

**Nonfunctional ingestion of plant miRNAs in silkworm revealed by digital droplet
PCR and transcriptome analysis**

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Supplementary Tables and Figures

Supplementary Table S1 TA-cloning and Sanger sequencing for mulberry miRNAs identified in silkworm tissues.

miRNA name	Fat body (C / T)		Silk gland (C / T)		Brain (C / T)	Prothoracic gland (C / T)	Salivary gland (C / T)	Gut (C / T)	Malpighian tubule (C / T)	Ovary (C / T)	Testis C / T)
	1st	2nd	1st	2nd							
	sequencing	sequencing	sequencing	sequencing							
mno-miR169a	1/38	0/38	0/28	0/42	0/35	1/46	0/35	0/39	0/38	1/43	0/45
mno-miR166b	15/73	32/42	42/74	22/37	22/41	13/41	3/33	3/35	4/37	10/40	6/45
mno-miR166c	20/37	42/44	26/36	40/43	25/42	21/34	5/24	10/41	16/38	14/44	12/32
mno-miR167e	5/40	1/44	5/37	1/43	10/39	4/41	2/30	3/37	8/40	3/41	4/49
mno-miR396b	30/41	31/47	24/43	20/44	41/47	26/39	14/38	13/41	20/41	15/44	11/49
mno-miR159a	0/40	-	1/20	-	-	-	-	-	-	-	-
mno-miR156c	1/12	-	1/46	-	-	-	-	-	-	-	-
mno-miR398	0/49	-	0/48	-	-	-	-	-	-	-	-
mno-miR162	0/42	-	0/40	-	-	-	-	-	-	-	-

C / T means correct clones / Total clones. “-” indicates that we did not clone the miRNA in the fat body, silk gland, brain, prothoracic gland, salivary gland, gut, malpighian tubule, ovary, and testis.

Supplementary Table S2 The copy number counts of miR166b in silkworm hemolymph (Hem), fat body (FB), and silk gland (SL) before (0 h) and following ingesting synthetic miR166b (0.5 h, 3 h, 6 h, and 12 h).

Sample	miR166(counts/ μ l)		Average value of miR166(counts/ μ l)
	1 st well	2 nd well	
Hem-0 h	22.5	21.7	22.1
Hem-0.5 h	562	745	653.5
Hem-3 h	377	456	416.5
Hem-6 h	28.9	30.4	29.65
Hem-12 h	47.6	59.8	53.7
FB-0 h	358	381	369.5
FB-0.5 h	1160	1100	1130
FB-3 h	511	552	531.5
FB-6 h	206	217	211.5
FB-12 h	140	121	130.5
SL-0 h	192	212	202
SL-0.5 h	162	190	176
SL-3 h	114	84	99
SL-6 h	114	105	109.5
SL-12 h	109	108	108.5

Supplementary Table S3 The predicted information for miR166b target genes.

Potential target gene	Potential target gene annotation	Gene in derived tissues ^{1,*}	Score	Energy (kcal/mol)	Length (nt)	Position start	Position end	Match condition
fner47P07	PREDICTED: similar to GA15241-PA [Tribolium castaneum]	nerve system + brain, larva	170	-41.45	1258	305	327	Query: 3' ccCCTT--ACTTCGGACCAGGct 5' : Ref: 5' ggGGAATGTGTGGCCTGGTCCGa 3'
fwd-23F03	PREDICTED: similar to Cpsf6 protein, partial [Strongylocentrotus purpuratus]	wing disc	161	-30.66	1273	827	850	Query: 3' ccCCTTAC--TTCG-GACCAGGct 5' : Ref: 5' ggGGAATGCAAAGTGATGGTCCGc 3'
fprW24O02	PREDICTED: probable phosphorylase b kinase regulatory subunit alpha-like isoform 2 [Bombus terrestris]	prothoracic gland, W-stage	164	-29.99	5840	2633	2653	Query: 3' cccctTACTTCGGACCAGGct 5' : : : : : : : Ref: 5' aagctGTGGGGTCTGGTCCGg 3'
fmxd11G04	PREDICTED: calcium-binding mitochondrial carrier protein Aralar1-like isoform 1 [Nasonia vitripennis]	maxillary galea	172	-29.95	4344	1687	1708	Query: 3' ccccTTACTT-CGGACCAGGct 5' : Ref: 5' ccaaAGTGAAGCCTGGTCCGt 3'
fe8d35D03	GK17955 [Drosophila willistoni]	embryo day 8	157	-28.73	5755	644	664	Query: 3' ccccTTACTTCGGACCAGGct 5' : Ref: 5' tgacAGGGCTGCCTGGTCCGg 3'

fwgP16B11	olfactory receptor [Bombyx mori]	wing, pupa day 2-8	158	-27.79	5590	2091	2112	Query: 3' ccCCTTAC-TTCGGACCAGGct 5' : :	Ref: 5' ggGGTATGTGGGATTGGTCCGc 3'
fufe34A12	PREDICTED: PAP-associated domain-containing protein 5-like [Apis mellifera]	unfertilized egg	161	-27.44	4400	337	360	Query: 3' ccCCT-TACTTC--GGACCAGGct 5' :	Ref: 5' acGGAGACGGAGCACCTGGTCCGc 3'
NM_001043963.1	Bombyx mori yellow-fb mRNA, complete cds.	ovary/embryo o/fat body	157	-27.38	1516	996	1017	Query: 3' ccccttaCTTCGG-ACCAGGct 5' :	Ref: 5' gtggttgGGAGCCGTGGTCCGc 3'
fwgP08C14	hook protein [Aedes aegypti]	wing, pupa day 2-8	163	-27.08	5275	4181	4199	Query: 3' cccCTTACTTCGGACCAGGct 5' :	Ref: 5' tctGAA--AGGCCTGGTCCGg 3'
fdpe24B10	REDICTED: similar to AGAP010191-PA [Tribolium castaneum]	diapaused egg	155	-26.85	2500	508	528	Query: 3' ccCCTTACTTCGGACCAGGct 5' : :	Ref: 5' cgGGTCTGTGACTTGGTCCGa 3'
DQ311279	Bombyx mori double-stranded RNA-binding zinc finger protein JAZ mRNA, complete cds.	testis/head/integument/mid gut/silk gland	160	-26.71	1593	446	466	Query: 3' ccCCTTACTTC--GGACCAGGct 5' : : :	Ref: 5' tcGGAA-GAGGATTTGGTCCGg 3'

* indicateds the genes from the derived tissues in the silkworm full-length cDNA libraries. Score and energy mean the parameters of miR166b with its target predicted by miranda software. Position start and position end indicate the positions of target gene matching to miR166b. Query and Ref mean the sequences of miR166b and their target genes.

Supplementary Table S4 The expression level of 72 potential target genes for miR166b before and after silkworm ingested synthetic miR166b.

GeneID	Gene Length	FED_control-Expression	FED_166b-Expression	FED_control-RPKM	FED_166b-RPKM	log2 Ratio(FED_166b/FED_control)	Up-Down-Regulation(FED_166b/FED_control)	P-value	FDR
DQ311279_KBtr3102131	1293	0	8	0.0010	0.2348	7.8750	Up	0.0042	0.0105
fphe18H11_KBtr3008861	630	0	4	0.0010	0.2409	7.9123	Up	0.0652	0.1116
fe8d35D03_KBtr3011035	2115	2	6	0.0365	0.1076	1.5601	Up	0.1883	0.2900
NM_001046951.1_KBtr3103491	1821	2	12	0.0424	0.2500	2.5601	Up	0.0081	0.0190
fmxcg11G04_KBtr3006016	1812	2	6	0.0426	0.1256	1.5601	Up	0.1883	0.2959
e10025H07_KBtr3008407	1617	2	2	0.0477	0.0469	-0.0248	Down	0.9839	0.9968
fdpe27A10_KBtr3005579	801	1	0	0.0482	0.0010	-5.5907	Down	0.4914	0.6103
NM_001134242.1_KBtr3104129	1251	2	0	0.0617	0.0010	-5.9475	Down	0.2436	0.3473
MFB-22J01_KBtr3006608	1293	6	4	0.1791	0.1174	-0.6098	Down	0.5296	0.6371
fwgP08A16_KBtr3004462	2037	10	17	0.1895	0.3166	0.7407	Up	0.2006	0.3028
NM_001135202.1_KBtr3104178	1734	12	4	0.2671	0.0875	-1.6098	Down	0.0451	0.0868
fufe05M06_KBtr3003435	2742	22	27	0.3097	0.3736	0.2706	Up	0.5187	0.6340
fwgP08C14_KBtr3004464	4116	62	50	0.5815	0.4609	-0.3352	Down	0.2222	0.3290
fcaL50E18_KBtr3007892	1464	24	22	0.6328	0.5702	-0.1504	Down	0.7259	0.8101
ftes09J21_KBtr3002070	3774	67	48	0.6853	0.4826	-0.5060	Down	0.0629	0.1127
ftes01O21_KBtr3001806	3957	106	110	1.0340	1.0547	0.0286	Up	0.8852	0.9088
fe8d26E10_KBtr3000564	966	26	31	1.0390	1.2176	0.2289	Up	0.5551	0.6476
AB383140_KBtr3100772	738	20	10	1.0461	0.5141	-1.0248	Down	0.0636	0.1113

ftes40N05_KBtr3003068	138	4	0	1.1189	0.0010	-10.1278	Down	0.0599	0.1097
fner48F12_KBtr3001725	1995	63	59	1.2190	1.1221	-0.1195	Down	0.6483	0.7341
fcaL46I03_KBtr3010924	1296	41	45	1.2212	1.3174	0.1095	Up	0.7279	0.8007
bmmt22O18_KBtr3009956	885	29	22	1.2649	0.9432	-0.4234	Down	0.3016	0.4148
fcaL26A22_KBtr3007341	183	6	3	1.2656	0.6220	-1.0248	Down	0.3298	0.4455
ffbm37M15_KBtr3000868	3384	116	147	1.3232	1.6482	0.3168	Up	0.0768	0.1285
fwgP16B11_KBtr3004585	219	8	5	1.4101	0.8663	-0.7029	Down	0.4053	0.5201
EU073423_KBtr3101484	3834	128	221	1.5433	2.6191	0.7631	Up	0.0000	0.0000
fufe39C19_KBtr3004101	1521	67	80	1.6985	1.9934	0.2310	Up	0.3351	0.6312
fprW23F16_KBtr3009502	2085	95	127	1.7588	2.3111	0.3940	Up	0.0437	0.0885
fprW24O02_KBtr3009532	3675	178	185	1.8697	1.9100	0.0308	Up	0.8396	0.8979
fmgV52F14_KBtr3001347	1233	62	70	1.9410	2.1541	0.1502	Up	0.5525	0.6545
fufe34A12_KBtr3003983	1713	87	103	1.9605	2.2814	0.2187	Up	0.2989	0.4185
fwd-23F03_KBtr3000242	589	30	29	1.9661	1.8681	-0.0738	Down	0.8447	0.8789
fphe27I11_KBtr3009051	279	17	9	2.3520	1.2239	-0.9424	Down	0.1116	0.1829
fner46B19_KBtr3001701	1737	112	128	2.4890	2.7960	0.1678	Up	0.3698	0.4909
fphe06C05_KBtr3008553	1422	102	70	2.7689	1.8678	-0.5680	Down	0.0107	0.0236
fprW16K18_KBtr3009416	1698	125	118	2.8417	2.6367	-0.1080	Down	0.5601	0.6437
fufe11D11_KBtr3003523	5334	421	509	3.0467	3.6206	0.2490	Up	0.0087	0.0197
fner44J23_KBtr3001686	1515	125	97	3.1849	2.4293	-0.3907	Down	0.0449	0.0887
fcaL15I24_KBtr3007105	2349	199	246	3.2702	3.9735	0.2810	Up	0.0408	0.0849
fphe05O06_KBtr3008545	1458	160	176	4.2361	4.5801	0.1127	Up	0.4756	0.6004
fwgP14B05_KBtr3004550	855	105	110	4.7405	4.8814	0.0423	Up	0.8310	0.9013
fdpe24B10_KBtr3005478	987	132	134	5.1625	5.1512	-0.0032	Down	0.9853	0.9853
fdpe20E17_KBtr3005343	3027	485	467	6.1849	5.8536	-0.0794	Down	0.3959	0.5167
fcaL11N10_KBtr3007021	192	33	23	6.6346	4.5451	-0.5457	Down	0.1642	0.2634

AB177622_KBtr3102492	2160	408	406	7.2833	7.1238	-0.0319	Down	0.7520	0.8880
fufe21M15_KBtr3003731	258	52	41	7.7801	6.0296	-0.3677	Down	0.2230	0.3240
e10013A10_KBtr3008211	2379	523	628	8.4861	10.0158	0.2391	Up	0.0051	0.0122
e10004B12_KBtr3008032	1662	1010	1131	23.4580	25.8198	0.1384	Up	0.0267	0.0570
fnr47P07_KBtr3001722	1032	1112	1235	41.5474	45.3551	0.1265	Up	0.0339	0.1887
fphe05E06_KBtr3008533	489	653	657	51.5473	50.9774	-0.0160	Down	0.8404	0.8864
MFB-15M01_KBtr3006466	1884	4735	4624	97.0153	93.1232	-0.0591	Down	0.0476	0.0895
fphe22F14_KBtr3008946	909	3795	3811	160.9781	158.8964	-0.0188	Down	0.5703	0.7876
AB064522_KBtr3102562	396	0	0	-	-	-	-	-	-
AB196705_KBtr3101600	738	0	0	-	-	-	-	-	-
AB330437_KBtr3101259	1251	0	0	-	-	-	-	-	-
AB425069_KBtr3100742	1734	0	0	-	-	-	-	-	-
DQ358082_KBtr3101752	1257	0	0	-	-	-	-	-	-
DQ443256_KBtr3101964	1821	0	0	-	-	-	-	-	-
fphe15F13_KBtr3011069	1293	0	0	-	-	-	-	-	-
ftes24P18_KBtr3002516	270	0	0	-	-	-	-	-	-
ftes49O09_KBtr3003295	2166	0	0	-	-	-	-	-	-
GU322819_KBtr3100270	909	0	0	-	-	-	-	-	-
MFB-15M07_KBtr3006467	396	0	0	-	-	-	-	-	-
NM_001043418.1_KBtr3102696	396	0	0	-	-	-	-	-	-
NM_001043453.1_KBtr3102731	2160	0	0	-	-	-	-	-	-
NM_001043521.1_KBtr3102794	738	0	0	-	-	-	-	-	-
NM_001043963.1_KBtr3103046	1257	0	0	-	-	-	-	-	-
NM_001046730.1_KBtr3103271	1293	0	0	-	-	-	-	-	-
NM_001110342.1_KBtr3103872	3834	0	0	-	-	-	-	-	-
NM_001172724.1_KBtr3104383	909	0	0	-	-	-	-	-	-

fe8d22B10_KBtr3000534	435	0	0	-	-	-	-	-	-
ftes10A11_KBtr3002088	4407	0	0	-	-	-	-	-	-

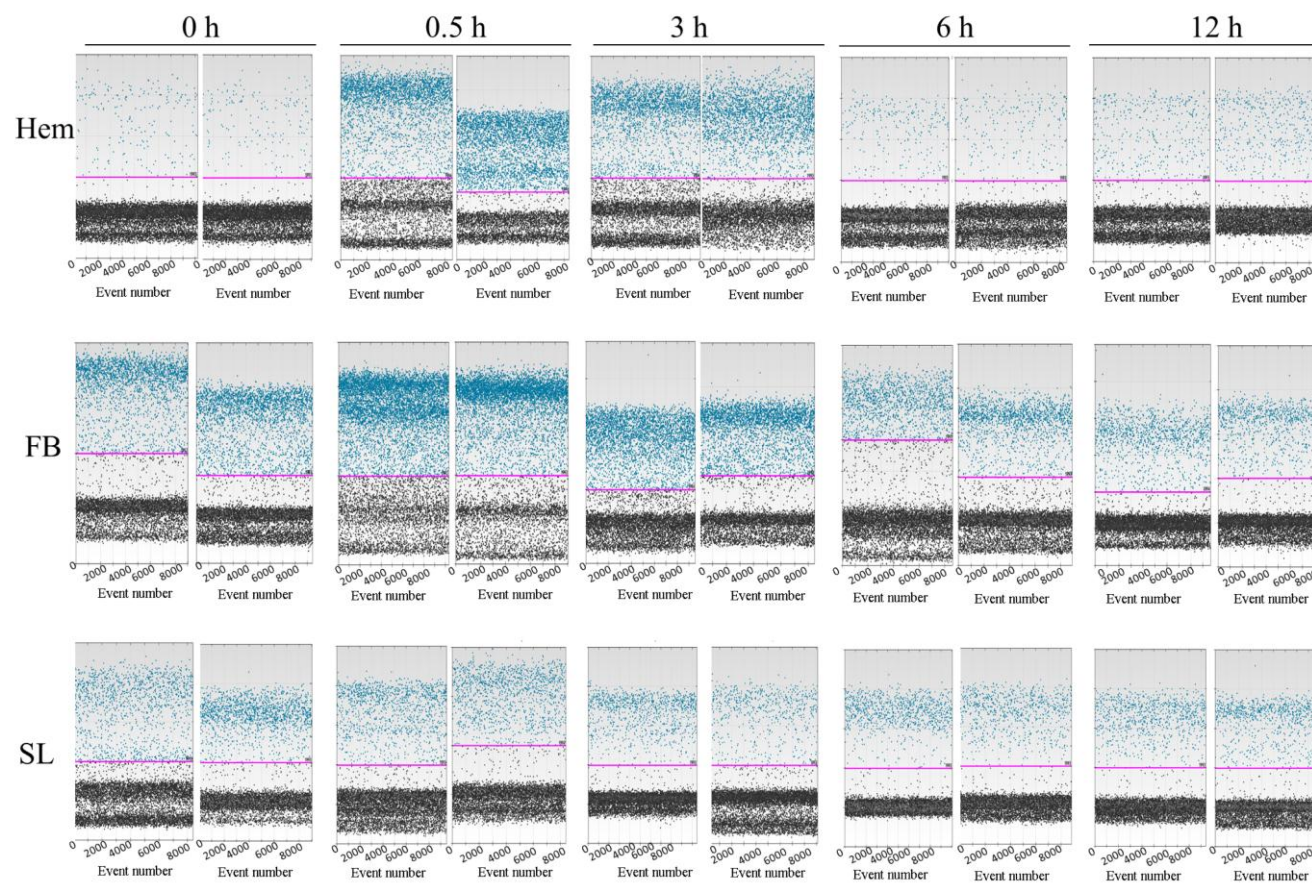
Gene expression levels (reads per kb per million reads, RPKM) were determined prior to feeding (FED-control) or after feeding of synthetic miR166b (FED-miR166b).

Supplementary Table S5 Primers for stem-loop PCR of miRNAs.

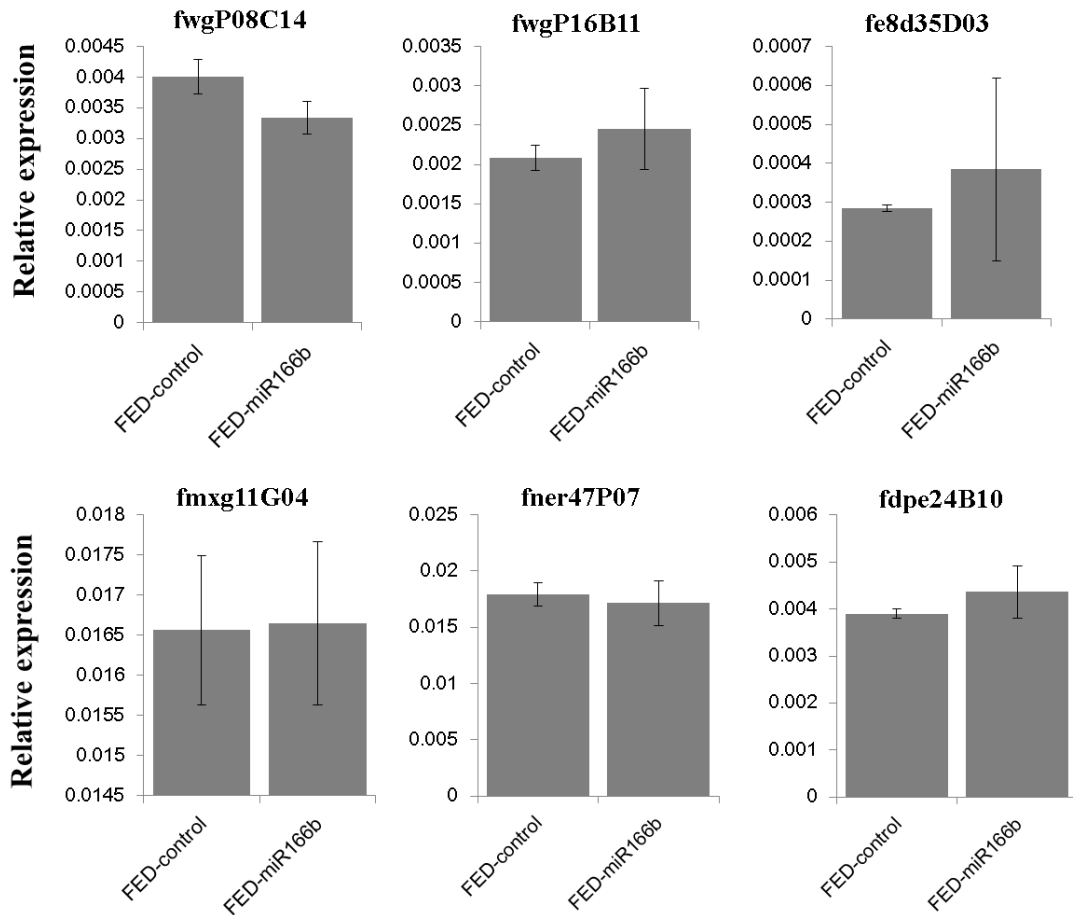
miRNA name	Sequence (5'-3')	Reverse transcriptive primer	Forword primer (5'-3')	Reverse primer(5'-3')
mno-miR166c	TCTCGGACCAGGCTTCATTCC	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACggaatg	GGCGTCTCGGACCAGGCT	CAGTGCAGGGTCCGAGGTAT
mno-miR167e	TGAAGCTGCCAGCATGATCTG	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACcagatc	TCGAGAGTGAAGCTGCCAGC	CAGTGCAGGGTCCGAGGTAT
mno-miR156c	TTGACAGAAGATAGAGAGCAC	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACgtgctc	GCCGAGGAGATTGACAGAAGATA	CAGTGCAGGGTCCGAGGTAT
mno-miR398	TGTGTTCTCAGGTCGCCCTG	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACcagggg	CCGAGGAGATGTGTTCTCAGGT	CAGTGCAGGGTCCGAGGTAT
mno-miR166b	TCGGACCAGGCTTCATTCCCC	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACggggaa	CGAGTTCGGACCAGGCTTCA	CAGTGCAGGGTCCGAGGTAT
mno-miR159a	TTTGGATTGAAGGGAGCTCTG	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACcagagc	CGAGAGTTTGGATTGAAGGGA	CAGTGCAGGGTCCGAGGTAT
mno-miR162	TCGATAAACCTCTGCATCCAG	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACctggat	CGAGGAGATCGATAAACCTCTGC	CAGTGCAGGGTCCGAGGTAT
mno-miR396b	TTCCACAGCTTTCTTGAAGT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACcagttc	GCAGCAGTCCACAGCTTTCTT	CAGTGCAGGGTCCGAGGTAT
mno-miR535	TGACAACGAGAGAGAGCACGC	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACgcgtgc	GCGAGTGTGACAACGAGAGAGA	CAGTGCAGGGTCCGAGGTAT
mno-miR172a	AGAATCTTGATGATGCTGCAT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACatgcag	GCACGCAGAGAATCTTGATGATG	CAGTGCAGGGTCCGAGGTAT
mno-miR168b	TCGCTTGGTGCAGGTCGGGAA	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACttcccg	CGAGTTCGCTTGGTGCAGGT	CAGTGCAGGGTCCGAGGTAT
mno-miR169a	CAGCCAAGGATGACTTGCCGG	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACccggca	CAGGAGACAGCCAAGGATGACT	CAGTGCAGGGTCCGAGGTAT

Supplementary Table S6 Primers for RT-PCR of miR166b potential target genes and immunity-relatively genes.

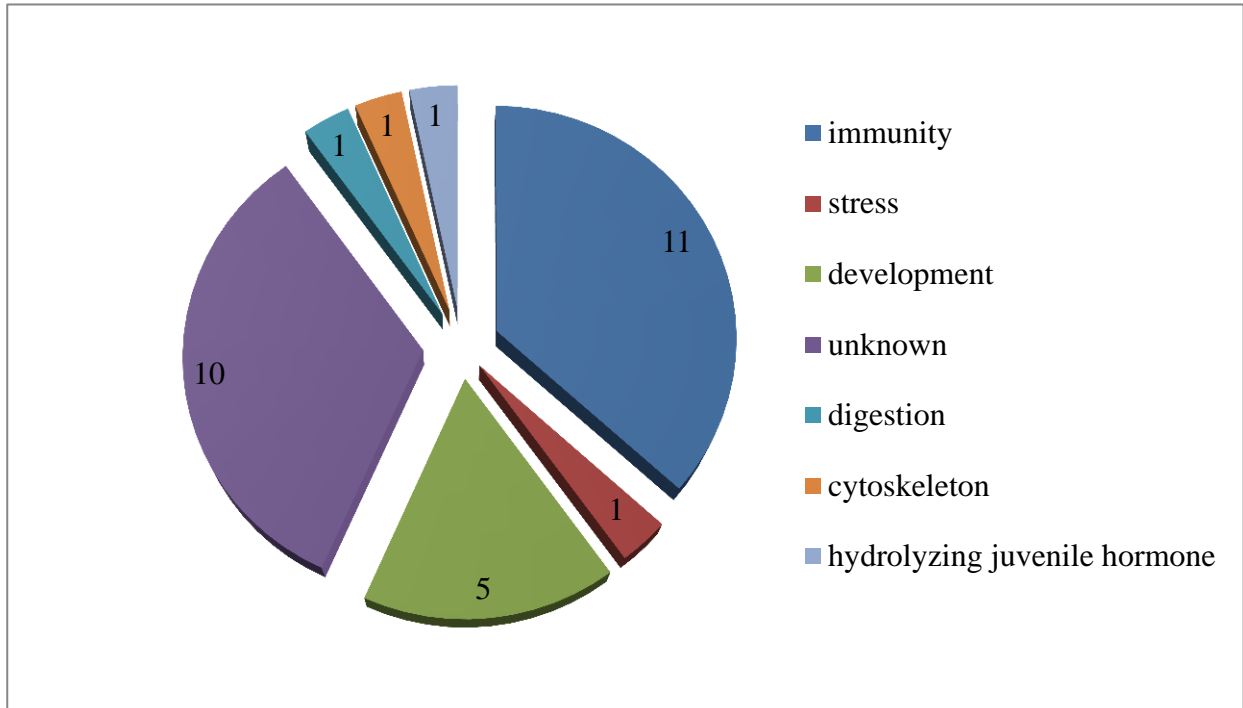
Gene name	Forward Primer (5' to 3')	Reversed Primer (5' to 3')
fwgP08C14	TACACCGATAAAAGAGAACCCGA	CGTGTCTTGCTTCATTTCTATCTTG
fwgP16B11	ATTCCAGTGCCCATTAATCGTCA	AGGATGCTAAAATATCCATGGATCC
fe8d35D03	GGCTCTGAACCACCCACG	GCCACAGACTATCCAATAATCG
fmng11G04	GGGCGGTTCTCAAGTGGTGT	GGAAGTATATGGCGCTGAAGGGC
fufe34A12	TGGCGAATACCTAATTATGCGT	CAGTACGGAATGAACCGAACAC
fdpe24B10	CTATCCAGAGAAGAGGCAGAAGAA	GACCACATCGCTTTCTCTTCTTG
NM_001043963.1	TGTCGACTCACGAGTACGCCA	CTCCTCGACGAGCGGTTTCT
fwd-23F03	CACCTGGGCTGAGACCACCTA	TCCTGGCGGACCATCACTTT
fprW24O02	ACGGTAAGGTGGCGACAGTG	AATCAGTAACGGCTTTAGCGAGA
fnr47P07	GGGATAAAATGCAGCGTCGAGTAGT	TGGCGGTGCTTGACCCTTTG
DQ311279	ATCCACCAAGACCCCCTCC	AGGAATGTTTTTCAAACCTGTCCTT
Bm_nscaf1071_24	TGTTTCGCTATTGTTTTTCGCC	CTTTTGCTTTTGCCAAGGTGT
Bm_nscaf2556_13	TACTCGGCCGGGGTAAG	ACATTGGGCTCCCACGAAGAT
Bm_nscaf2814_10	GTGTGACCAACAACCTGTGAGCCC	TACGGAAAATGCGAATGGACGA
Bm_nscaf2818_080	TATTGTATTGTTGTTTGTGTGGCA	GGTCGCAGAAGCACTGTGGAAC
Bm_nscaf3050_1	GCAGGTGCTATCAACTCTCCTCAAT	TAAAGGTAATGGAGATTTCAAAGGA
Bm_nscaf2970_047	CTATGGTAGCGTCCAAGGAGAGA	CTCTTTCGAACTCCTCACGTAGC
Translation initiation factor 4A	TTCGTA CTGGCTCTTCTCGT	CAAAGTTGATAGCAATTCCT



Supplementary Figure S1 Droplet digital PCR indicates the synthetic miR166b entered into the silkworm hemolymph, fat body, but not into silk gland after application of oral synthetic miR166b. The Y axis represents the signal intensity. The X axis represents the droplet numbers for a 20 μ l PCR reaction divided by QX100 droplet generator. Hem: Hemolymph, FB: fat body, SL: silk gland. The purple line below represents the signals of NTC (no template control).



Supplementary Figure S2 The expression levels of 6 potential miR166b target genes in fat body before and after silkworm larvae ingested synthetic miR166b. FED-control represents the silkworm larvae were only fed on mulberry leaves. FED-miR166b means the silkworm larvae were fed on a piece of mulberry leaf containing synthetic miR166b. Statistical significance was determined by Student's t test (*, $p < 0.05$).



Supplementary Figure S3 Categorization of 30 differentially expression genes before and after ingestion synthetic miR166b by silkworm. The values indicate the gene numbers in each category.

References

1. Suetsugu ,Y. et al. Large scale full-length cDNA sequencing reveals a unique genomic landscape in a lepidopteran model insect, *Bombyx mori*. *G3 (Bethesda)* **3**, 1481-1492 (2013).