Wing vein development in the sawfly *Athalia rosae* is regulated by spatial transcription of Dpp/BMP signaling components

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Abstract
Wing venation among insects serves as an excellent model to address how diversified patterns are produced. Previous studies suggest that evolutionarily conserved Decapentaplegic (Dpp)/Bone Morphogenetic Protein (BMP) signal plays a critical role in wing vein development in the Dipteran *Drosophila melanogaster* and the Hymenopteran sawfly *Athalia rosae*. In sawfly, *dpp* is ubiquitously expressed in the wing during prepupal stages, but Dpp/BMP signal is localized in the future vein cells. Since localized BMP signaling involves BMP binding protein Crossveinless (Cv), redistribution of BMP ligands appears to be crucial for sawfly wing vein formation. However, how ubiquitously expressed ligands lead to a localized signal remains to be addressed. Here, we found that BMP binding protein short gastrulation (Sog) is highly expressed in the intervein cells. Our data also reveal that BMP type I receptors thickveins (Tkv) and saxophone (Sax) are highly expressed in intervein cells and at lower levels in the vein progenitor cells. RNAi knockdown of *Ar-tkv* or *Ar-sax* indicates that both receptors are required for localized BMP signaling in the wing vein progenitor cells. Taken together, our data suggest that spatial transcription of core- and co-factors of the BMP pathway sustain localized BMP signaling during sawfly wing vein development.

Keywords
Sawfly *Athalia rosae*; wing vein development; BMP; Dpp

1. Introduction

Insect wings are highly diversified among species and provide a rich resource to address how morphological diversity manifests throughout evolution. Wing venations shows characteristic features in a species-specific manner due to the importance of aerodynamics associated with specific flight system (Wootton, 1992, Wootton et al., 2003). Although morphological diversity has been historically described among many different species (Comstock and Needham, 1898), the molecular mechanisms of wing vein development have been mostly investigated in the Dipteran *Drosophila melanogaster*. Therefore, very little is known about mechanisms underlying diversity in wing venation.

In *Drosophila*, wing vein development is established through two consecutive stages. The positional information of longitudinal veins (LVs) is determined by a morphogen gradient composed of Dpp and Hedgehog (Hh) signaling in the larval wing imaginal disc (Affolter and Basler, 2007, Shimmi et al., 2014). Formation of LVs is further established by the interactions of three growth factor signaling pathways: the Dpp, Epidermal Growth Factor (EGF) and Notch pathways (Blair, 2007). Then, during pupal stages, the positional information of crossveins appears to be established by inducing Dpp/BMP signaling (O'Connor et al., 2006, Shimmi et al., 2005, Ralston and Blair, 2005, Matsuda and Shimmi, 2012). Intriguingly, *dpp* expression is only observed in the LV progenitor cells during the early pupal stage, but Dpp/BMP signal is detected in all the future vein cells, including the crossveins (Matsuda and Shimmi, 2012, Ralston and Blair, 2005). Therefore, a combination of a short-range signaling in LVs and long-range signaling in crossveins is crucial for spatial regulation of BMP signaling during *Drosophila* pupal wing vein development.

The core components of BMP signaling pathway are highly conserved, including BMP ligands, BMP receptors, and downstream factor Smad proteins. In *Drosophila*, the following genes were identified as core components: the three ligands Dpp, Glass bottom boat (Gbb), and Screw (Scw); the two type I receptors Thickveins (Tkv) and Saxophone (Sax); the two type II receptors Punt (Put) and Wishful thinking (Wit); and the three Smad proteins Mothers against Dpp (Mad), Medea, and Daughters against Dpp (Dad) (Kahlem and Newfeld, 2009). Furthermore, co-factors are often utilized for spatial regulation of BMP signaling in a context-dependent manner. For example, two BMP binding proteins, Sog and Cv, are required for BMP ligand trafficking from LVs into crossveins to sustain long-range signaling (Matsuda and Shimmi, 2012). Interestingly, a similar mechanism comprising BMP and Sog (or Chordin in vertebrates) is widely utilized among species ranging from Cnidaria to all phyla of Bilateria to establish dorsal-ventral patterning during early embryogenesis (De Robertis, 2008, Bier and De Robertis, 2015). Therefore, gene sets involved in this network may have been co-opted for insect wing vein formation (Shimmi et al., 2014).

The sawfly *Athalia rosae* is a primitive member of Hymenoptera that is the most basal group of the Holometabola (Misof et al., 2014). The sawfly has two pairs of wings with different patterns of venation (Fig. 1A, B). Previous observations suggest that vein formation largely takes place in both fore- and hindwings during prepupal stages (Matsuda et al., 2013b). Furthermore, conserved Dpp/BMP signaling is needed for wing vein development in sawfly. Intriguingly, in contrast to the localized expression of *Dm-dpp* in the *Drosophila* pupal wing, *Ar-dpp* is ubiquitously expressed in the sawfly prepupal wing to produce localized BMP signaling in the wing vein progenitor cells. BMP binding protein Ar-Cv is needed for localized BMP signaling (Matsuda et al., 2013b). These facts indicate that sawfly wing vein formation may need long-range BMP signaling regulated by BMP binding proteins, which is a circumstance similar to crossvein formation in the *Drosophila* wing (Shimmi et al., 2014). However, how spatial distribution of BMP signal is regulated, and what components are required for localizing BMP signal, remain to be addressed.

In this study, to understand further how Dpp/BMP signal is regulated in sawfly wing vein development, we investigate how *Ar-sog*, BMP type I receptors *Ar-tkv* and *Ar-sax*, and BMP ligand *Ar-gbb* are expressed in sawfly prepupal wing by in situ hybridization. Our data reveal that *Ar-sog*,

Ar-tkv and *Ar-sax* are highly expressed in the future intervein cells. Functional analysis of Ar-Tkv and Ar-Sax by RNAi knockdown indicate that both receptors are needed for wing vein formation. Furthermore, biochemical analysis supports that Ar-Tkv encodes functional BMP receptor. Taken together, our data suggest that wing vein development in sawfly is regulated by spatial transcription of components involved in the BMP signaling pathway.

2. Materials and methods

2.1. Sawfly

Laboratory stocks of the sawfly, *Athalia rosae* were maintained at 25°C under 16L-8D light conditions. General biology and staging were described previously (Oishi et al., 1993). The last instar larvae used for injection of double-strand RNAs (dsRNAs) were identifiable by their body color and their wondering behavior to dig into soil to make cocoons. Prepupal stages last about four days, and were staged as PCF (post-cocoon formation) 1-4.

2.2. Plasmid construction

The information of sawfly orthologues encoding *gbb*, *sog*, *tkv* and *sax* was retrieved from the genome database of the i5k initiatives (https://www.hgsc.bcm.edu/arthropods/turnip-sawfly-genome-project) and gene annotation by NCBI (BioProject: PRJNA282653). Based on the sequences of predicted transcripts, gene-specific primers to amplify their open reading frame (ORF) were designed. The primers used are listed at Table S1. DNA fragments corresponding to each ORF were amplified using the gene-specific primers and the cDNA constructed from embryonic RNA pool by using a SMARTer PCR cDNA synthesis kit (Clontech). Each PCR product was cloned into pCR4Blunt-TOPO vector using a Zero Blunt TOPO PCR cloning kit for sequencing (Invitrogen). The inserts were sequenced and confirmed to match the sequences in database. These cDNA clones were now termed *Ar-gbb* (LC373544), *Ar-sog* (LC373543), *Ar-tkv* (LC373545) and *Ar-sax* (LC373546). *Ar-tkv* cDNA with GFP tag at the C-terminus was amplified using a primer set listed at Table S1 and cloned into pUAST-attB vector (Invitrogen).

2.3. Immunostaining and in situ hybridization

Immunostaining and in situ hybridization were performed as previously described (Matsuda et al., 2013b). Briefly, prepupal tissues during late PCF3 or PCF4 stages were fixed with 4% formaldehyde in PBS (nacalai tesque) overnight at 4°C. Samples were then washed 3 x 10 minutes in PBT (PBS / 0.1% Tween 20), permeabilized for 15 minutes with PBT, and blocked for 1 hour with 5% normal goat serum / PBT at room temperature. The primary antibody was rabbit anti-pMad (Cell signaling) and the secondary antibody was Goat anti-rabbit IgG Alexa 568 (Life Technologies). Digoxigenin-labeled probes for *Ar-sog*, *Ar-gbb*, *Ar-tkv* or *Ar-sax* RNA were used for in situ hybridization.

2.4. Imaging and image analysis

Fluorescent images were captured with a Leica TCS SP8 confocal microscopy. Images of in situ hybridization were obtained with a Nikon Eclipse 90i microscope. Dark field images were taken with Zeiss Lumar V12 Stereoscope. All images were processed and analyzed with ImageJ.

2.5. BMP signaling assay and Western blotting

A cell-based BMP signaling assay was conducted as described previously (Tauscher et al., 2016). Briefly, *Drosophila* S2 cells were transfected with indicated genes and *tubp-GAL4*. Three days after transfection, the cells were collected. The cells were then spun down and resuspended into 80 μl of 1x SDS-PAGE sample buffer. The BMP signals were then measured by Western blotting probing with the following antibodies: primary antibodies, mouse anti-Flag M2 (Sigma), mouse anti-GFP (Merck) and rabbit anti-pMad antibodies; secondary antibodies, anti-mouse-680

(LI-COR) and anti-rabbit-800 (LI-COR) antibodies; then analyzed with the Odyssey Infrared Imaging System (LI-COR).

2.6. Preparation of dsRNA and RNAi

Short cDNA fragments corresponding to a part of *Ar-tkv* (351 bp), *Ar-sax* (370 bp) and the *enhanced green fluorescent protein* (*egfp*) gene (298 bp) were PCR-amplified using PrimeStar HS DNA polymerase (Takara) and the gene-specific primers to which the T7 polymerase promoter sequence was incorporated at each 5´end. The primers used are listed at Table S1. Templates used were plasmids carrying the full-length cDNAs of *Ar-tkv* and *Ar-sax*, respectively and the *egfp*-carrying pPIGA3GFP/hspGFP S65T transformation vector (Sumitani et al., 2003). The dsRNAs were synthesized using a MEGAScript T7 kit (Ambion) and the T7 promoter sequence-flanking short cDNAs as templates. Synthesized dsRNAs were suspended in RNase-free distilled water in a concentrated solution (3μg/μl) and stored at –20˚C until used. dsRNAs were injected into hemocoel of the last instar larvae anesthetized by chilling on ice for 30 min as described previously (Yoshiyama et al., 2013).

2.7. Reverse transcription (RT)-PCR

Total RNA was extracted from a pair of wing primordia attaching with thoracic tissues of prepupae using an RNeasy mini kit (Qiagen). Transcripts of *Ar-tkv*, *Ar-sax*, *Ar-dpp* (AB121072) and *A. rosae elongation factor-1α* (*Ar-ef-1α*, AB253792) were amplified using a OneStep RT-PCR kit and gene-specific primer sets with 50 ng of total RNA as template. The primers used are listed at Table S1. The PCR cycles performed for amplification were 25 cycles for fragments of *Ar-tkv* (expected length: 440 bp), *Ar-sax* (733 bp), and *Ar-dpp* (499 bp), and 20 cycles for that of *Ar-ef-1α* (339 bp).

3. Results

3.1. Dpp/BMP signal is localized in the wing vein progenitor cells

Previous studies show that BMP signaling readout by phospho-Smad1/5/8 (pMad) antibody staining reflects the location of wing vein progenitor cells during the prepupal PCF3 and PCF4 stages (Matsuda et al., 2013b). To further investigate how BMP is induced at the cellular level, fluorescent images of pMad accumulation were captured by confocal microscopy. Similar to previous observations, pMad positive cells largely reflect the future wing vein cells when images were processed through maximal projection (Fig. 1C, D). To understand what cells accumulate pMad signaling, pMad staining on the Medial vein of the forewing was further investigated. Optical cross-sections around the Medial vein indicate that pMad signal is largely observed in the nucleus of cells forming the lumen (Fig. 1E-E'''). pMad accumulation in the intervein cells, including the flanking regions, appears to be below the threshold of detection. These results suggest that widely expressed Ar-Dpp may traffic from intervein cells to future vein cells for formation of a localized BMP signal, consistent with a long-range signaling model in sawfly wing development.

3.2. BMP ligand Gbb is expressed in the sawfly prepupal wing

In *Drosophila*, the BMP5/6/7/8 type ligand Gbb as well as Dpp are expressed in the pupal wing to sustain long-range Dpp:Gbb signaling into crossveins (Matsuda and Shimmi, 2012, Ray and Wharton, 2001). Thus, we investigated whether Gbb is also expressed in the sawfly prepupal wing. *Ar-gbb* cDNA is 1320bp long, encoding 439 amino acids, and Gbb orthologues share homology among insects (Fig. S1A). During PCF4, *Ar-gbb* is ubiquitously expressed in wing tissue, although *Ar-gbb* appears to be expressed at higher levels around the flanking regions of the future vein cells in both fore- and hindwings (Fig. 2A, B). These results together with our previous findings suggest that the two BMP ligands Ar-Dpp and Ar-Gbb are expressed in the wing progenitor cells, but do not provide the positional information for signaling.

3.3. Ar-sog is expressed in the prepupal wing

In *Drosophila*, long-range signaling from LVs into crossveins requires the BMP binding protein Sog, which is predominantly expressed in intervein cells (Matsuda and Shimmi, 2012). To address whether sawfly Sog is expressed in a similar manner, we first isolated *Ar-sog* cDNA. *Ar-sog* cDNA is 2874bp long, encoding 957 amino acids, and Sog orthologs are conserved among insects (Fig. S1B). We then examined *Ar-sog* expression in the prepupal wings by in situ hybridization. During late PCF3/early PCF4, our data reveal that *Ar-sog* is highly expressed in the future wing intervein cells and much less in future longitudinal veins in both fore- and hindwings (Fig. 2C, D). Similar pattern is seen during late PCF4 (Fig. 2E, F). Although these results suggest that *Ar-sog* may be weakly expressed in the future vein cells, *Ar-sog* expression shows complementary patterns of pMad accumulation during insect wing vein development, serving to provide positional information of vein cells (Shimmi et al., 2014).

3.4. BMP receptors Tkv and Sax are expressed in the prepupal wing and are required for wing vein formation.

We next asked how BMP signal is interpreted by BMP receptors. As there are two type I receptors in *Drosophila*, we performed a BLAST search to find orthologous genes in the sawfly. We found that there exist two type I receptor-like genes in the sawfly, which are similar to Tkv- and Sax-type receptors, and isolated the cDNAs. *Ar-tkv* cDNA is 1536 bp, encoding 511 amino acids (Fig. S2A). *Ar-sax* cDNA is 1812bp, encoding 603 amino acids (Fig. S2B). We then investigated their expression in the prepupal wing. Interestingly, *Ar-tkv* is highly expressed in the center of the intervein region and much lower in future vein and flanking cells in both fore- and hind-wings (Fig. 3A, B). *Ar-sax* expression is also highly expressed in the intervein cells and lower in the future vein cells (Fig. 3C, D). Since both Ar-Tkv and Ar-Sax expression show complementary patterns to pMad signal in the prepupal wing, one interpretation is that they encode non-functional receptors, e.g. scavenger receptors that trap the ligands (Luo et al., 2015), and other receptors may contribute to BMP signaling in the future vein cells. If this is the case, loss-of-function of *Ar-tkv* or *Ar-sax* may lead to expansion of BMP signaling, resulting in ectopic wing vein formation. To test this idea, RNAi knockdown of *Ar-tkv* or *Ar-sax* was performed. When dsRNA against *Ar-tkv* or *Ar-sax* was injected into the dorsal hemocoel during the final larval instar, transcripts of *Ar-tkv* or *Ar-sax* in prepupal PCF4 wings were significantly reduced, respectively (Fig. 4A). Wing vein formation during PCF4 was substantially disrupted by injection of 1 μg or 3 μg of dsRNAi against *Ar-tkv* or *Ar-sax* (Fig. 4B-G). We then addressed how BMP signal is regulated. When *Ar-tkv* was knocked down (1 μg of dsRNA), pMad staining largely disappears in both fore- and hindwings during PCF4 (Fig. 4H-K). This happens even when a lower concentration of dsRNA against *Ar-tkv* was injected (Table 1). pMad staining in *Ar-sax* RNAi (3 μg of dsRNA) is substantially reduced but partially remains or is ectopically produced in both fore- and hindwings during PCF4 (Fig. 4L, M and Table 1). To further test whether Ar-Tkv encodes functional BMP receptor, GFP tagged Ar-Tkv was expressed in *Drosophila* S2 cells. Ar-Tkv:GFP proteins are observed at the molecular weights expected and induce pMad accumulation in cell lysates when co-expressed with Mad and Dm-Dpp (Fig. 5). Taken together, these results indicate that both Ar-Tkv and Ar-Sax are required for wing vein development but presumably each in a distinct manner.

4. Discussion

This study shows the involvement of core- and co-factors of BMP signaling in sawfly wing vein development. Although previous studies indicate that BMP signal is crucial for wing vein formation during prepupal stages in the sawfly, and BMP ligands are likely to be redistributed for pMad accumulation (Matsuda and Shimmi, 2012), it remains to be addressed how patterned BMP

signal is produced. Here, our findings suggest that spatial transcription of core- and co-factors of BMP signaling is likely one of the means to bring about the patterned BMP signal.

4.1. Co-factors of BMP trafficking in sawfly wing vein development

Provided that a long-range trafficking of BMP ligands is needed for localized BMP signal in all wing vein progenitor cells in sawfly, it would be interesting to investigate which *Drosophila* orthologs in sawfly are involved in BMP signal induction in crossvein progenitors during pupal stages. In *Drosophila*, in addition to BMP binding proteins Sog and Cv, various molecules have been shown to be required for crossvein development. Crossveinless-2, another BMP binding protein, is needed for BMP signaling in future crossvein cells by serving as a co-receptor of BMP signal (Serpe et al., 2008, Conley et al., 2000). Heparan sulfate proteoglycan (HSPG) Dally has been shown to be required for BMP signaling in the PCV region through interaction with Crossveinless-2 (Serpe et al., 2008). Tolloid-related protease functions as a Sog processing enzyme to induce BMP signal by releasing BMP ligands from ligand-Sog complex (Serpe et al., 2005). Crossveinless-c, encoding a Rho GTPase-activating protein, is also required for BMP signaling in crossveins by down-regulating the Rho GTPase-like pathway at the basal side of wing epithelial cells (Matsuda et al., 2013a). Furthermore, Crossveinless-d, encoding a vitellogenin-like lipoprotein, is expressed in fat body during pupal stages and is required for BMP signaling in crossvein progenitor cells of the pupal wing (Chen et al., 2012). Besides, differential cleavage of Dpp precursor may affect signaling range in the wing tissues (Kunnapuu et al., 2009). These observations indicate that various factors are involved in sustaining long-range BMP signaling from LVs into crossveins in different contexts.

Our previous observations indicate that Ar-Cv plays a significant role in inducing localized BMP signaling in wing vein progenitor cells (Matsuda et al., 2013b). This study further suggests that Ar-Sog expression appears to provide the positional information of vein progenitor cells (Fig. 2). Functional analysis of Ar-Sog using RNAi knockdown is anticipated to reveal whether Ar-Sog is required for BMP signaling during sawfly wing vein development. Furthermore, investigation of Crossveinless-2, Crossveinless-c, Crossveinless-d or Dally orthologs in sawfly will help reveal the molecular mechanisms underlying sawfly wing vein formation.

Besides co-factors involved in BMP signaling, other growth factors may regulate wing vein formation in sawfly as well. In *Drosophila*, both Notch and EGF are crucial for wing vein differentiation by interacting with the Dpp/BMP signaling pathway (Sotillos and De Celis, 2005, Blair, 2007). Notch has been shown to play a crucial role in establishing the vein – intervein boundary in *Drosophila* pupal wings (de Celis et al., 1997). Future study will reveal how they contribute to sawfly wing vein development.

4.2. Spatial transcription of BMP type I receptors and localized BMP signaling

In *Drosophila*, BMP type I receptor Tkv distribution is tightly modulated in pupal wings as well as in larval wing imaginal discs to regulate ligand mobility (Lecuit and Cohen, 1998, Matsuda and Shimmi, 2012). Thus, we hypothesized that localized BMP signaling may involve transcriptional regulation of BMP receptors in sawfly wing vein development. Interestingly, in contrast to Dm-Tkv, which is highly expressed at the flanking region of longitudinal veins of the pupal wing, Ar-Tkv expression is very high in intervein cells but very low around future vein cells, including the flanking region (Fig. 3). RNAi analysis suggests that Ar-Tkv in wing vein cells is needed for BMP signaling (Fig. 4). Biochemical analysis further supports that Ar-Tkv encodes a functional BMP receptor when expressed in *Drosophila* S2 cells (Fig. 5). Although it remains to be addressed how Ar-Tkv in intervein cells is involved in wing development, Ar-Tkv appears to serve as a primary BMP type I receptor during wing vein development.

Ar-sax RNAi phenotypes are different from *Ar-tkv* RNAi. Loss of *Ar-sax* shows partial loss or even ectopic expression of BMP signal (Fig. 4). These phenotypes may be interpreted by invoking a supporting role for Ar-Sax signaling during wing vein development. In *Drosophila,* loss of *Dm-sax* results in abnormal or ectopic vein formation (Singer et al., 1997). It has been proposed that Dm-Sax is capable of both promoting and inhibiting BMP signaling in *Drosophila* wing imaginal disc (Bangi and Wharton, 2006). Similar mechanisms may be utilized in sawfly wing development.

4.3. Evolutional insights of insect wing venations

Although insect wing venation is a rich resource to address how morphological diversity is produced, very little is known about molecular mechanisms behind wing vein development among species. We have previously proposed that changes in Dpp/BMP signal are key mechanisms for creating vein patterning (Shimmi et al., 2014). How are patterns of Dpp/BMP signal produced? Understanding transcriptional regulation of BMP ligand Dpp and BMP binding protein Sog may be key to address these questions. Although spatial regulation of Dpp ligands must be a crucial process for producing wing vein patterns, *dpp* expression does not always imitate vein patterns. *Drosophila Dm-dpp* expression manifests the positional information of longitudinal veins, yet in contrast, sawfly *Ar-dpp* expression does not provide positional information due to its ubiquitous expression. On the other hand, both *Dm-sog* and *Ar-sog* expressions are consistently observed in intervein regions. Importantly, *sog* expression (or *chordin* in vertebrates) has been proposed to be evolutionary conserved in early embryogenesis to instruct spatial BMP activity (Bier and De Robertis, 2015). In contrast, temporal but not spatial regulation of BMP ligand transcription is crucial in this process (Reversade and De Robertis, 2005, Lapraz et al., 2009, Genikhovich et al., 2015). Therefore, co-option of conserved systems comprising of BMP and Sog/Chordin may lead to diversified insect wing venation. Further studies concerning *sog* transcription in wing development among various insect species should help uncover the mechanisms behind diversified vein patterning.

In summary, we found that BMP type I receptors Ar-Tkv and Ar-Sax, and BMP binding protein Ar-Sog, are highly expressed in intervein cells in both fore- and hindwings during prepupal stages. Our data further reveal that both Ar-Tkv and Ar-Sax are required for localized BMP signaling in wing vein progenitor cells. Taken together, our observations provide insights that spatial transcription of core- and co-factors of BMP signal are required for sawfly wing vein development.

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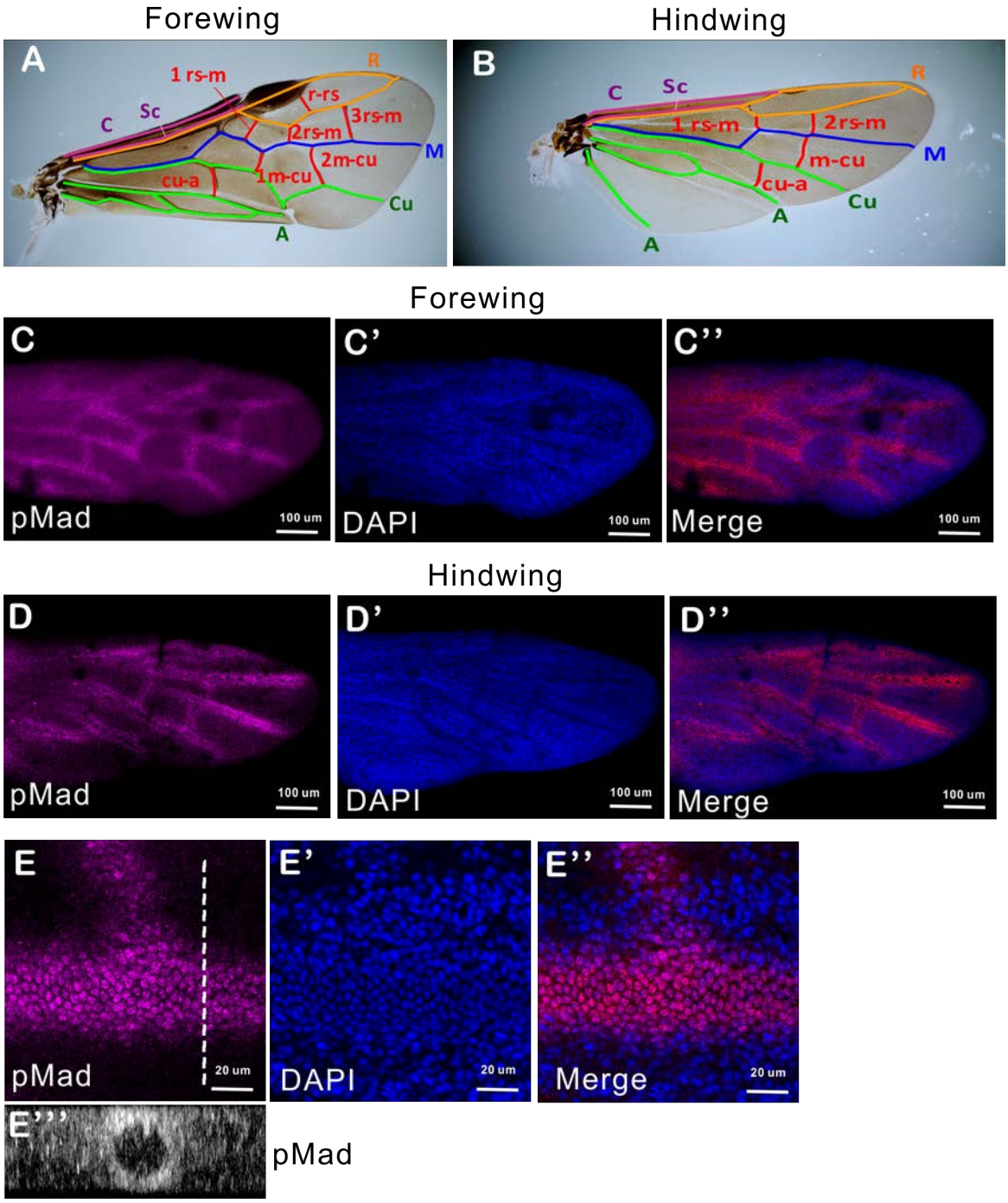

Fig. 1. BMP signal reflects wing vein formation in sawfly. (A, B) Fore- and hindwings of adult sawfly. LVs are marked in capital letters and crossveins in small letters. LVs: Costa (C), Subcosta (Sc), Radius (R), Media (M), Cubitus (Cu), Anal (A). Name of crossveins reflect the connected LVs and are numbered sequentially from proximal to distal. (C, D) pMad accumulation (C, D), DAPI (C', D') and merged image (C'', D'') of forewing (C-C'') and hindwing (D-D'') at prepupal stage PCF4. (E) Higher magnification image of pMad (E), DAPI (E') and merged image (E'') on Medial vein (M) of forewing at prepupal stage PCF4. Optical cross section of pMad accumulation on the dashed line is shown in the lower panel (B/W image) (E''').

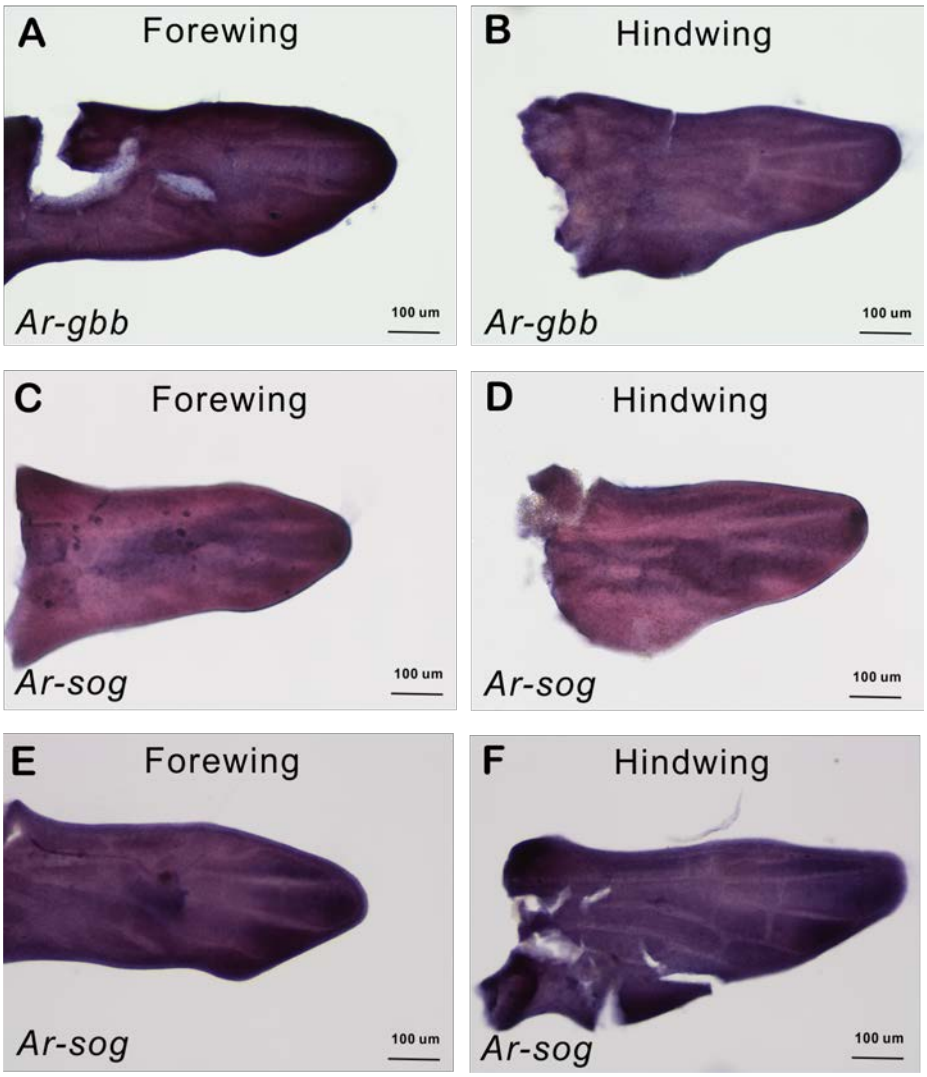

Fig. 2. Sawfly *gbb* and *sog* are expressed in prepupal wing. (A, B) in situ hybridization of *Ar-gbb* at prepupal early PCF4 fore- and hindwings. Sawfly *gbb* is ubiquitously expressed in prepupal wing. (C-F) in situ hybridization of *Ar-sog* at prepupal early PCF4 (C, D) and late PCF4 (E, F) forewing (C, E) and hindwing (D, F).

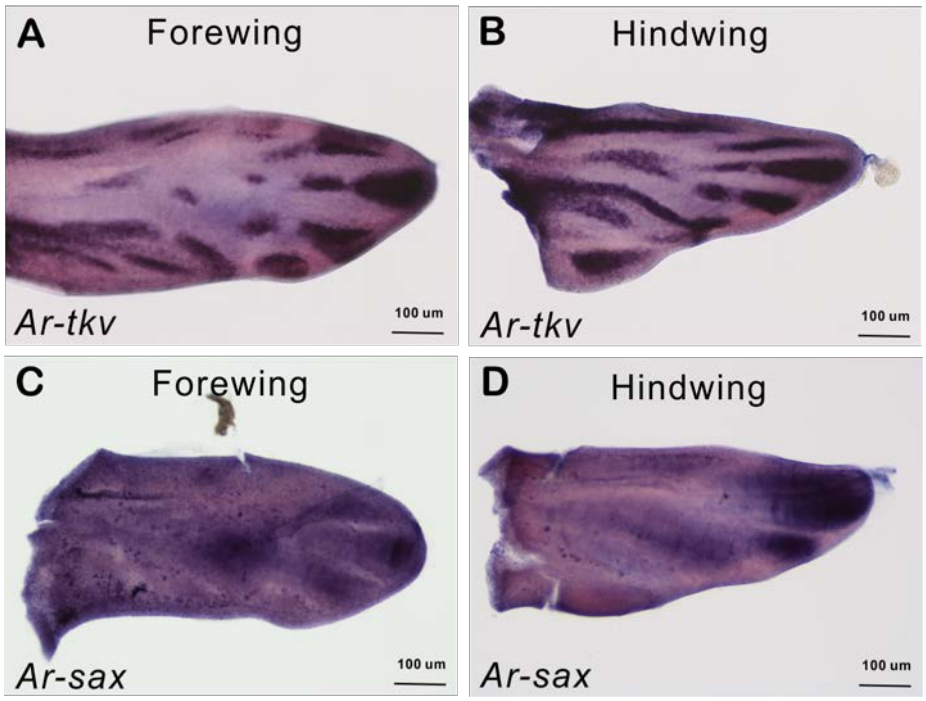

Fig. 3. BMP type I receptors *tkv* and *sax* are expressed in sawfly prepupal wings. (A, B) in situ hybridization of *Ar-tkv* at prepupal late PCF4 fore- and hindwings. (C, D) in situ hybridization of *Ar-sax* at prepupal early PCF4 fore- and hindwings.

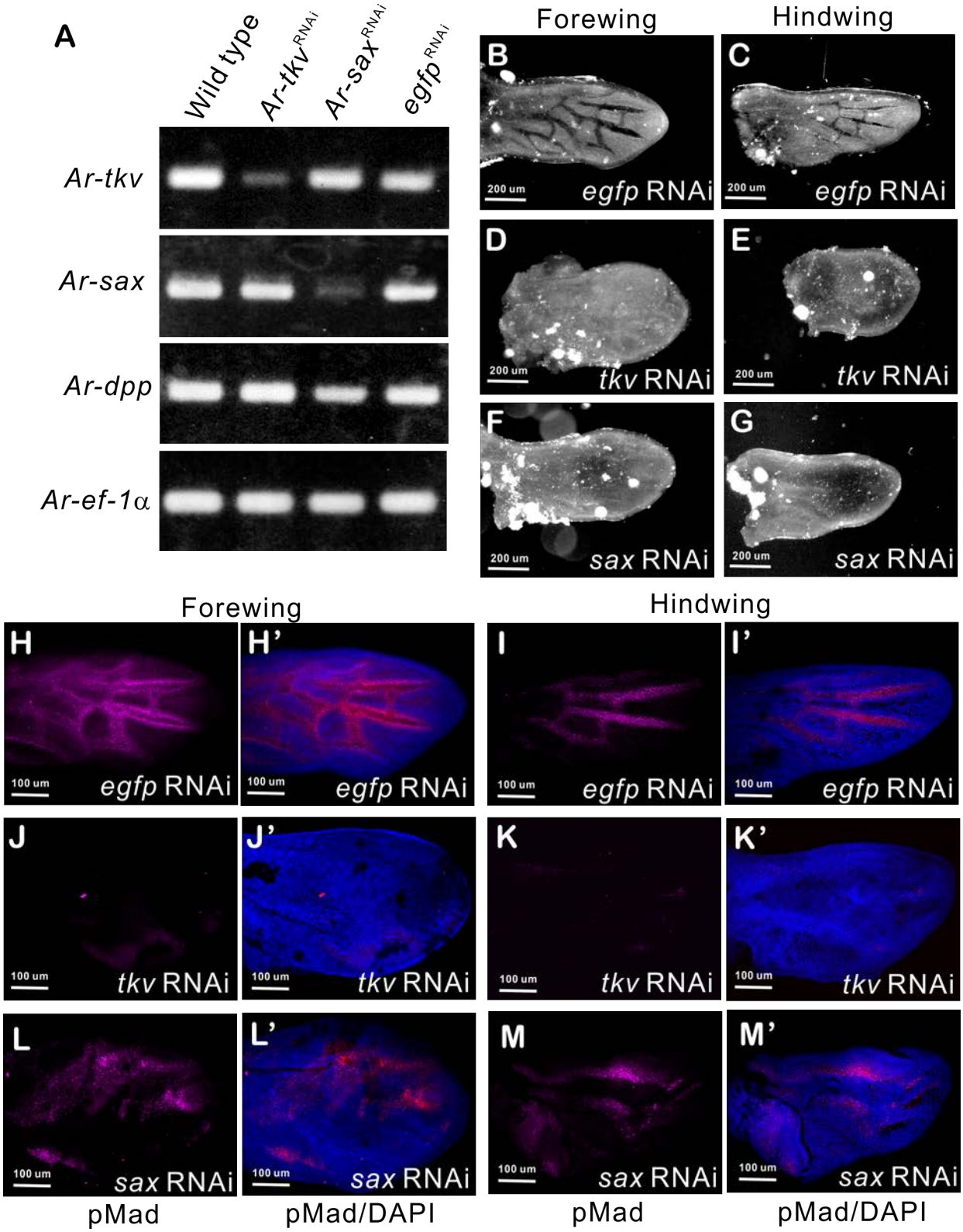

Fig. 4. Both Tkv and Sax are required for localized BMP signaling in sawly prepupal wings. (A) Effectiveness of *Ar-tkv* RNAi and *Ar-sax* RNAi in the sawfly. Expression of *Ar-tkv*, *Ar-sax*, *Ar-dpp*, and *Ar-ef-1α* is determined by RT-PCR. Lane 1: wild type wings from uninjected individuals. Lane 2: wings from individuals injected with 1 μg of *Ar-tkv* dsRNA. Lane 3: wings from individuals injected with 3 μg of *Ar-sax* dsRNA. Lane 4: wings from individuals injected with 3 μg of *egfp* dsRNA (RNAi control). (B-G) Prepupal PCF4 forewing (B, D, F) and hindwing (C, E, G) injected

with *egfr* dsRNA (B, C), *Ar-tkv* dsRNA (D, E) or *Ar-sax* dsRNA (F, G). (H-M) pMad accumulation and pMad/DAPI staining of prepupal PCF4 forewing and hindwing injected with *egfr* dsRNA (H, I), *Ar-tkv* dsRNA (J, K) or *Ar-sax* dsRNA (L, M).

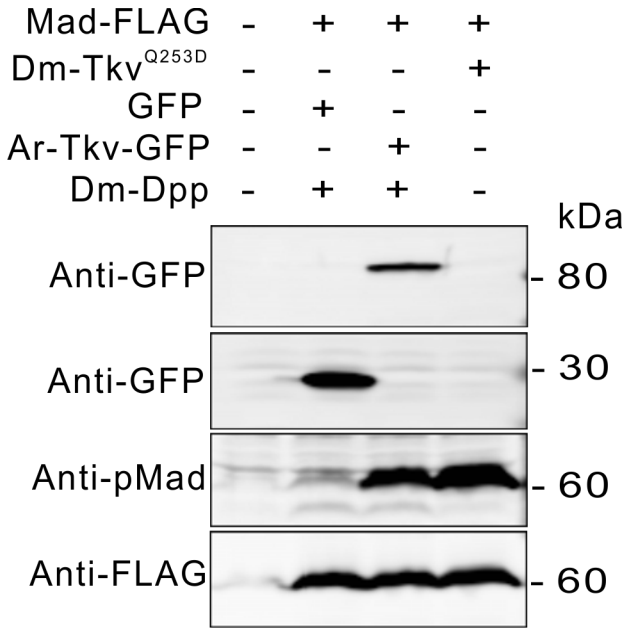

Fig. 5. Sawfly Tkv encodes functional BMP receptors in *Drosophila* S2 cells. *Ar-tkv-GFP*, *Dm-tkv^{Q253D}* (constitutive active form of Tkv), *Mad-FLAG*, *Dm-dpp*, or *gfp* are co-expressed in *Drosophila* S2 cells. Three days after transfection, cell lysates were analyzed by Western blotting probing with anti-GFP, anti-pMad and anti-FLAG antibodies.

Supplemental figures

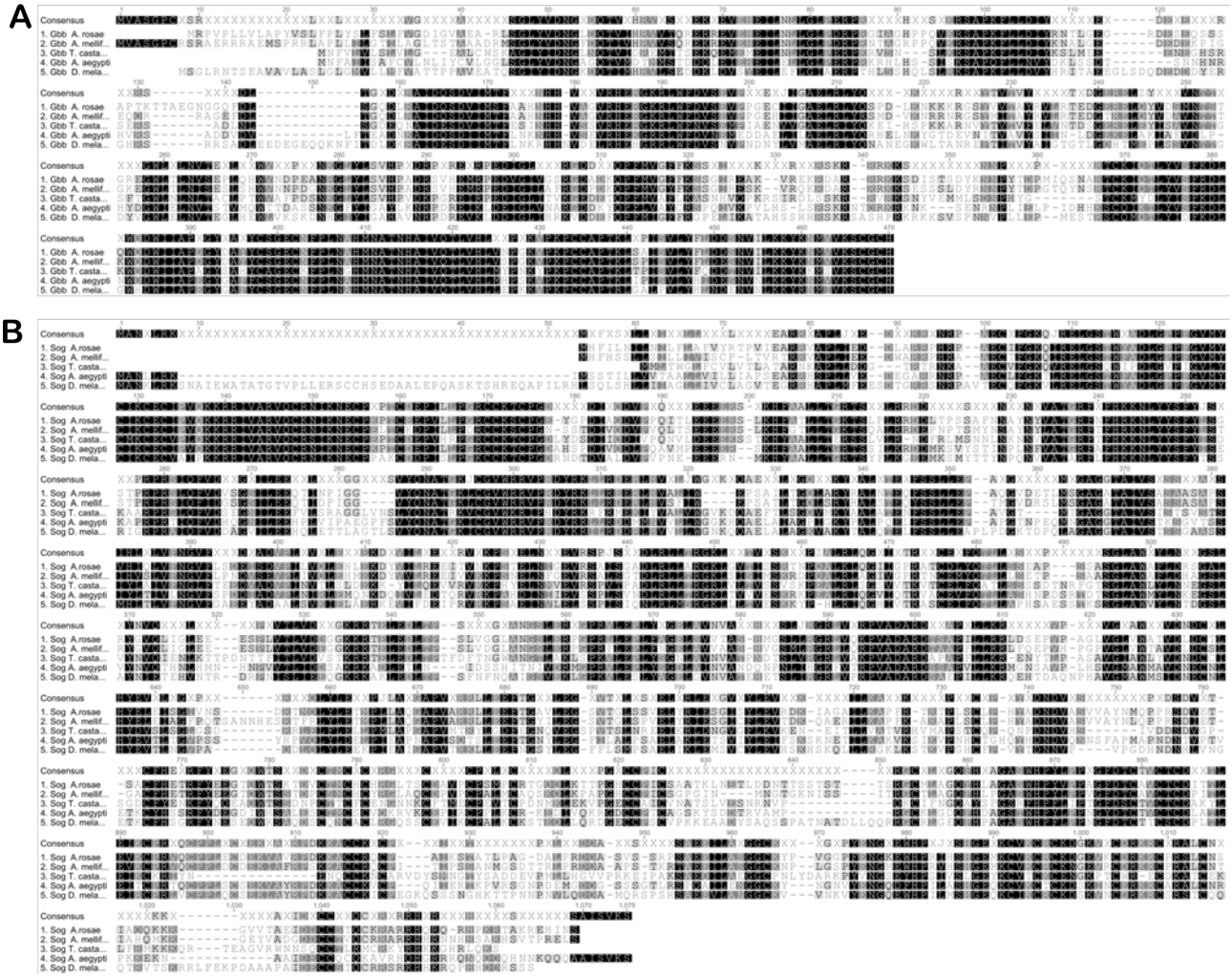

Fig. S1. (A) Amino acid alignment of Gbb proteins from various insect species. Amino acid numbers are indicated above the alignment. Identical amino acids are shaded black and similar ones are shaded gray. Gaps introduced to optimize the extent of amino acid identity are indicated by dashes. Consensus amino acids at positions that are identical or similar are shown above the alignment. Accession numbers are as follows: Gbb *Athalia rosae* LC373544.1, Gbb *Apis mellifera* XP_394252.2, Gbb *Tribolium castaneum* NP_001107813.1, Gbb *Aedes aegypti* XP_001659788.1, Gbb *Drosophila melanogaster* NP_477340.1. (B) Amino acid alignment of Sog proteins from various insect species. Amino acid numbers are indicated above the alignment. Identical amino acids are shaded black and similar ones are shaded gray. Gaps introduced to optimize the extent of amino acid identity are indicated by dashes. Consensus amino acids at positions that are identical or similar are shown above the alignment. Accession numbers are as follows: Sog *Athalia rosae* LC373543.1, Sog *Apis mellifera* XP_393520.4, Sog *Tribolium castaneum* XP_015838931.1, Sog *Aedes aegypti* XP_021699929.1, Sog *Drosophila melanogaster* NP_476736.1.

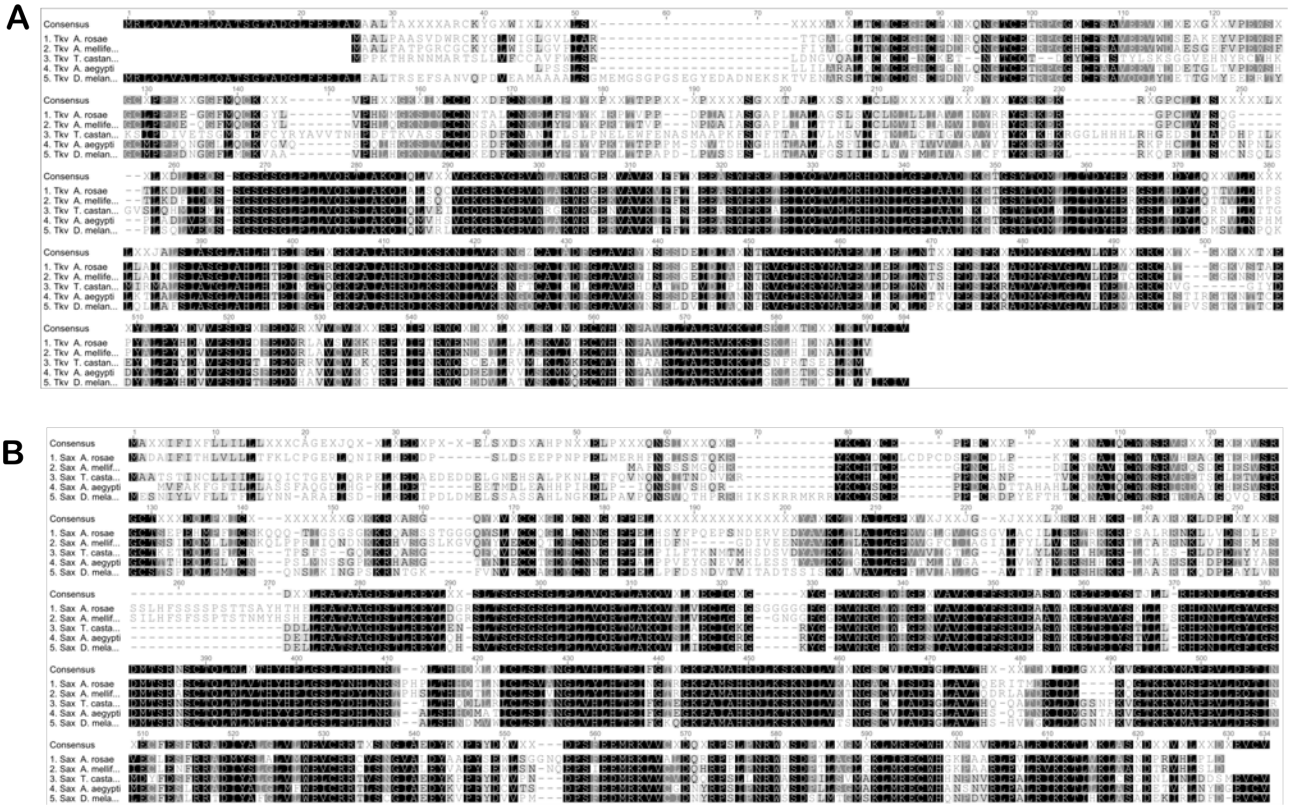

Fig. S2. (A) Amino acid alignment of Tkv proteins from various insect species. Amino acid numbers are indicated above the alignment. Identical amino acids are shaded black and similar ones are shaded gray. Gaps introduced to optimize the extent of amino acid identity are indicated by dashes. Consensus amino acids at positions that are identical or similar are shown above the alignment. Accession numbers are as follows: Tkv *Athalia rosae* LC373545.1, Tkv *Apis mellifera* XP 391989.2, Tkv *Tribolium castaneum* XP 008190529.1, Tkv *Aedes aegypti* XP 001653798.1, Tkv *Drosophila melanogaster* NP 787990.1. (B) Amino acid alignment of Sax proteins from various insect species. Amino acid numbers are indicated above the alignment. Identical amino acids are shaded black and similar ones are shaded gray. Gaps introduced to optimize the extent of amino acid identity are indicated by dashes. Consensus amino acids at positions that are identical or similar are shown above the alignment. Accession numbers are as follows: Sax *Athalia rosae* LC373546.1, Sax *Apis mellifera* XP_016772990.1, Sax *Tribolium castaneum* NP_001164080.1, Sax *Aedes aegypti* XP_021696048.1, Sax *Drosophila melanogaster* NP_001246193.1.