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Supplemental Information

Cell Cycle Progression Determines Wing Morph in the Polyphenic Insect *Nilaparvata lugens*

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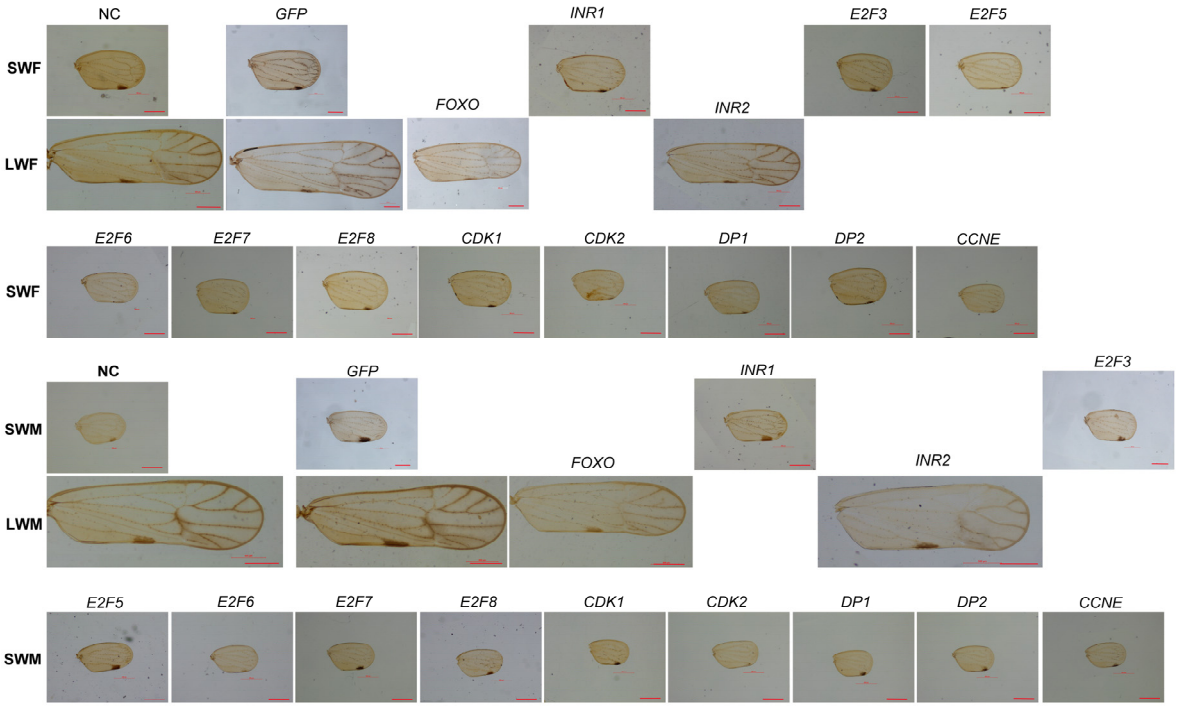

Figure S1: Representative long and short wings after treatment at the 4th instar nymph stage, Related to Figure 4.

SWF: short-winged female, SWM: short-winged male, LWF: long-winged female, LWM: long-winged male. Scale bar = 500 μm.

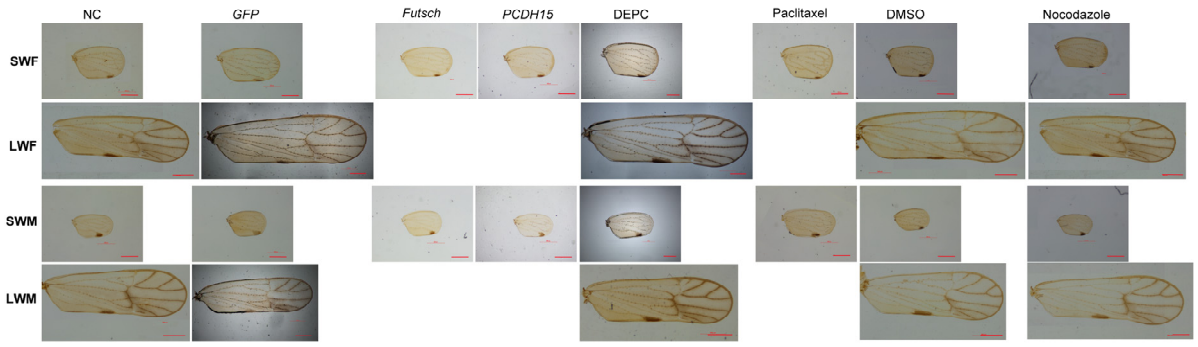

Figure S2: Representative long and short wings after treatment at the 5th instar nymph stage, Related to Figure 5.

SWF: short-winged female, SWM: short-winged male, LWF: long-winged female, LWM: long-winged male. Scale bar = 500 μm.

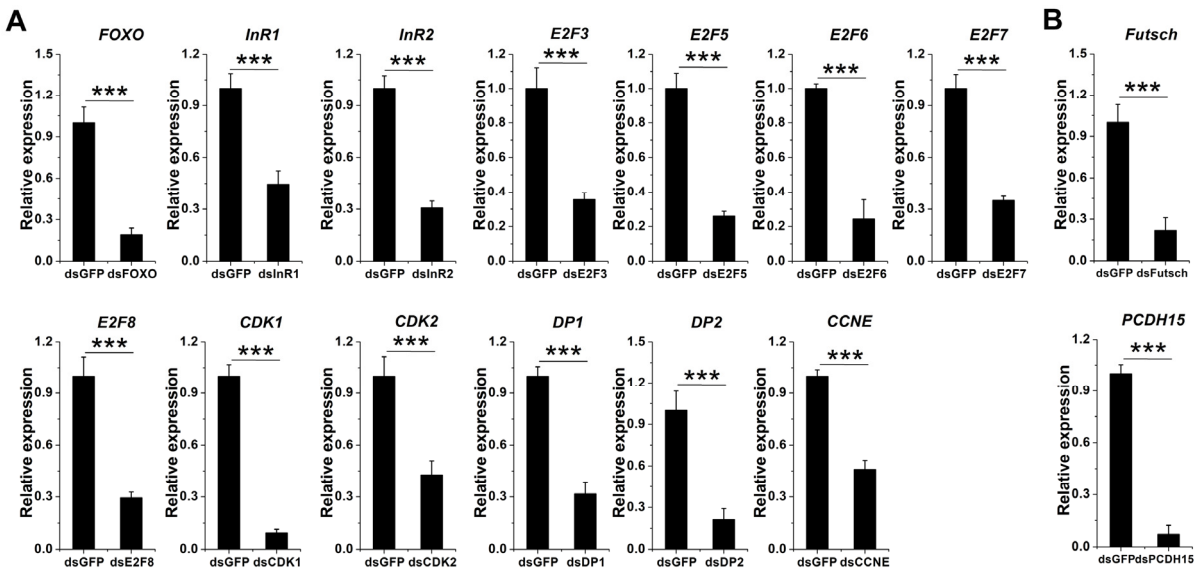

Figure S3: Relative expression of genes down-regulated by RNAi, Related to Figure 4 , 5 and 6.

dsRNA of *InR1*, *InR2* and *FOXO*, *E2F3*, *E2F5*, *E2F6*, *E2F7*, *E2F8*, *CDK1*, *CDK2*, *DP1*, *DP2* and *CCNE* were injected into 4th instar nymphs (A) and dsRNA of *PCDH15* and *Futsch* were injected into the 5th instar nymphs (B). Relative abundance of each transcript three days after dsRNA injection was compared with that from control nymphs injected with GFP dsRNA. Twelve biological replicates were used for each treatment. Bars represent means of three separate measurements. Student's *t*-test was used for statistical comparison. *: P<0.05, **: P<-0.01, ***: P<0.001. The primers for RT-PCR were listed in Table S3.

Table S1: Primers for cloning, Related to Figure 1, 2, 4, 5, 6 and 7.

Name	Sequence (5'-3')	GenBank Accession Number
E2F3F	CACCTCTCCTACAAGTGCAC	XM_022345983.1
E2F3R	CAAGGCCACTGAGAGGAATC	
E2F5F	GTGCTTGAAGGAATCGGTCT	MT240933
E2F5R	TCTTCTGCGCAAACTCAGTT	
E2F6F	GTGCCAATCGAGTCCACATC	MT240934
E2F6R	TCCTGTGGACTATCAGCAGC	
E2F7F	AGACAGGCTCTTCGAGATCT	XM_022328840.1
E2F7R	CAACCCCAATAACTCGAGCA	
E2F8F	TCATTCCTAACCCACCATCCA	XR_002606046.1
E2F8R	ATGAATGATGGGTGGGTGGA	
DP1F	GACTCCTGCCAACAACCAAG	XM_022342425.1
DP1R	TTCCTGCACCGAGTTTGTTG	
DP2F	CACAGCGCCTTCATGATCTC	MT240935
DP2R	CATCGTCGGAGTAGTCGTGA	
CDK1F	GCTTGAGAAGATCGGTGAGG	XM_022336874.1
CDK1R	AACACCCGAGCAATACTTCG	
CDK2F	TACCGCGCGCCTGAGATA	XM_022351965.1
CDK2R	GGGAATAGCGGCTTGTAATCC	
CCNEF	GGACGAGACCTTCTACGAGG	XM_022344075.1
CCNER	CATTGCAGGCTCCATCAGTC	
FutF	GCAGTGTCAAGAGTTGGTCG	XM_022348003.1
FutR	CTCTCTGGTTCCTGTGCAGA	
PCDH15F	GCCATTGATGCTGATGAGGG	MT240936
PCDH15R	CAGTGTGATCGGTCTGGGTA	

FOXOF	CTGTTCCCTGAATCGCCGCT	KM250122
FOXOR	CGTTGCAGTCGAATCCGTCG	
InR1F	GCAGTGGCTCAACAACAAGA	KF974333.1
InR1R	CCTCGCTGAAGAAGTCCAAC	
InR2F	GGAGCTATGGTGTTGTCTTGTG	XM_022333236
InR2R	CTTGAGTTGGCCTCATTGGT	

Table S2: Primers for dsRNA synthesis, Related to 1, 2, 4, 5, 6 and 7.

Name	Sequence (5'-3')
dsE2F3F	TAATACGACTCACTATAGGGAGACCACCACCTCTCCTACAAGTGCAC
dsE2F3R	TAATACGACTCACTATAGGGAGACCACCAAGGCCACTGAGAGGAATC
dsE2F5F	TAATACGACTCACTATAGGGAGACCACGTGCTTGAAATCGGTCT
dsE2F5R	TAATACGACTCACTATAGGGAGACCACTCTTCTGCGCAAACTCAGTT
dsE2F6F	TAATACGACTCACTATAGGGAGACCACGTGCCAATCGAGTCCACATC
dsE2F6R	TAATACGACTCACTATAGGGAGACCACTCCTGTGGACTATCAGCAGC
dsE2F7F	TAATACGACTCACTATAGGGAGACCACAGACAGGCTCTTCGAGATCT
dsE2F7R	TAATACGACTCACTATAGGGAGACCACCAACCCCAATAACTCGAGCA
dsE2F8F	TAATACGACTCACTATAGGGAGACCACTCATTCCTAACCCACCATCCA
dsE2F8R	TAATACGACTCACTATAGGGAGACCACATGAATGATGGGTGGGTGGA
dsDP1F	TAATACGACTCACTATAGGGAGACCACGACTCCTGCCAACAACCAAG
dsDP1R	TAATACGACTCACTATAGGGAGACCACTTCCTGCACCGAGTTTGTTG
dsDP2F	TAATACGACTCACTATAGGGAGACCACCACAGCGCCTTCATGATCTC
dsDP2R	TAATACGACTCACTATAGGGAGACCACCATCGTCGGAGTAGTCGTGA
dsCDK1F	TAATACGACTCACTATAGGGAGACCACGCTTGAGAAGATCGGTGAGG
dsCDK1R	TAATACGACTCACTATAGGGAGACCACAACACCCGAGCAATACTTCG
dsCDK2F	TAATACGACTCACTATAGGGAGACCACGGACGAGACCTTCTACGAGG
dsCDK2R	TAATACGACTCACTATAGGGAGACCACCATTGCAGGCTCCATCAGTC
dsCCNEF	TAATACGACTCACTATAGGGAGACCACTACCGCGCGCCTGAGATA
dsCCNER	TAATACGACTCACTATAGGGAGACCACGGGAATAGCGGCTTGTAATCC
dsFutF	TAATACGACTCACTATAGGGAGACCACGCAGTGTCAAGAGTTGGTCG
dsFutR	TAATACGACTCACTATAGGGAGACCACCTCTCTGGTTCCTGTGCAGA
dsPCDH15F	TAATACGACTCACTATAGGGAGACCACGCCATTGATGCTGATGAGGG
dsPCDH15R	TAATACGACTCACTATAGGGAGACCACCAGTGTGATCGGTCTGGGTA
dsFOXOF	TAATACGACTCACTATAGGGAGACCACCTGTTCCCTGAATCGCCGCT

dsFOXOR	TAATACGACTCACTATAGGGAGACCACCGTTGCAGTCGAATCCGTCG
dsInR1F	TAATACGACTCACTATAGGGAGACCACGCAGTGGCTCAACAACAAGA
dsInR1R	TAATACGACTCACTATAGGGAGACCACCCTCGCTGAAGAAGTCCAAC
dsInR2F	TAATACGACTCACTATAGGGAGACCACGGAGCTATGGTGTTGTCTTGTG
dsInR2R	TAATACGACTCACTATAGGGAGACCACCTTGAGTTGGCCTCATTGGT
dsGFPF	TAATACGACTCACTATAGGGAGACCACTTTGTATAGTTCATCCATGCCATGT
dsGFPR	TAATACGACTCACTATAGGGAGACCACATGAGTAAAGGAGAAGAACTTTTCA

Table S3: Primers for RT-PCR, Related to Figure 7 and S3.

Name	Sequence (5'-3')	Size (bp)
E2F3QF	CCGAGCTGCTCTTCATGTT	95
E2F3QR	AAATGTGCCAATCGAGTCCA	
E2F5QF	CCTGCCGACGACATCAATAT	105
E2F5QR	TCTTTTCGACTTTTCCGCCT	
E2F6QF	TCTAAGATGGGAGGCCAAGT	105
E2F6QR	ATTCCTCTCAGTGGCCTTGA	
E2F7QF	CGTCAATCACAAGGGACACA	105
E2F7QR	GGCTGTTTTGCTGATGAGATG	
E2F8QF	ACATGTCTGCTATTCCCCAAC	103
E2F8QR	ATGAAACGTTGTGGAGGGTG	
DP1QF	TGCTGATGAGTTGGTCGAAG	97
DP1QR	TGCATCGTACACCCTTCTTC	
DP2QF	CTGATTGCCGAGGAAGTGAG	98
DP2QR	TCGTCGGAGTAGTCGTGAG	
CDK1QF	CAAGATTCTCAGCACACCGA	102
CDK1QR	TGATTGAGAGTGTTGGTGGTC	
CDK2QF	TGTCGAATCGCAAACCTCTT	96
CDK2QR	GCCAAGTCGTTTCATCAGGA	
CCNEQF	GTTCGTGAGGTTGGTTCAGT	95
CCNEQR	GGTAGACACAAGTAGCTGCC	
FutQF	AAGCAGCAAAGTGTGGAGAA	99
FutQR	CTTGGCAGCTGTCACTTTTG	
PCDH15QF	CTTATGAGTTTGTGGTGCGC	102
PCDH15QR	ACACTTCGGCATTGATACCC	

FOXOQF	ACCGGTTCATGCGCGTACAG	96
FOXOQR	CTCGACGGCGAGCTGATTTG	
InR1QF	GTCGGAGGAGATCAGCAGTC	101
InR1QR	CCACGTCTCTGTGCACGTAT	
InR2QF	GGAGCTATGGTGTTGTCTTG	97
InR2QR	CCTGCAAGTACGTAGGCTAA	
RPS15QF	TAAAAATGGCAGACGAAGAGCCCAA	150
RPS15QR	TTCCACGGTTGAAACGTCTGCG	

Table S4: Shapiro-Wilk normality test（P-value）, Related to Figure 7.

Gene Template	*CCNE*	*CDK1*	*CDK2*	*DP1*	*DP2*	*E2F3*	*E2F5*	*E2F6*	*E2F7*	*E2F8*	*FOXO*	*InR1*	*InR2*	*Futsch*	*PCDH15*
dsGFP	0.468	0.96	0.353	0.102	0.957	0.633	0.214	0.278	0.369	0.353	0.831	0.643	0.294	0.741	0.875
dsCCNE	0.149														
dsCDK1		0.298													
dsCDK2			0.492												
dsDP1				0.082											
dsDP2					0.27										
dsE2F3						0.361									
dsE2F5							0.468								
dsE2F6								0.313							
dsE2F7									0.181						
dsE2F8										0.31					
dsFOXO											0.89				
dsInR1												0.084			
dsInR2													0.603		
dsFutsch														0.401	
dsPCDH15															0.114

Table S5: Shapiro-Wilk normality test（P-value）, Related to Figure 7.

Gene / Template	*CCNE*	*CDK1*	*CDK2*	*DP1*	*DP2*	*E2F3*	*E2F5*	*E2F6*	*E2F7*	*E2F8*	*Futsch*	*PCDH15*
dsGFP+H_2O	0.975	0.08	0.505	0.604	0.808	0.294	0.998	0.578	0.124	0.502	0.078	0.391
dsGFP+PTX	0.405	0.632	0.935	0.883	0.447	0.929	0.183	0.615	0.799	0.09	0.122	0.669
dsFOXO+H_2O	0.191	0.053	0.136	0.216	0.574	0.871	0.814	0.194	0.286	0.664	0.544	0.158
dsFOXO+PTX	0.986	0.35	0.423	0.893	0.285	0.216	0.887	0.125	0.38	0.104	0.074	0.269

Transparent Methods

Insect rearing

Brown planthoppers were maintained in the lab and were originally a gift of Z.R. Zhu (Institute of Insect Science, Zhejiang University, China). The long- and short-winged adults were classified based on wing morphology as previously reported (Lin et al., 2016a; Lin et al., 2016b; Lin et al., 2016c). Seedlings of rice variety IIyou-023 (*Oryza sativa* L. cv.) cultured with nutrient solution (Yoshida et al., 1976) were used to feed the insects. Insects were maintained at 28℃, light:dark = 14h:10h, 70%-80% humidity.

Wing-bud dissection and staining

Brown planthopper nymphs were developmentally staged and collected. Wing pads were dissected in cold (4°C) PBS. Dissected wing pads were then fixed in 4% paraformaldehyde in PBS (Sangon Biotech, Shanghai, China) and washed three times in PBS (15 min./wash). For visualization of the nucleus, DAPI (1:1000, Sangon Biotech, Shanghai, China) was used. Phalloidin-iFluor 488 (1:100, Abcam, USA) was used for visualization of the actin cytoskeleton. Samples were incubated with stain for 1 hour at room temperature, then washed four times in PBS (15 min./wash) and mounted on slides in Anti-Fade Mounting Medium (Sangon Biotech, Shanghai, China).

Light Microscopy

A Zeiss confocal microscope (Zeiss LSM800, Zeiss, Germany) was used to visualize nuclei (DAPI) and actin cytoskeletons (Phalloidin-iFluor 488, Abcam, USA) of the wing pads. The distal region of the wing-pads was imaged. Images were acquired using Axion Vision and were processed using Adobe Photoshop 10.

Transmission Electron Microscopy (TEM)

Dissected wing-pads were fixed in 2.5% glutaraldehyde for 12 h，rinsed with 0.1M PBS (pH7) 3 times for 15 min each，fixed in 1% osmium tetroxide for 1.5 h, then rinsed twice in PBS (15 min each). Wing pads were then sequentially dehydrated with 50%, 70%, 80%, 90%, 95% ethanol, 15min each wash, 100% ethanol for 20 min and then 100% acetone for 20 min. The sample was treated with embedding agent and acetone at a volume ratio of 3:1 for 1 h, followed by a volume ratio of 1:1 for 3 h, and then with pure embedding agent for 12 h. The sample was placed in a heating polymerization apparatus at 70° C for 48 h, the excess resin was removed, and the sample was sliced using a Leica EM UC7 slicer, to a thickness of 70nm per slice. Slices were stained using uranyl acetate 50% ethanol saturated solution for 1 h, double distilled water elution, citric acid lead staining for 15 min, and the sample was observed using a Hitachi H-7650 electron microscopic.

Cloning of genes for dsRNA synthesis

Total RNA was extracted using the Trizol-based RNAiso Plus total RNA extraction kit (Takara, Dalian, China), and the Roche Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Shanghai, China) was used to synthesize first strand cDNA. Sequences of *NlInR1*, *NlInR2* and *NlFOXO* were identified from previously published *N. lugens* sequences (Lin et al., 2016b; Lin et al., 2016c; Xu et al., 2015). The previously cloned *InR1* and *FOXO* DNA fragments used as templates for dsRNA synthesis were amplified by PCR using

Ex-Taq polymerase (Takara, Dalian, China). *E2F3*, *E2F5*, *E2F6*, *E2F7*, *E2F8*, *CDK1*, *CDK2*, *DP1*, *DP2* *CCNE*, *Futsch* and *PCDH15* were identified from the transcriptome sequence (Xue et al., 2010) and the NCBI database (www.ncbi.nlm.nih.gov). All the primers are listed in Table S1. Genes were then cloned, confirmed by sequencing, and used as template for double stranded RNA (dsRNA) synthesis.

Injection of dsRNA and chemical inhibitors

RiboMAX™ Large Scale RNA Production System-T7 (Promega, Beijing, China) was used to synthesize dsRNA. dsRNAs of *InR1*, *FOXO*, *E2F3*, *E2F5*, *E2F6*, *E2F7*, *E2F8*, *CDK1*, *CDK2*, *DP1*, *DP2* and *CCNE*, were synthesized. The procedure was the same as described in Technical Bulletin TB166 (Promega, Beijing, China) except different templates were used to synthesize dsRNAs and different dsRNA primers (with a 27-base T7 sequence at the 5' end, Table S2) were used. Nocodazole (0.1 mM, dissolved in DMSO, MedChem Express, Shanghai, China) and Paclitaxel (0.1 mM, dissolved in DEPC-treated water, Sangon Biotech, Shanghai, China) were injected into 5^{th} instar nymphs in a total volume of 0.l μl per nymph. 4^{th} and 5^{th} instar brown planthopper nymphs were anesthetized by CO_2 before injection. A Nikon microscope and Narishige injection system (MN-151, Narishige) were used for injection and the procedure was the same as previously described (Lin et al., 2014). Each nymph was injected with 0.1 μg dsRNA, and afterwards the nymphs were allowed to recover for 2 hours before being returned to rice seedlings.

RT-PCR

Total RNA was isolated from dissected wing pads as above. 12 biological replicates were used for each treatment and twenty brown planthoppers were pooled for each replicate. Three technical replicates were used for each biological replicate and were averaged for statistical analysis. Smple sizes were indicated in the figure legend. Roche SYBR® Green PCR Master Mix and SYBR® Green RT-PCR Reagents kits (Roche Applied Science, Shanghai, China) were used for RT-PCR and first-strand cDNA synthesis, which was diluted 20 times after synthesis. For the first strand cDNA synthesis, we used 25 μl reaction, and 2 μl of diluted cDNA was used as template. Roche SYBR® Green PCR Master Mix and SYBR® Green RT-PCR Reagents kits (Roche Applied Science, Shanghai, China) were used for RT-PCR and first-strand cDNA synthesis, which was diluted 20 times after synthesis. For the first srand cDNA synthesis, we used 25 μl reaction, and 2 μl of diluted cDNA was used as template. $2^{-\Delta\Delta Ct}$ relative expression method was used for the expression level comparison (Livak and Schmittgen, 2001). The reference gene (*RPS15*) used was selected according to a previous study (Yuan et al., 2014). The primers used are shown in Table S3.

Measuring the number of nuclei

Image J (National Institutes of Health, USA) was used for nuclei number counting. The area was delineated, which was 475 μm^2 per block. The number of blocks used was not less than 9. The images were transformed into 8-bit TIFF format, i.e., the color picture was changed into black and white.

Flow cytometry

Wings were removed from newly emerged brown planthopper adults and placed in a 1.5 mL centrifuge tube in PBS. The wings were ground with a pestle. The ground samples were centrifuged (10000 g, 4°C, 3 min), the supernatant was discarded, the samples were resuspended in ethanol (100%) and placed on ice for 1 h, and then centrifuged (10000 g, 3 min, 4°C), the supernatant was discarded, the samples were resuspended in 1X PBS plus 0.25% Triton, and then held at 4°C for 10 min. The sample was centrifuged (10000g, 3min), the supernatant was discarded, and DAPI (1:1000, Sigma Aldrich, Shanghai, China) was then added. Samples were then measure in a Beckman CytoFLEX flow cytometer. Cell cycle analysis was carried out using FlowJo (FlowJo, LLC, USA).

Statistical analysis

SPSS 20.0 was used for statistical analyses. Chi-square test was used for comparison of the wing-morph ratios after treatment with dsRNAs. For independent sample t test, we first analyzed whether the data was normally distributed. Then independent sample t tests were carried out.

For one-way ANOVA, we first checked that the data of each group was normally distributed (Table S4, S5). Then ANOVA was carried out, and multiple comparison methods were selected based on the homogeneity test results. If the variance was homogeneous, we selected the LSD method under "Assumed Homogeneity of Variance" in the multiple comparisons; if the variance was not homogeneous, we selected Dunnett's T3 under "Unhypothesized Homogeneity of Variance". Different letters indicate significant differences ($P<0.01$).

Supplemental References

Lin, X., Xu, Y., Yao, Y., Wang, B., Lavine, M.D., and Lavine, L.C. (2016a). JNK signaling mediates wing form polymorphism in brown planthoppers (Nilaparvata lugens). Insect Biochem Mol Biol *73*, 55-61.

Lin, X., Yao, Y., Jin, M., and Li, Q. (2014). Characterization of the Distal-less gene homologue, NlDll, in the brown planthopper, Nilaparvata lugens (Stal). Gene *535*, 112-118.

Lin, X., Yao, Y., Wang, B., Emlen, D.J., and Lavine, L.C. (2016b). Ecological Trade-offs between Migration and Reproduction Are Mediated by the Nutrition-Sensitive Insulin-Signaling Pathway. Int J Biol Sci *12*, 607-616.

Lin, X., Yao, Y., Wang, B., Lavine, M.D., and Lavine, L.C. (2016c). FOXO links wing form polyphenism and wound healing in the brown planthopper, Nilaparvata lugens. Insect Biochem Mol Biol *70*, 24-31.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Xu, H.J., Xue, J., Lu, B., Zhang, X.C., Zhuo, J.C., He, S.F., Ma, X.F., Jiang, Y.Q., Fan, H.W., Xu, J.Y.*, et al.* (2015). Two insulin receptors determine alternative wing morphs in planthoppers. Nature *519*, 464-467.

Xue, J., Bao, Y.-Y., Li, B.-l., Cheng, Y.-B., Peng, Z.-Y., Liu, H., Xu, H.-J., Zhu, Z.-R., Lou, Y.-G., Cheng, J.-A.*, et al.* (2010). Transcriptome Analysis of the Brown Planthopper Nilaparvata lugens. PLoS One *5*, e14233.

Yoshida, S., Forno, D.A., and Cock, J.H. (1976). Laboratory manual for physiological studies of rice (International Rice Research Institute).

Yuan, M., Lu, Y., Zhu, X., Wan, H., Shakeel, M., Zhan, S., Jin, B.R., and Li, J. (2014). Selection and evaluation of potential reference genes for gene expression analysis in the brown planthopper, Nilaparvata lugens (Hemiptera: Delphacidae) using reverse-transcription quantitative PCR. PLoS One *9*, e86503.