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Decapentaplegic function in wing vein development and wing morph transformation in brown planthopper, *Nilaparvata lugens*

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ABSTRACT

The *decapentaplegic* (*dpp*) gene plays a variety of roles in diverse cellular and molecular processes of the growth and development. In insects, *dpp* is mainly required for dorsal-ventral patterning and appendage formation. The brown planthopper (BPH) *Nilaparvata lugens*, a major pest of rice, possesses two distinct wing morphs described as long-winged (LW) and short-winged (SW) morphs. With our lab-maintained stable strains of LW and SW BPH, RNA interference (RNAi) was used to research the functions of *N. lugens dpp* (*Nldpp*) on wing development. Silencing of *Nldpp* in the SW strain led to the significant lengthening of the forewing, while *Nldpp*-knockdown in the LW strain resulted in distorted wings. Moreover, knockdown of *Nldpp* caused the complete absence of wing veins. During the development of wing-pads, the *Nldpp* abundance in the terga of the SW strain was significantly higher than that of the LW strain. Through controlling the direction of wing morph transformation, we found that the expression level of *Nldpp* increased in the *NlInR1*-knockdown BPH (tending to SW) and abundance of *Nldpp* declined after ds*NlInR2* injection (tending to LW). Our results showed that *Nldpp* is mainly responsible for the formation and development of veins in BPH. Also, *Nldpp* can be regulated by *NlInR1/2* and participate in the wing morph transformation.

1. Introduction

The brown planthopper (BPH), *Nilaparvata lugens*, is the most destructive rice pest in Asia (Kong et al., 2015). This pest has two distinct wing morphs described as long-winged (LW) and short-winged (SW). LW BPHs can migrate to search for suitable habitats, whereas SW BPHs have higher fertility and cause more losses of rice crops (Supplementary Fig. S1) (Harrison, 1980). This wing dimorphism of BPH exacerbates the extent of damage and makes this pest more difficult to control (Kong et al., 2015). Understanding the mechanisms underlying wing morph differentiation can help inform and improve pest management strategies to control the BPH. Therefore, scholars have conducted a great deal of research on the development of the BPH wing (Bertuso et al., 2002; Iwanaga et al., 2011; Xue et al., 2010; Zhang, 1983). In recent years, two insulin receptor genes, *NlInR1* and *NlInR2*, have been revealed as switch genes in wing morph transformation in BPH (Xu et al., 2015). During the process of wing formation and development, however, many of the downstream events from *NlInR1/2* require more in-depth study.

Decapentaplegic (*dpp*), belonging to transforming growth factor β (TGF-β) superfamily, is a homolog of bone morphogenetic protein (BMP) and widely conserved among species. It plays a variety of roles in diverse cellular and molecular processes of growth and development in insects, similar to the growth and differentiation factors (GDFs), Activin and Nodal (Hogan, 1996; Massagué, 1998; Massagué and Chen, 2000). The function of insects' *dpp* gene is most thoroughly studied in *Drosophila melanogaster*. In the early stages of embryonic development, *dpp* is mainly involved in the formation of the embryonic septum and the differentiation of the dorsal-ventral axis (Irish and Gelbart, 1987). Subsequently, Dpp, cooperating with Hedgehog and Wingless signaling pathways, acts as a morphogen that determines the location and differentiation of appendages including wing, leg, and antenna by the presence of different concentration gradients of Dpp (Bryant, 1988; Morimura et al., 1996). Also, previous reports have shown that *dpp* functions in oogenesis and is responsible for the patterning of anterior eggshell structures (Twombly et al., 1996). Furthermore, *dpp* can also function in eye development by promoting the proliferation and motility of subretinal glia (Rangarajan et al., 2001; Yoshida et al., 2005).

Previous reports on the role of *dpp* in wing development are

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concentrated on *D. melanogaster*, while the studies performed on other insects is very limited. During the development process of *D. melanogaster* wing imaginal disc, *dpp* forms different concentration thresholds in the central stripe of the anterior-posterior compartment boundary and functions in a dose-dependent manner to regulate the expression levels of downstream genes (Nellen et al., 1996; Barrio and Milán, 2017). Subsequently, *dpp* determines the final wing size by controlling the growth and proliferation rates of cells in the developing wings (Barrio and Milán, 2017; Harmansa et al., 2015). In *D. melanogaster*, *dpp* expresses highly along the veins, and loss-of-function of *dpp* results in partial deletion or incomplete development of venation (Sotillos and de Celis, 2006; Yu et al., 1996). Similar to *D. melanogaster*, the *dpp* gene of sawfly *Athalia rosae* is ubiquitously expressed in both the forewing and hindwing, and *dpp*-knockdown caused a complete lack of vein development (Matsuda et al., 2013). In butterfly *Precis coenia*, *dpp* is involved in the formation of rays and eyespots on wings (Carroll et al., 1994). Also reported is *dpp*'s participation in wing bud regeneration of black cutworm *Agrotis ipsilon* (Wei, 2014). In summary, the functions of *dpp* on wing development are similar but slightly different among different species.

Two different wing morphs make BPH a good subject for exploring wing formation mechanisms. Characterization of *dpp* in BPH can help to deepen our understanding of wing dimorphism and expand our cognition of *dpp* function in hemipteran insects. In the current study, we first cloned the full-length cDNA of *Nldpp* using a rapid amplification of cDNA ends (RACE) method and carried out sequence analyses. With our lab-maintained LW and SW BPH strains, RNAi was used to research the functions of *Nldpp* on wing development. Then we performed quantitative PCR (qPCR) to detect and compare the differences in expression of *Nldpp* between the two strains. To further reveal the roles of *Nldpp* in wing morph formation, we controlled the direction of wing morph transformation by silencing of the switch genes *NlInR1/2* and measured the variation in expression level of *Nldpp*. Finally, we tested the effect of *Nldpp*-knockdown on seven wing development genes, and studied the function of these genes through RNAi.

2. Materials and methods

2.1. Insects

Wild BPHs were collected from rice fields in Huazhong Agricultural University in Wuhan, China and they were fed with susceptible rice variety Taichuang Native 1. Individuals were kept in a chamber at a temperature of 28 ± 1 °C, a relative humidity of 70 ± 5%, and a photoperiod of 14 h: 10 h (light: dark). Newly-eclosed adults (fewer than 12 h) of LW or SW morphs were maintained separately for more than 90 generations to obtain morph-stable sub-populations of the LW (proportion of LW ≈ 85%) and SW strains (proportion of SW ≈ 100%) according to the method of Morooka and Tojo (2008) .

2.2. Nldpp full-length cDNA cloning

Total RNA was isolated and extracted with the TransZol reagent (TransGen, China). cDNA was reverse transcribed with a PrimeScript™ RT reagent Kit (Takara, Japan). The full-length sequence of *Nldpp* was obtained from a RACE method according to a SMARTer® RACE 5'/3'Kit (Takara, Japan). The primers used in this study are presented in Supplementary Table S1.

2.3. Sequence analysis of Nldpp

The BPH genome sequences were referenced to Xue et al. (2014). Gene structure of *Nldpp* was analyzed with Expasy (http://web.expasy.org). The protein functional domain was predicted with InterProScan (http://www.ebi.ac.uk/interpro/) (Zdobnov and Apweiler, 2001).

2.4. dsRNA synthesis and microinjection

To avoid off-target effects, two dsRNAs that targeted different regions of *Nldpp* (Fig. 1) were synthesized with a MEGAscript RNAi Kit (Thermo Fisher Scientific, USA). Either 150 ng ds*Nldpp* or 100 ng ds*NlInR1/2* were injected into 3rd-instar nymph (1 d after ecdysis) from the ventral side of the thorax with a Nanoliter 2010 microinjector (WPI, USA). An equivalent amount of ds*GFP* (GenBank accession No. U76561) was injected as the control. The primers used for dsRNA synthesis are presented in Supplementary Table S1. The survival numbers of injected BPHs were recorded daily. Three replicates were set up for each treatment, and each replicate contained 50 nymphs. The whole bodies of treated-BPH were sampled on the 1st, 3rd, and 5th d after injection (corresponding to 3rd, 4th, and 5th instar nymphs) for evaluating the RNAi delivery efficiency, while the terga (containing wing pads) of treated-BPH were sampled on the 4th day after injection (corresponding to initial stage of the 5th instar) to detect the effect of *Nldpp*- or *NlInR1/2*-knockdown on other genes. Adult phenotypes were observed and recorded using a stereomicroscope (Olympus szx16, Japan).

2.5. qPCR

Total RNA of each sample was isolated and extracted using the TransZol reagent (TransGen, China). One microgram of total RNA in each sample was used for reverse transcription using a PrimeScript™ RT reagent Kit (Takara, Japan). qPCR was performed with a SYBR Premix Ex Taq (Takara, Japan) using a Bio-Rad CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA). Independent reactions were performed in quadruplicate, and the signal intensity of the target gene was presented as the average value. *Nlactin1* (GenBank accession No. EU179846.1) was used as the housekeeping gene for quantifying relative expression levels of test genes as described by Livak and Schmittgen (2001). The qPCR primers are listed in Supplementary Table S1.

2.6. Statistical analyses

Statistical analyses were carried out with SPSS 20.0. The differences between treatments were compared using the Student's *t*-tests or one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. $P < 0.05$ was considered statistically significant. All results are expressed as means ± SEM.

3. Results

3.1. Sequence analysis of Nldpp

The genome sequence of *Nldpp* contains 5 exons and 4 introns, while the full-length cDNA of *Nldpp* is 2, 217 bp, with a 5′-untranslated region (UTR) of 293 bp, a 3′ UTR of 755 bp and a poly(A) tail of 29 bp (GenBank accession No. MH633740). A total of 379 amino acid residues were encoded by *Nldpp* with a predicted protein molecular weight of 41.7 kDa and a pI of 8.41. The deduced amino acid sequence contains a TGF-β propeptide and a TGF-β ligand domain (Fig. 1).

3.2. RNA interference (RNAi) of Nldpp

ds1*Nldpp* and ds2*Nldpp* were designed to target different regions of *Nldpp* mRNA to avoid off-target effects. After knockdown of *Nldpp* at the 3rd instar, there was a significant reduction to varying degrees of *Nldpp* endogenous transcription on the 1st, 3rd, and 5th days. Results of the qPCR analysis showed that the expression levels of *Nldpp* were significantly reduced to 52.17%–80.37% in the LW strain while that of the SW strain decreased by 32.47%–57.81% (Supplementary Fig. S2). However, there was no significant difference in the RNAi efficiency between the two dsRNAs, as well as the *Nldpp*-knockdown phenotypes. Thus we randomly selected ds2*Nldpp* for follow-up experiments.

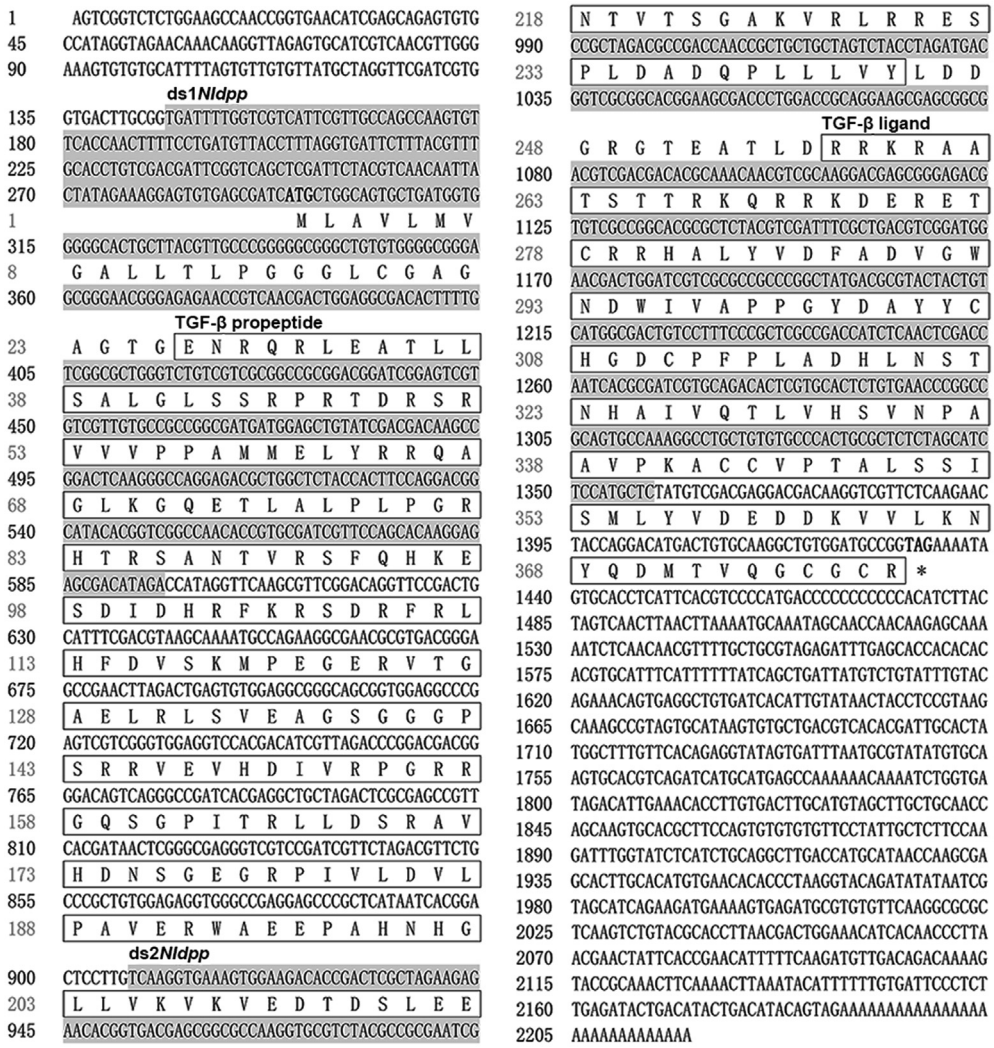


Fig. 1. Sequence analyses of *Nldpp*. Nucleotide and deduced amino acid sequences of *Nldpp*. A TGF-β propeptide and a TGF-β ligand domain are boxed. The corresponding sequences of ds1*Nldpp* and ds2*Nldpp* were shaded in grey.

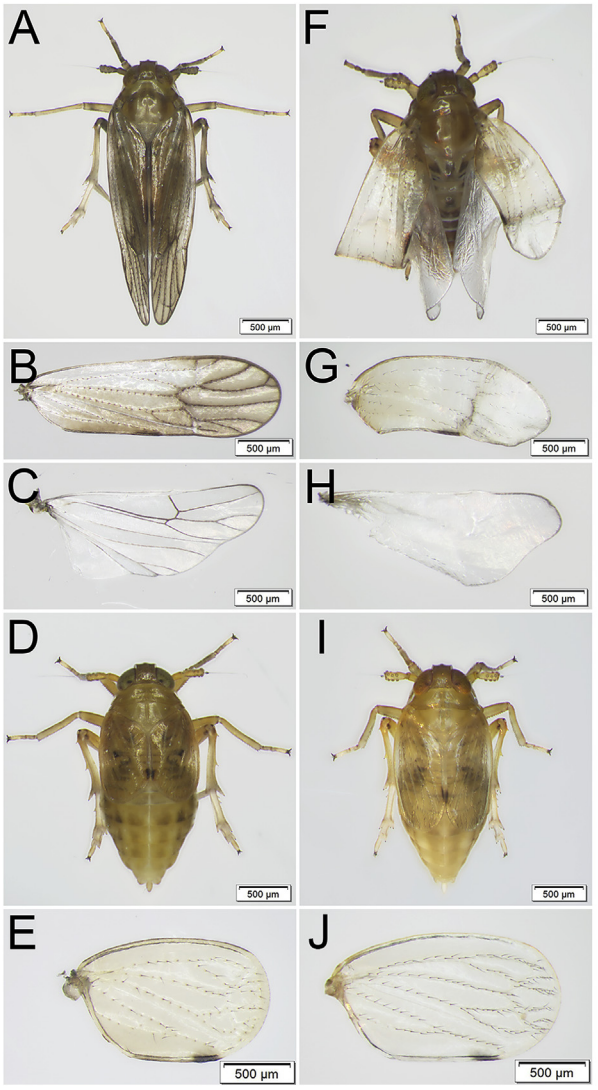
When the ds*Nldpp*-injected nymphs emerged as adults, the forewing and hindwing of LW BPH were distorted (phenotype rate was 100%) (Fig. 2A, F); but the wings of SW BPH was not (Fig. 2D, I). The forewing length of SW BPH (hindwing of SW BPH is not developed) was significantly longer while there was no obvious difference in wing length of LW BPH compared to that of the control group (Fig. 2K and L). More than 80% of the SW BPHs' forewing length significantly differed due to the knockdown of *Nldpp* (Fig. 2M, N). In addition, silencing of *Nldpp* caused the wing veins to be completely absent in almost all of the treated BPHs in both strains. However, the bristles typically found adhered to wings along the veins were present and unchanged. Notably, the bristles of the SW BPHs in the apex angle of the remigium were denser. After knockdown of *Nldpp*, the number of bristles in the forewing of SW BPH increased significantly from 96.3 (control, n = 10) to 174.8 (*Nldpp*-RNAi, n = 10) (Fig. 2A–J). Both the LW and SW strains showed extremely low survival rates (eclosion rates) of 7.33% (LW) and 8.67% (SW) after injection of ds*Nldpp*, which was significantly lower than that of BPHs treated with ds*GFP* (about 70%) (Supplementary Fig. S3).

3.3. Expression analysis of Nldpp

To further understand the functional mechanism of *Nldpp* in wing development, we measured the expression levels of *Nldpp* in terga of the 3rd, 4th, and 5th-instar nymphs using our lab-maintained stable LW and SW strains. The qPCR results showed significantly different expression characteristics between the two strains; especially in 5th-instar nymphs, the abundance of *Nldpp* in terga of the SW strain was nearly two times that of the LW strain (Fig. 3).

3.4. Effect of NlInR1/2-knockdown on the expression level of Nldpp

Two insulin receptors, *NlInR1* and *NlInR2*, have been shown to function as master regulators of wing morphs. They play opposite roles in the wing-morph determination in BPH. Usually, nymphal knockdown of *NlInR2* led to a strong bias towards LW-morph adults; in contrast, silencing of *NlInR1* led to a strong bias towards SW-morph adults (Xu et al., 2015). *NlInR1/2*-RNAi was conducted with 3rd-instar nymphs of the wild BPH population. Almost 100% of BPHs developed into LW morphs after silencing of *NlInR2*, while knockdown of *NlInR1* caused 100% of nymphs to develop into SW adults (Fig. 4A and B). Considering that *dpp* has multiple functions in insect development, we specifically dissected the tergum of the initial stage of 5th instar referring to a report in which this stage was considered as critical stage for wing morph differentiation and active period for wing-pad development (Jiang, 2016). In the terga of 5th-instar nymph, the expression level of *Nldpp* increased by 31.10% in *NlInR1*-knockdown BPH, and *Nldpp* abundance was reduced by 16.89% after ds*NlInR2* injection (Fig. 4C and D). These results indicate that during the process of wing morph formation, *Nldpp* may



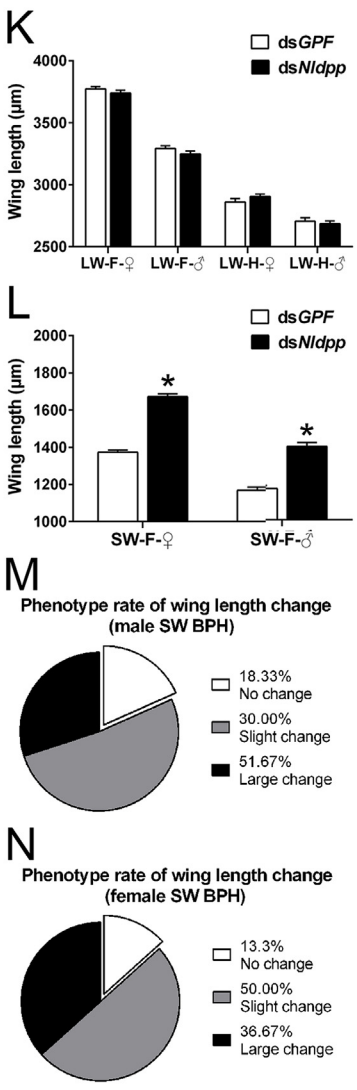


Fig. 2. Wing phenotypes of BPH adults developed from nymphs treated with ds*Nldpp* at the 3rd instar. A–E: Wing phenotypes of BPH treated with ds*GFP*. A: Normal LW BPH; B, C: Normal forewing (B) and hindwing (C) of LW BPH. D: Normal SW BPH; E: Normal forewing of SW BPH. F–J: Wing phenotypes of BPH treated with ds*Nldpp*. F: ds*Nldpp*-treated-BPH of LW BPH; G, H: Distorted forewing (G) and hindwing (H) without vein of LW BPH. I: ds*Nldpp*-treated-BPH of SW BPH; J: Forewing without vein of SW BPH. K, L: Wing lengths of BPH after ds*Nldpp* injection at the 3rd instar. ds*GFP* was injected as the control. Each value is the mean of twenty wing lengths. Error bars represent the standard errors. Bars labelled with an asterisk indicate significant differences in wing length between ds*GFP* and ds*Nldpp* injection treatments according to Student's *t*-tests ($P < 0.05$). K: The wing lengths of LW strain after ds*Nldpp* injection; L: The wing lengths of SW strain after ds*Nldpp* injection. M, N: Phenotype rate of wing length change in SW BPH after ds*Nldpp* injection at the 3rd instar. ds*GFP* was injected as the control. Each value is the mean of sixty wing lengths. The average wing length of the control was used as a reference. The wing length after *Nldpp*-RNAi was compared with the reference for calculating the change rate. No change: the change rate of wing length after ds*NlDpp* injection was less than 5% when compared to the control reference. Slight change: the change rate was between 5% and 10%. Large change: the change rate was more than 10%. M: Phenotype rate of male SW BPH; N: Phenotype rate of female SW BPH.

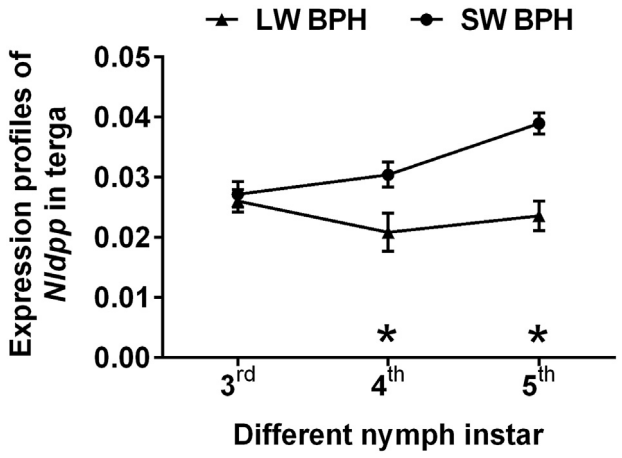


Fig. 3. Expression profiles of *Nldpp* in the nymph terga of LW and SW BPHs. One hundred terga of 3rd-, 4th-, or 5th-nymphs were used in each sample for RNA extraction and qPCR. Each value is the mean of four replicate samples. *Nlactin1* was used as a housekeeping gene for quantitation of relative expression. Error bars represent standard errors. Asterisk indicates significant differences in expression level between LW and SW strains according to Student's *t*-tests ($P < 0.05$).

function as a downstream gene and under the regulation of *NlInR1/2*. Moreover, we obtained similar results in wing morph proportions, and similar but stronger changes in *Nldpp* abundance due to the knockdown of *NlInR1* in our lab-maintained LW strain and knockdown of *NlInR2* in the SW strain (Supplementary Fig. S4).

3.5. Effect of Nldpp-knockdown on seven wing development genes

To further clarify the pathway of *Nldpp* regulating wing development in BPH, we detected the effect of *Nldpp*-knockdown on the expression level of seven wing development genes. Three of the seven genes, *spalt* (*sal*), *optomotor-blind* (*omb*), and *vestigial* (*vg*), were reported to act as downstream target genes of *dpp*, which are involved in the formation of wing structure and the position of wing veins (Zimmerman and Padgett, 2000). The other four genes that we screened, *Brinker* (*Brk*), *engrailed* (*en*), *hedgehog* (*hh*), and *scalloped* (*sd*), are closely related to *dpp* according to a wing development network (Abouheif and Wray, 2002). The terga of 5th-instar nymphs were sampled for detection. After injection of ds*Nldpp*, qPCR analyses showed that abundance of endogenous transcripts of *Nldpp* in BPH tergum reduced by about 50% (Fig. 5A). The expression levels of *Nlen*, *Nlhh*, *Nlomb*, *Nlsd*, and *Nlsal* were significantly lower than that of their respective control by varying degrees in both LW and SW strains (Fig. 5C–G), while *NlBrk* as greater by more than two-fold (Fig. 5B). However, *Nlvg* abundance was not affected (Fig. 5H).

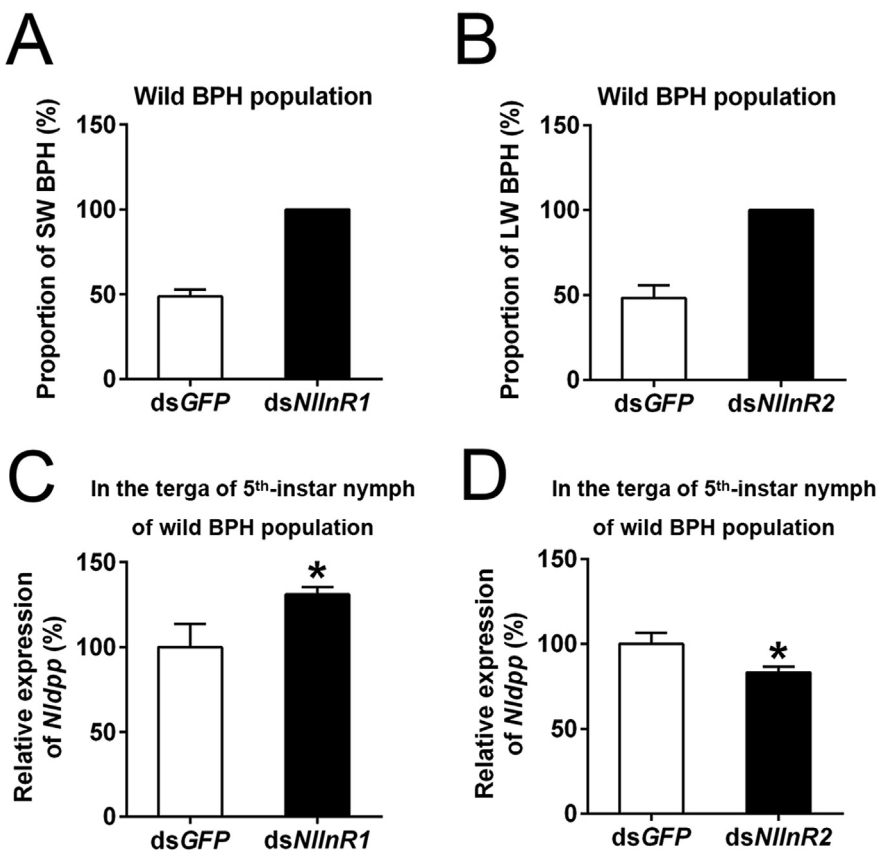


Fig. 4. Effect of *NlInRs*-knockdown on the wing morph of adults and the expression level of *Nldpp*. The amount of 100 ng ds*NlInR1* or ds*NlInR2* was injected into 3rd-instar nymphs of wild BPH population. ds*GFP* was injected as the control. Error bars represent standard errors. A: The proportion of SW BPH after injection of ds*NlInR1*. B: The proportion of LW BPH after injection of ds*NlInR2*. Each value is the mean of three replicates with 50 BPHs for each replicate. C: The expression level of *Nldpp* after injection of ds*NlInR1*. D: The expression level of *Nldpp* after injection of ds*NlInR2*. One hundred terga of 5th-instar nymphs per sample were used for RNA extraction and qPCR. Each value is the mean of four replicates. *Nlactin1* was used as a housekeeping gene for quantitation of *Nldpp* relative expression. Bars labelled with an asterisk indicate significant differences in expression levels between ds*GFP* and ds*NlInRs* injection treatments according to Student's *t*-tests ($P < 0.05$).

We then performed RNAi treatment with 3rd-instar nymphs of LW or SW strains to detect whether phenotypes caused by knockdown of these seven genes are identical or similar to those of *Nldpp*-RNAi. We found no obvious change in the adults of BPH after injection ds*Nlen*, ds*Nlhh*, or ds*NlBrk* when compared to ds*GFP* treatment (Fig. 6A–H). In contrast, *Nlsal*-, *Nlomb*-, *Nlvg*-, and *Nlsd*-RNAi caused different forms of defects in the wing (Fig. 6I-P). After knockdown of *Nlvg* and *Nlsal*, the forewing length of SW BPH increased significantly (increased by 11.24% and 8.59%, respectively, n = 10), which were consistent with the phenotype of *Nldpp*-RNAi. For LW strain, *Nlsal*-RNAi caused distorted forewings and hindwings (distorted wing rate was 100%, n = 19) (Fig. 6K and L), while *Nlvg*-RNAi resulted in blisters at the end of the wings (blister rate = 77.14%, n = 35) (Fig. 6O, P). Knockdown of *Nlomb* also caused blisters on the wing of LW BPH (blister rate = 30.95%, n = 42), which in addition caused the wings to roll up (distorted wing rate was 100%) (Fig. 6I and J). When the expression level of *Nlsd* or *Nlvg* was inhibited, the veins in both LW and SW BPHs became lighter (Fig. 6K and L), although this was a more subjective judgement.

4. Discussion

The diversity of wing morphology is one of the important reasons for population growth and development of insects. In recent years, a large number of studies have revealed that gene can play the same, similar, or even different roles in the wing development of various insects (Abouheif and Wray, 2002; Belleghem et al., 2012; Brisson et al., 2010; Brook and Diaz, 1996; Cadigan, 2002; Tomoyasu et al., 2009). As the research depth and breadth has expanded for this topic, knowledge and understanding of the diversity in gene function have gradually been deepened (Angelini and Kaufman, 2005; Linz and Tomoyasu, 2015). The research subjects have also gradually expanded from model insects to important pests (Corona et al., 2016; Martin et al., 2012; Xu et al., 2015). In our study, BPH was selected as the research subject mainly because of its wing dimorphism and as well as the fact that *dpp* has not been studied in this Hemipteran insect. Our results showed that *Nldpp* is mainly responsible for the formation of the veins during the wing development process. Also, *Nldpp* can respond to the regulatory signals from wing morph switch genes (*NlInR1* and *NlInR2*) and participate in wing morph transformation.

Silencing of *Nldpp* caused the complete absence of wing veins in both forewings and hindwings of LW and SW BPHs. This phenotype of *dpp*-knockdown in BPH was same with that of *A. rosae*, and was similar to that of *D. melanogaster*. In *A. rosae*, knockdown of *dpp* cause a lack of whole veins. Also, overexpression of *dpp* in *D. melanogaster* caused the development of extra veins (Capdevila and Guerrero, 1994), while *dpp* depletion resulted in a partial loss of veins (Sotillos and de Celis, 2006; Yu et al., 1996). The similar phenotypes in veins of *dpp*-knockdown suggested that *dpp* might somehow conserve its function in vein development in insects.

In addition to vein absence, *Nldpp*-knockdown also resulted in two distinct phenotypes in LW and SW BPH. After ds*Nldpp* injection, the wing of SW BPH grew significantly longer, while LW adults' forewings and hindwings were distorted. By detecting the expression profiles of *Nldpp* in morph-stable strains, we found significant differences of *Nldpp* abundances in LW and SW BPHs. Considering that *dpp* likely relies on different expression levels to perform its various functions (Bryant, 1988; Morimura et al., 1996), the differences in levels of *dpp* expression in LW and SW strains provide a possibility for *Nldpp* to play different roles. Given that the RNAi efficiency of ds*Nldpp* injection were almost the same in the two strains, we speculate that the differences of remaining *Nldpp* abundances after *Nldpp*-knockdown in LW and SW BPHs are likely to be the cause of the different phenotypes. Although the expression level of *Nldpp* decreased due to the *Nldpp*-knockdown in the SW strain, the remaining transcript abundance of *Nldpp* was still high and close to the level of normal individuals of the LW strain. When the ds*Nldpp*-treated nymphs developed into adults, their forewing grew longer significantly, and the

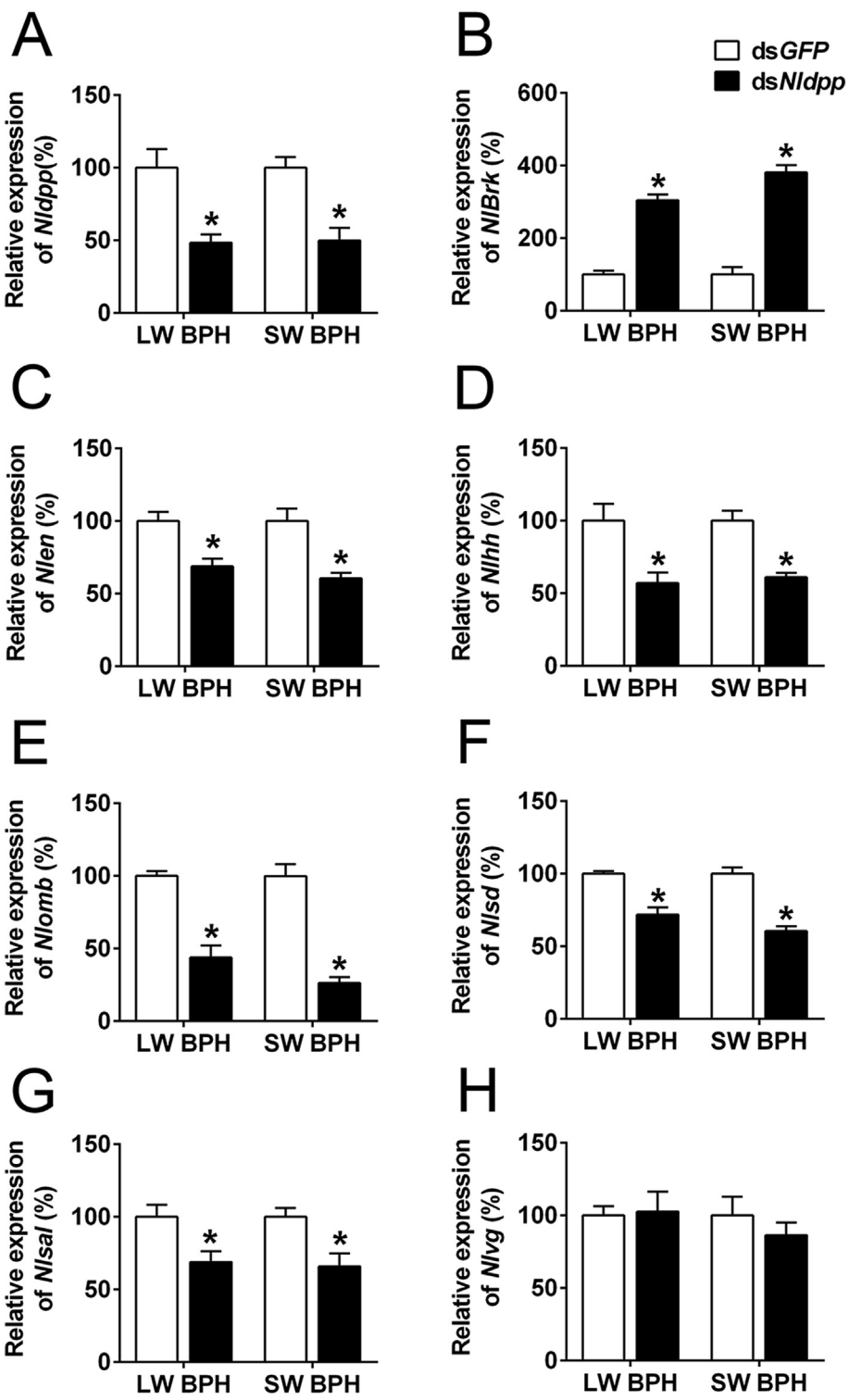


Fig. 5. Relative expression levels of wing development genes in 5th-instar nymph terga after ds*Nldpp* injection at the 3rd **instar.** ds*GFP* was injected as the control. For each sample, 100 terga of 5th-instar nymph were used for RNA extraction and qPCR. Each value is the mean of four replicates. *Nlactin1* was used as a housekeeping gene for quantitation of relative expression. Error bars represent standard errors. Bars labelled with an asterisk indicate significant differences in expression levels between ds*GFP* and ds*Nldpp* injection according to Student's *t*-tests ($P < 0.05$).

bristles in the apex angle of remigium clearly became thicker. Thus the forewing of SW BPH after *Nldpp*-knockdown showed a tendency toward to the LW BPH's forewing. However, there was no noticeable change in the hindwing of SW BPH. We also attempted to test in this experiment, a higher dosage of the ds*Nldpp* injection from 150 ng to 200 ng; however, all the tested BPHs died soon after treatment. The function of *Nldpp* is involved in many aspects of development (Irish and Gelbart, 1987; Morimura et al., 1996; Twombly et al., 1996; Yoshida et al., 2005). Down-regulation of *Nldpp* might affect some important physiological processes such as nerve cell development (Rangarajan et al., 2001), resulting in serious malformation or death. On the other hand, silencing of *Nldpp* in the LW strain reduced the *Nldpp* transcripts to a very low expression level. This low level of *Nldpp* was insufficient to maintain the normal development of wings. Consequently, both forewings and hindwings were distorted after eclosion.

Nldpp-RNAi can significantly alter the expression of several wing development genes, indicating that in the process of *Nldpp* regulating wing formation, more downstream genes are needed to participate and

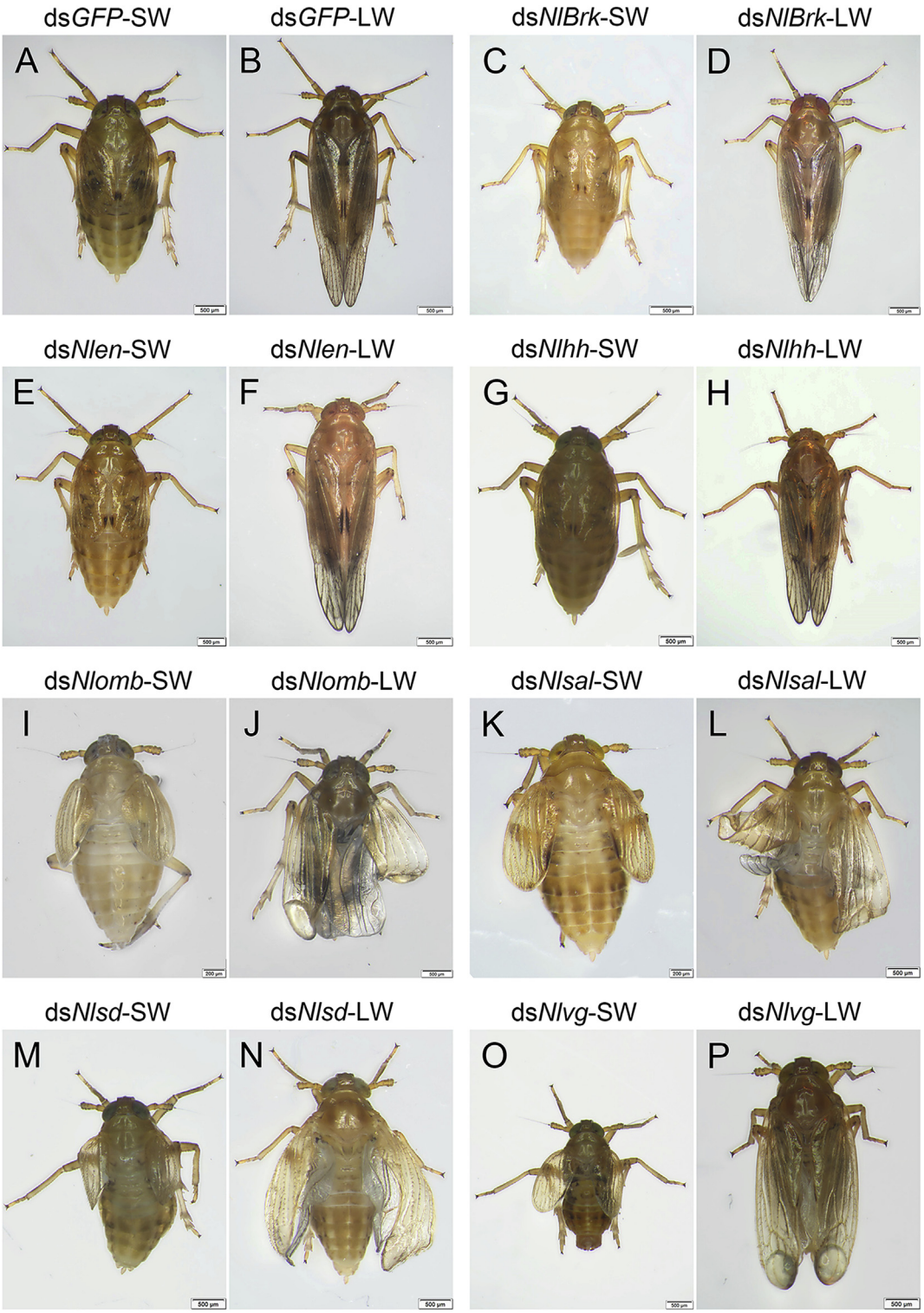


Fig. 6. Wing phenotypes of BPH adults developed from nymphs treated with dsRNA of seven wing development genes. The amount of 100 ng dsRNA of each gene was injected into 3rd-instar nymphs of LW or SW strain. ds*GFP* was injected as the control. qPCR was performed to measure and ensure that the expression level of each gene was significantly down-regulated after RNAi treatment. A, B: Wing phenotypes of SW BPH (A) and LW BPH (B) treated with ds*GFP*; C, D: Wing phenotypes of SW BPH (C) and LW BPH (D) treated with ds*NlBrk*; E, F: Wing phenotypes of SW BPH (E) and LW BPH (F) treated with ds*Nlen*; G, H: Wing phenotypes of SW BPH (G) and LW BPH (H) treated with ds*Nlhh*; I, J: Wing phenotypes of SW BPH (I) and LW BPH (J) treated with ds*Nlomb*; K, L: Wing phenotypes of SW BPH (K) and LW BPH (L) treated with ds*Nlsal*; M, N: Wing phenotypes of SW BPH (M) and LW BPH (N) treated with ds*Nlsd*; O, P: Wing phenotypes of SW BPH (O) and LW BPH (P) treated with ds*Nlvg*.

synergize. Through RNAi treatment, we found some similarities between the phenotypes of *Nldpp*-RNAi and the defects caused by RNAi of the wing development genes we tested. Note that phenotypic differences suggest that there might be more downstream genes of *Nldpp*, which need further study and verification. Restricted by technology and other factors, we have not been able to construct transgenic lines to specifically reduce or increase the expression level of *Nldpp* in wing tissue. To further verify the roles of *Nldpp* in wing morph differentiation, we measured the variations of *Nldpp* expression levels after injecting ds*NlInR1/2*. In the BPHs, which will develop into LW adults, the expression level of *Nldpp* decreased, while the *Nldpp* transcript abundance increased in those that are about to grow into SW adults. Taking into account the effect of *Nldpp*-knockdown on the expression levels of other genes, we concluded that *Nldpp* responds to upstream signals from *NlInR1/2* and participates in the wing morphs development by interacting with wing development genes.

5. Conclusion

Our results showed that *Nldpp* is mainly responsible for the formation and development of veins in BPH. Also, *Nldpp* can respond to the regulatory signals from wing morph switch genes (*NlInR1* and *NlInR2*) and participate in the development of the wing morphs via a potentially dose-dependent response and the interactions with wing development genes. This study deepens the understanding of wing dimorphism and expands the cognition of *dpp* function.

Conflicts of interest

The authors have declared that no competing interest exists.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.02.016.

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