR171a	1.62	3.24	osa-miR168a	-1.47	-2.94
ppt-miR1026a	1.57	3.14	ath-miR156h	-1.55	-3.1
osa-miR169d	1.49	2.98	smo-miR156c	-1.61	-3.32
pta-miR947	1.41	2.82	ath-miR168b	-1.84	-3.68
ptc-miR169aa	1.31	2.62	pta-miR482a	-1.85	-3.7
osa-miR169n	1.22	2.44	pta-miR951	-1.88	-3.76
ppt-miR901	1.2	2.4	sof-miR168b	-1.9	-3.8
ppt-miR395	1.12	2.24	ath-miR157a	-1.9	-3.8
osa-miR166m	1.11	2.22	osa-miR168b	-1.91	-3.82
ath-miR854a	1.11	2.22	pta-miR156b	-1.95	-3.9
ppt-miR903	1.1	2.2			

templates including the reference gene using QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA). Calculations for the relative expression levels were as described above.

Results

Differentially expressed conserved miRNAs

MiRNA array analysis revealed that of the 653 unique land plant miRNA sequences examined, 208 were detected in both mature and germinated loblolly pine pollen, but the expression levels of most of these were not significantly different. However, the expressions of 47 individual miRNAs showed significant changes between mature and germinated pollen (Table 1). Of this, 28 individual miRNAs were up-regulated in germinated pollen compared to mature pollen with fold changes that ranged from 2.00 to 6.3 (Table 1). On the other hand, 19 individual miRNAs were down-regulated in germinated pollen as compared to

Table 2 Representative differentially expressed conserved miRNAs in the male gametophyte of loblolly pine based on qRT-PCR analysis

	Mean C_T	ΔC_T	$\Delta\Delta C_T$		Fold change	
	Mature	Germinated	Mature	Germinated		
ath-miR390a	25.73	23.79	-7.06	-10.90	-3.84	14.3
tae-miR1125	23.47	23.03	-7.15	-10.31	-3.16	8.9
ppt-miR903	33.63	33.50	0.83	-1.18	-2.01	4.0
ath-miR395b	36.19	36.05	0.41	-1.40	-1.81	3.5
ath-miR854a	28.11	28.74	-5.52	-6.44	-0.92	1.9
ppt-miR901	32.24	33.04	-3.54	-4.41	-0.87	1.8
ptc-miR397b	36.12	37.12	0.35	-0.33	-0.68	1.6
pta-miR950a	31.33	35.20	-3.19	-2.65	0.54	-1.5
ath-miR844	38.56	37.68	3.37	4.34	0.97	-2.0
pta-miR946a	33.65	32.88	-1.56	-0.46	1.10	-2.1
osa-miR168b	35.39	37.10	1.32	2.93	1.61	-3.1
pta-miR156b	36.11	34.48	-2.48	-0.71	1.77	-3.4

mature pollen with fold change reductions that ranged from -2.0 to -3.9 (Table 1). The expressions of 12 individual miRNAs representing 12 different families were verified through qRT-PCR (Table 2). The results showed the same miRNAs that were up-regulated and down-regulated as those obtained through microarray analysis, with fold change values of 1.6–14.3 and -1.5 to -3.4, respectively (Table 2). The 47 differentially expressed conserved miRNAs represent a total of 22 families, where 14 families were up-regulated (Tables 1, 3), while eight families were down-regulated (Tables 1, 4).

Potential targets of conserved miRNAs

To gain insights on the target genes and functions of the conserved miRNAs which were differentially expressed in the male gametophyte of loblolly pine, the web-based program psRNATarget was utilized to predict their potential targets using the *Pinus* DFCI Gene Index as source of mRNA sequences. For the 28 up-regulated individual miRNAs, which represent 14 families, 54 target genes were predicted (Table 3). Although information on the identities of target proteins and functions were not known for all the target genes, target protein identity was available for at least one of the target genes from most of the miRNA families, except for miR901, miR903, and miR1125 (Table 3). For 19 down-regulated individual miRNAs, which represent eight families, 32 target genes were predicted (Table 4). Information on the identities of target genes and functions were available for all of the families (Table 4). For both types of differentially expressed miRNAs, the majority of the functions were related to defense/stress response, followed by metabolism, gene regulation, and signaling. Two of the potential targets were verified through qRT-PCR, and the results showed

that their expression patterns were inversely proportional with those of their corresponding miRNAs (Fig. 1).

Discussion

This study has revealed 47 conserved miRNAs representing 22 families that are differentially expressed between the two stages of the male gametophyte of loblolly pine that have been examined. The change in the expressions of these miRNAs highlights their dynamic nature and implies their involvement in the transition of the male gametophyte from mature (ungerminated) to germinated phase. Many of the 22 miRNA families are also expressed in the mature pollen of rice and/or Arabidopsis including miR156/157, miR162, miR166, miR168, miR169, miR171, miR390, miR395, miR396, miR397, miR844, and miR854 (Chambers and Shuai 2009; Grant-Downton 2010; Le Trionnaire and Twell 2010; Borges et al. 2011; Le Trionnaire et al. 2011; Peng et al. 2012). Interestingly, miR156/157 and miR168, which have been shown to be two of the most highly expressed miRNAs in the mature pollen of rice and Arabidopsis (Chambers and Shuai 2009; Grant-Downton et al. 2009; Borges et al. 2011; Le Trionnaire et al. 2011), are also the two most highly expressed miRNAs in the mature loblolly pine pollen. Whereas the level of the expression of miR156/157 and miR168 is not known in germinated rice or Arabidopsis pollen, these two miRNAs and miR844 showed reduced expression in germinated loblolly pine pollen. This implies that in loblolly pine, these three miRNAs exert greater roles in the formation of the mature pollen than in germinated pollen. Beside these three miRNAs, the eight other miRNAs indicated above are all up-regulated in germinated loblolly pine pollen. Interestingly, the expression pattern of the target gene of one of

Table 3 Predicted targets of up-regulated conserved miRNAs expressed in germinated loblolly pine pollen using the psRNA Target program (the dashes indicate that no target gene function can be inferred)

miRNA	Target gene	Target protein	Score	Target function
miR162	TC400535	Endoribonuclease Dicer homolog (<i>Arabidopsis thaliana</i>)	2.0	Gene regulation
	TC365441	Cytochrome P450-like protein (<i>Arabidopsis thaliana</i>)	3.0	Metabolism
miR166	TC189536	Class III HD-Zip protein HDZ31 (<i>Pinus taeda</i>)	2.0	Gene regulation
	TC183302	Class III HD-Zip protein HDZ32 (<i>Pinus taeda</i>)	2.0	Gene regulation
	CX713974	Class III HD-Zip protein HDZ33 (<i>Pinus taeda</i>)	2.0	Gene regulation
	BX253120	Class III homeodomain-leucine zipper protein (<i>Marchantia polymorpha</i>)	2.0	Gene regulation
miR169	TC173151	CCAAT-binding transcription factor (<i>Oryza sativa</i>)	2.5	Gene regulation
	CF390605	Aminotransferase 3 chloroplast precursor (<i>Arabidopsis thaliana</i>)	2.5	Metabolism
	TC184461	Predicted protein (<i>Physcomitrella patens</i>)	2.5	–
	TC162405	Chloroplast threonine deaminase (<i>Pinus taeda</i>)	3.0	Metabolism
	TC159690	Delta-Selinene-like synthase (<i>Picea sitchensis</i>)	3.0	Defense/stress response
	TC165799	Predicted protein (<i>Physcomitrella patens</i>)	3.0	–
	TC172291	Predicted protein (<i>Physcomitrella patens</i>)	3.0	–
TC156602	Predicted protein (<i>Physcomitrella patens</i>)	3.0	–	
miR171	TC154597	F6N15.20 protein/SCL-like (<i>Arabidopsis thaliana</i>)	0.5	Gene regulation
miR390	CT579103	No description	1.5	–
	TC164578	No description	1.5	–
	TC174360	No description	2.5	–
	GT234481	Harpin-induced protein 1 containing protein (<i>Oryza sativa</i>)	3.0	Defense/stress response
miR395	TC172139	ATP sulfurylase/APS kinase (<i>Arabidopsis thaliana</i>)	2.5	Signaling
	TC163088	SET domain-containing protein-like (<i>Oryza sativa</i>)	3.0	Gene regulation
miR396	TC154673	Chromosome undetermined scaffold_358 (<i>Vitis vinifera</i>)	2.0	–
	TC170132	Heat-shock protein 81 (<i>Arabidopsis thaliana</i>)	2.5	Defense/stress response
	TC158937	Chromosome chr3 scaffold_8 (<i>Vitis vinifera</i>)	2.5	–
	TC159334	Cysteine-rich repeat secretory protein 55 precursor (<i>Arabidopsis thaliana</i>)	2.5	–
miR397	TC191439	Laccase (<i>Pinus taeda</i>)	1.5	Defense/stress response
	TC196348	Laccase (<i>Pinus taeda</i>)	1.5	Defense/stress response
	TC159420	Laccase (<i>Pinus taeda</i>)	1.5	Defense/stress response
	TC188042	Laccase (<i>Pinus taeda</i>)	1.5	Defense/stress response
	TC156315	Laccase (<i>Pinus taeda</i>)	1.5	Defense/stress response
	TC167989	Laccase-like multicopper oxidase (<i>Ginkgo biloba</i>)	2.0	Defense/stress response
	TC172735	Laccase (<i>Pinus taeda</i>)	2.5	Defense/stress response
	TC194089	Laccase (<i>Pinus taeda</i>)	2.5	Defense/stress response
	TC183930	Laccase (<i>Pinus taeda</i>)	2.5	Defense/stress response
	BX680150	Laccase (<i>Pinus taeda</i>)	2.5	Defense/stress response
	CD022020	Laccase-like multicopper oxidase (<i>Ginkgo biloba</i>)	2.5	Defense/stress response
TC196093	Laccase (<i>Pinus taeda</i>)	3.0	Defense/stress response	
miR854	AW755221	Os11g0287000 protein (<i>Oryza sativa</i>)	2.0	–
	T C179619	Formin-like protein 1 precursor (<i>Arabidopsis thaliana</i>)	2.5	Metabolism
	TC187328	Transaldolase ToTAL2 (<i>Solanum lycopersicum</i>)	3.0	Metabolism
	GT253886	Transaldolase-like protein (<i>Arabidopsis thaliana</i>)	3.0	Metabolism
	TC159839	Transaldolase ToTAL2 (<i>Arabidopsis thaliana</i>)	3.0	Metabolism
miR901	TC189512	Chromosome chr4 scaffold_83 (<i>Vitis vinifera</i>)	3.0	–
	TC169943	Os04g0378200 protein (<i>Oryza sativa</i>)	3.0	–
miR903	TC190174	Chromosome chr8 scaffold_88 (<i>Vitis vinifera</i>)	3.0	–
	TC158355	Predicted protein (<i>Physcomitrella patens</i>)	3.0	–

Table 3 continued

miRNA	Target gene	Target protein	Score	Target function
miR947	TC177502	Lipase (<i>Vibrio parahaemolyticus</i>)	0.0	Metabolism
	TC163947	No description	1.5	–
	TC180959	No description	2.0	–
	TC198377	No description	2.0	–
	TC191249	Metalloprotease (<i>Aspergillus oryza</i>)	3.0	–
miR1026	CT581705	Predicted protein (<i>Physcomitrella patens</i>)	2.0	–
	TC165721	Plastidial delta-12 oleate desaturase (<i>Olea europaea</i>)	2.0	Metabolism
miR1125	TC160125	Chromosome chr12 scaffold_18 (<i>Vitis vinifera</i>)	3.0	–

these, i.e., pta-miR166, which encodes a Class III HD-Zip protein, was inversely proportional to the expression pattern of its miRNA. The expression patterns of these eight miRNAs in germinated rice and/or Arabidopsis are not known. Our results show that conserved miRNAs expressed in mature pollen are also expressed in germinated pollen, although their levels of expression vary. Nevertheless, most of those identified in this study show higher expression levels in germinated pollen.

It is worth noting that all of the miRNA families indicated above are also expressed in various sporophytic tissues from several species of gymnosperms (particularly conifers) (Qiu et al. 2009; Yakovlev et al. 2010) and angiosperms (Zhang et al. 2006a, b; Chambers and Shuai 2009). MiR166 and miR171 are also expressed in loblolly pine zygotic embryos and megagametophytes (Oh et al. 2008). Collectively, these results imply that the miRNAs detected in this study are not only conserved among species but also between organs and tissues representing the sporophyte and gametophyte phases, and at various stages of development. As suggested by Wei et al. (2011), conserved miRNAs could serve as integral regulators of both sporophytic and gametophytic development. On the other hand, there are several differentially expressed conserved miRNAs reported here for loblolly pine male gametophyte (i.e., miR474, miR482, miR901, miR903, miR946, miR947, miR950, miR951, miR1026, and miR1125) that have not yet been reported in rice or Arabidopsis pollen. Whereas all of these are also expressed in sporophytic tissues (needles and stems) of various conifers (Lu et al. 2007; Morin et al. 2008; Yakovlev et al. 2010; Wan et al. 2012b), only miR947 and miR951 (up- and down-regulated in germinated loblolly pine pollen, respectively) have not yet been reported from any sporophytic tissue in angiosperm. Therefore, our results at least suggest differences and similarities in the conserved miRNA profiles of the male gametophytes from the representative gymnosperms and angiosperms. Although more detailed analysis is necessary to confirm this generalization, the large-scale miRNA analysis by Peng et al. (2012) has already pointed out

several differences in the types and expression patterns in the male gametophytes of rice and Arabidopsis, both in terms of conserved and recently evolved miRNAs. Considering the many morphological and developmental differences in the male gametophytes of gymnosperms and angiosperms (Singh 1978; Gifford and Foster 1989; Fernando et al. 2010; Williams 2012), detailed comparative miRNA analysis will likely provide further differentiation between these two major groups of pollen-bearing plants. This will likely include miRNAs that relate to sperm formation since this process occurs in the formation of the mature pollen of rice and Arabidopsis, but not in the mature and germinated pine pollen until after about a year of pollen tube growth in the ovule (Fernando et al. 2010). As alluded to above, many recently evolved miRNAs will also serve to further differentiate the male gametophytes of gymnosperms and angiosperms.

The targets of the miRNAs identified in this study need to be experimentally verified, and thus, discussion on this subject has to be limited since functional analysis in gymnosperms is not feasible at the moment. With this limitation, computational prediction provides insights on the target genes and the functions of conserved miRNAs. The expression levels of the two representative potential target mRNAs (TC189536 and DR054135) were inversely proportional with the expression levels of their corresponding miRNAs (pta-miR166 and pta-miR951, respectively). Computational prediction shows that most of the targets of the differentially expressed miRNAs in the male gametophyte of loblolly pine appear to be involved in stress/defense response, followed by metabolism, gene regulation, and signaling. However, target functions based on the psRNATarget program are not available for miR901, miR903, and miR1125, which are all up-regulated in germinated pollen. Interestingly, these three miRNAs are among the significantly up-regulated miRNAs in response to drought stress in rice (Zhou et al. 2010). Stress/defense response-related transcripts are also preferentially expressed in rice pollen, which, according to Wei et al. (2010), indicates that the ability to deal with abiotic and

Table 4 Predicted targets of down-regulated conserved miRNAs expressed in germinated loblolly pine pollen using the psRNATarget program (the dashes indicate that no target gene function can be inferred)

miRNA	Target gene	Target protein	Score	Target function
miR156/157	EX437839	SBP-domain protein (<i>Physcomitrella patens</i>)	2.0	Gene regulation
	TC161194	SBP-domain protein (<i>Medicago truncatula</i>)	2.0	Gene regulation
	FD733579	Ubiquitin carrier protein (<i>Pinus resinosa</i>)	3.0	Metabolism
miR168	DR177325	TIR-NBS-LRR (<i>Pinus taeda</i>)	2.5	Defense/stress response
	TC173099	Expressed protein (<i>Oryza sativa</i>)	3.0	–
miR474	TC156754	F5D14.2 protein (<i>Arabidopsis thaliana</i>)	2.0	–
	TC174403	Peroxidase 2 precursor (<i>Spinacia oleracea</i>)	3.0	Defense/stress response
miR482	BM492069	Predicted protein (<i>Physcomitrella patens</i>)	3.0	–
	TC183199	No description	1.5	–
	GW762852	No description	2.0	–
	TC182294	Chromosome chr9 scaffold_7 (<i>Vitis vinifera</i>)	2.5	–
	TC156441	F3L24.22 protein (<i>Arabidopsis thaliana</i>)	3.0	–
	TC183038	Acetyl-Coenzyme A synthetase 2 (AMP forming)-like (<i>Bos Taurus</i>)	3.0	Metabolism
miR844	TC158637	Predicted protein (<i>Physcomitrella patens</i>)	2.5	–
	DR691785	Predicted protein (<i>Physcomitrella patens</i>)	2.5	–
	GT266419	Phosphatidate cytidyltransferase (<i>Vitis vinifera</i>)	3.0	Metabolism
	CV034846	No description	3.0	–
	CT581131	Predicted protein (<i>Physcomitrella patens</i>)	3.0	–
miR946	TC163753	No description	0.0	–
	DN627657	Chromosome undetermined scaffold_80 (<i>Vitis vinifera</i>)	0.0	–
	TC185886	Chromosome undetermined scaffold_2028, (<i>Vitis vinifera</i>)	1.5	–
	TC191927	Receptor-like kinase (<i>Marchantia polymorpha</i>)	2.5	Signaling
	CO198034	Proton pyrophosphatase (<i>Vigna radiata</i>)	2.5	Metabolism
miR950	TC187692	TIRR-NBS-LRR type disease resistance protein (<i>Populus trichocarpa</i>)	3.0	Defense/stress response
	TC159392	TIR (<i>Medicago truncatula</i>)	2.5	Defense/stress response
	TC159801	Chromosome undetermined scaffold_142, (<i>Vitis vinifera</i>)	3.0	–
	TC188428	Toll/interleukin-1 receptor (TIR)/X (<i>Pinus contorta</i>)	1.5	Defense/stress response
	TC193865	TIR/NBS (<i>Pinus contorta</i>)	1.5	Defense/stress response
miR951	TC194328	Toll/interleukin-1 receptor (TIR)/X (<i>Pinus taeda</i>)	1.5	Defense/stress response
	DR054135	TIR/P-LOOP/LRR (<i>Pinus taeda</i>)	2.5	Defense/stress response
	TC169880	TIR/NBS (<i>Pinus taeda</i>)	2.5	Defense/stress response
	TC155674	Predicted protein (<i>Physcomitrella patens</i>)	3.0	–

biotic stresses during pollen development may be essential to successful fertilization.

This study has identified several interesting conserved miRNAs that are expressed in the male gametophyte of loblolly pine. This study further demonstrates that expressions of

many conserved miRNAs are different between mature and germinated loblolly pine pollen, with most of the miRNAs are up-regulated in germinated pollen. This indicates that the transition from mature (ungerminated pollen) to germinated pollen is modulated at the miRNA level. This study also

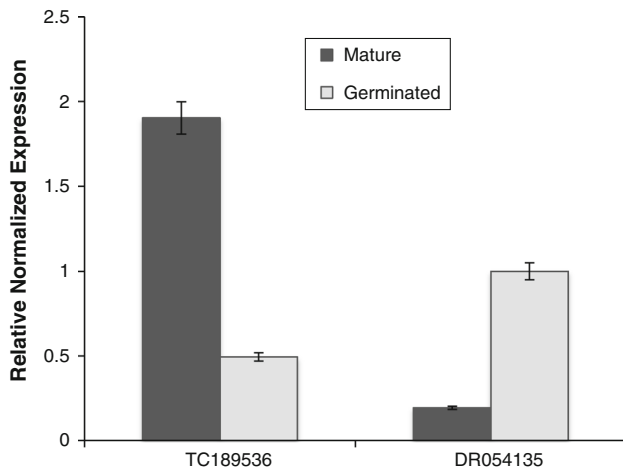


Fig. 1 Expression patterns of potential target genes of representative differentially expressed miRNAs (TC189536 and DR054135 are sequences obtained from the Pine Gene Index, which are the potential targets of *pta-miR166* and *pta-miR951*, respectively. TC189536 encodes a Class III HD-Zip protein, whereas DR054135 encodes Toll/interleukin-1 receptor/P-LOOP/LRR protein)

allowed comparisons of the type and expression patterns of some conserved miRNA between the male gametophytes of loblolly pine with those of rice and *Arabidopsis*. Although some similarities and differences have been identified, it is premature to identify any specific miRNA at this point. Nevertheless, it is interesting that such differentiation may be achieved even with just the use of conserved miRNAs. Since it was not the aim of the current study, further studies are needed to verify the miRNA targets and the specific roles they play in phase transitions, particularly from ungerminated to germinated phase of the male gametophyte. However, loblolly pine (and gymnosperms in general) is currently not amenable to molecular genetic analysis because of its long generation time. Therefore, functional analysis has to be delayed until alternative approaches become available including perhaps, the use of direct pollen or protoplast transient transformation as a system for single-cell analysis which is emerging as the new frontier in genomic research.

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