DOI: 10.1534/genetics.109.102848

Homology of Dipteran Bristles and Lepidopteran Scales: Requirement for the *Bombyx mori achaete-scute* Homologue *ASH2*

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Manuscript received March 15, 2009
Accepted for publication July 29, 2009

ABSTRACT

Lepidopteran wing scales and Drosophila bristles are considered homologous structures on the basis of the similarities in their cell lineages. However, the molecular mechanisms underlying scale development are essentially unknown as analysis of gene function in Lepidoptera is sorely limited. In this study, we used the *Bombyx mori* mutant *scaleless* (*sl*), which displays a nearly complete loss of wing scales, to explore the mechanism of lepidopteran wing-scale formation. We found that *Bm-ASH2*, one of four Bombyx *achaete-scute* homologs, is highly expressed in early pupal wings of wild-type silkworms, but its expression is severely reduced in *sl* pupal wings. Through molecular characterization of the mutant locus using luciferase and gel shift assays, genetic analysis of recombining populations, and *in vivo* rescue experiments, we provide evidence that a 26-bp deletion within the *Bm-ASH2* promoter is closely linked to the *sl* locus and leads to loss of *Bm-ASH2* expression and the scaleless-wings phenotype. Thus, the *Bm-ASH2* appears to play a critical role in scale formation in *B. mori*. This finding supports the proposed homology of lepidopteran scales and dipteran bristles and provides evidence for conservation of the genetic pathway in scale/bristle development at the level of gene function.

THE wing surface of lepidopteran adults is covered by wing scales, which function in heat preservation, mimesis, touch, etc. (Nijhout 1991). Lepidopteran scales and Drosophila bristles are considered homologous structures on the basis of the similarities in their cell lineages and the expression of a few molecular markers (Overton 1966, 1967; Galant *et al.* 1998). Development of the Drosophila bristle is regulated by the basic helix-loop-helix (bHLH) transcription factors of the Achaete-Scute Complex (*AS-C*). Within the clusters of cells expressing *achaete* (*ac*) and *scute* (*sc*), some are selected to become sensory organ precursors, which then form and innervate the mature bristles (Skeath and Carroll 1991; Jan and Jan 1994). In *ac/sc* double-mutant flies, the majority of the bristles are lost; on the contrary, ectopic expression of *ac* and/or *sc* can induce extra bristles (Garcia-Bellido 1979; Campuzano *et al.* 1986; Balcells *et al.* 1988; Rodriguez *et al.* 1990). In the butterfly *Precis coenia*, the *AS-C* homolog *B-ASH1* is indeed expressed in scale-forming cells during pupation (Galant *et al.* 1998), and as we reported, the four *AS-C* homologs of the silkmoth *Bombyx mori* are also expressed during wing development, particularly at the early pupal stage when scale formation begins (Zhou *et al.* 2008). By studying the genomic organization and the evolution of *ac/sc* genes of multiple distant insect species, Negre and Simpson (2009) have recently argued that the independent evolution of the homologs might have contributed to morphological diversity in Diptera and Lepidoptera.

We show here that a small deletion in the *Bm-ASH2* gene underlies the *scaleless* (*sl*) mutant phenotype in *B. mori*. As previously described, this mutant displays a severe reduction in wing scales (Zhou *et al.* 2004, 2006). We find that *Bm-ASH2* expression is strongly reduced in the *sl* pupal wing. At the molecular level, we identify a 26-bp deletion within the promoter region of *Bm-ASH2*, which is linked to the mutant phenotype. Using an electrophoresis mobility shift assay, we show that the 26-bp region contains a *cis*-regulatory element. Finally, we show that targeted expression of *Bm-ASH2* in the *sl* pupal wing can partially rescue the lack of wing scales, confirming the central role of *Bm-ASH2* downregulation in the etiology of this mutant. Thus, at least one *ASH* factor is fundamentally involved in scale formation in *B. mori*. This finding supports the proposed homology of lepidopteran scales and Drosophila bristles and provides evidence for conservation of the genetic pathway in the formation of these structures.

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.109.102848/DC1.

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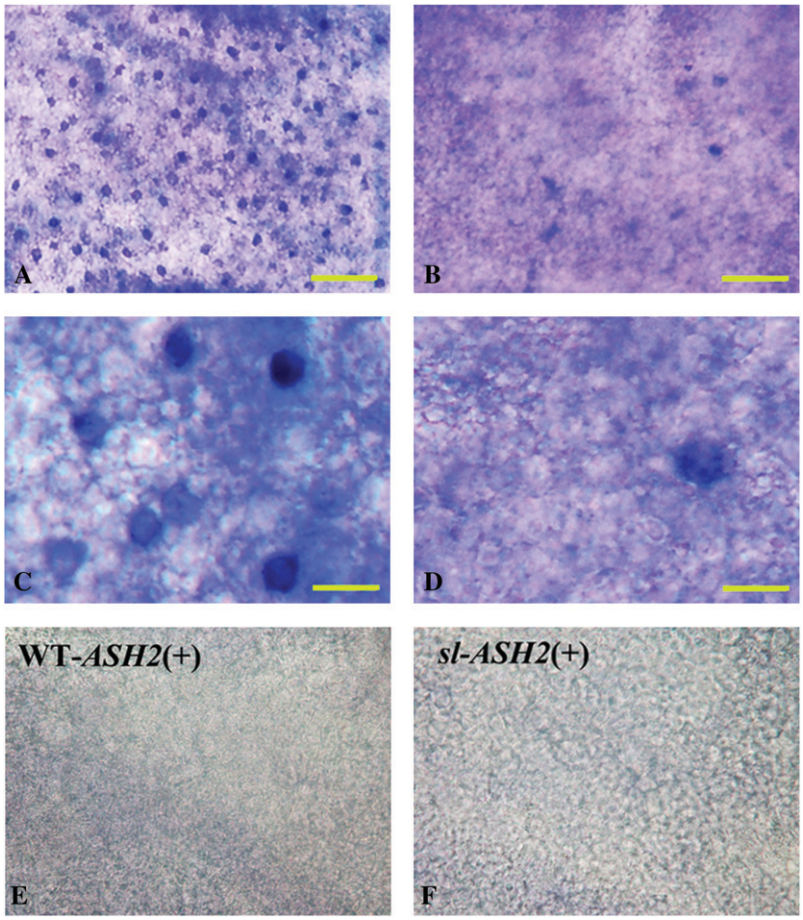

Figure 1.—The expression pattern of *Bm-ASH2* is abnormal in the *scaleless* pupal wing. Cells expressing *Bm-ASH2* are regularly located on the wing surface of wild-type pupa (A and C, blue dots). By contrast, in *sl* pupa, only a very few cells were detected expressing *Bm-ASH2* (B and D). C and D are enlargements of a partial area in A and B. No staining was observed in the sense-strand control for wild type [(E)WT-*ASH2* (+)] or *scaleless* [(F) *sl-ASH2* (+)]. Distal is to the top. Bars, 100 μm in A, B, E, and F, and 20 μm in C and D.

MATERIALS AND METHODS

Animals: Silkworms used in the experiments were the wild-type strains 7532 and Furong and the transparent wings mutant strain *scaleless* (Zhou *et al.* 2004). The larvae were cultured on mulberry leaves under 25° with 70–80% relative humidity.

***In situ* hybridization:** DIG-labeled RNA probes were generated by *in vitro* transcription, using the ORF region of a gene as the template. One-day-old pupal wings were dissected in cold 0.75% NaCl and fixed in fresh 4% formaldehyde in 100 mm HEPES (pH 7.9), 2 mm $MgCl_2$, and 1 mm EDTA for 2–3 hr at room temperature or overnight at 4°. The tissues were washed for 3× for 5 min each time in $PBST_{0.2}$ (0.2% Tween20) and then digested with Proteinase K (20 μg/ml) for 5 min, followed by treatment with 0.2% glycine in PBS. The wings were refixed in 4% polyformaldehyde for 30 min and washed 2× for 5 min each time in $PBST_{0.2}$. Prehybridization was processed in hybridization solution (50% formamide, 5× SSC, 2% blocking powder, 10 mg/ml yeast tRNA, 5 mg/ml salmon sperm DNA, and 20 mg/ml heparin) for 2 hr. An antisense RNA probe (100–500 ng) was used for each experiment, and hybridization was done overnight. Then the tissues were washed 4× with $PBST_{0.2}$, for 10, 20, 30, and 30 min, respectively. Subsequently, they were dipped in 5% goat serum and 2% BSA in $PBST_{0.2}$ for 2 hr and in anti-DIG-AP Fab fragment for 2 hr. The samples were washed 3× for 10 min each time in $PBST_{0.2}$ and stained with NBT (nitro blue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidine salt) in the dark until a signal appeared. The issues were dehydrated successively with 30%, 50%, 70%, and 100% methanol for 15 min each, followed by a successive 1-hr treatment with 50% and 80% glycerol. Sense-strand RNA was used as the control for each experiment.

Cell culture and dual-luciferase reporter assay: Cell culture and dual-luciferase reporter assay were processed as described (Zhou *et al.* 2008). One pupal wing was dissected and lysed with 50 μl of the passive lysis buffer, and 10 μl of the lysate was used for the dual-luciferase reporter assay according to the manufacturer's protocol (Dual-luciferase Reporter Assay System, Promega). Luciferase activity was determined with a 20/20^n Luminometer (Turner BioSystems).

Electrophoretic mobility shift assay: Probes for the electrophoretic mobility shift assay (EMSA) were prepared by annealing two partially overlapping oligos (supporting information, Table S1), synthesized to have two sticky ends to introduce dATP by filling the gaps. The probes were labeled with [α-^{32}P]dATP. Nuclear extracts were prepared from 1-day-old pupal wings as described (Feng *et al.* 1998; Blough *et al.* 1999), and the protein concentration was determined by the Bradford Assay (Bradford 1976). Five micrograms of nuclear proteins were incubated with a probe at a concentration of 10^4 cpm in 0.04 pmol DNA in a final volume of 20 μl containing 50 mm HEPES–KOH (pH 7.9), 250 mm KCl, 20 mm $MgCl_2$, 5 mm DTT, 5 mm EDTA, 0.5 mg/ml BSA, 0.25 μg/μl Poly (deoxyinosinic-deoxycytidylic) (Sigma), and 50% glycerol. After incubation for 30 min at 25°, the binding reactions were analyzed on 7% polyacrylamide gels. The gels were dried and exposed to a phosphoscreen (Bio-Rad) for 3 hr. For competition analysis, 100-fold excess of unlabeled double-stranded oligos was used.

Genomic DNA extraction from silkmoth: The head and thorax of a moth was cut and homogenized in 2 ml solution A [0.25 m sucrose, 10 mm EDTA, 30 mm Tris–HCl (pH 7.5)] on

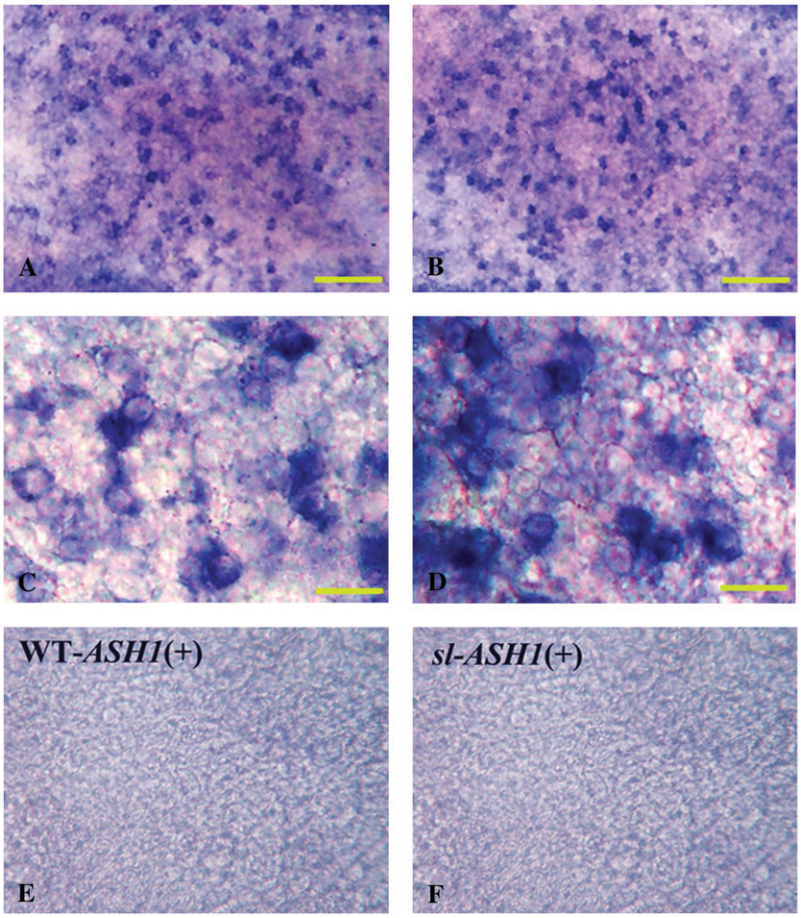

FIGURE 2.—The expression patterns of *Bm-ASH1* in wild-type and *scaleless* pupal wings are similar. There are no significant differences in *Bm-ASH1* expression patterns between wild type (A and C, blue dots) and *sl* (B and D). Unlike those expressing *Bm-ASH2*, cells expressing *Bm-ASH1* form special clusters but are not arrayed singly on the wing surface. C and D are enlargements of a partial area in A and B. No staining was observed in the sense-strand control for wild type [(E)WT-*ASH1* (+)] or *scaleless* [(F)*sl-ASH1* (+)]. Distal is to the top. Bars, 100 μm in A, B, E, and F, and 20 μm in C and D.

ice. After centrifugation for 3 min at 1500 × *g*, the supernate was discarded and the pellet was resuspended in 600 μl solution B [10 mM Tris–HCl (pH 7.5), 10 mM EDTA, 0.15 M NaCl, 1% sarcosyl] and incubated on ice for 15 min. The sample was treated successively with 1 vol phenol/chloroform to denature the proteins. Genomic DNA was precipitated with 2 vol ethanol, and the pellet was washed with 70% ethanol, dried, and dissolved in TE with 20 μg/ml RnaseA and stored at −20° for further use.

Ectopic expression of a foreign gene in the silkworm pupal wing by a transient expression system: The ORF region of a candidate gene was cloned into a modified pBacPAK8 vector with an *ie-1* promoter and a *hr3* enhancer (CHEN *et al.* 2004). Ten microliters of the recombinant plasmid (5 μg) with 5 μl of lipofectin were micro-injected into the pupal wing. Injection of 10 μl of double-distilled water with 5 μl of lipofectin was used as the control.

RESULTS AND DISCUSSION

Expression of *Bm-ASH2* is severely reduced in *scaleless* pupal wings: The *scaleless* mutant loses nearly all wing scales. Given that *AS-C* genes play critical roles in the development of Drosophila bristles and that *AS-C* homologs (*ASH*) are expressed in the developing wings of butterflies and silkmoths (JAN and JAN 1994; GALANT *et al.* 1998; ZHOU *et al.* 2008), we decided to investigate the potential involvement of these bHLH-type of transcription factors in the etiology of this mutant. We used semiquantitative RT–PCR to assess the expression level of *Bm-ASH* genes in wild-type and *sl* wings. The levels of *Bm-ASH1*, *Bm-ASH3*, and *Bm-ase* in *sl* pupal wings were similar to those from two different wild-type lines, Furong and 7532. On the contrary, the *Bm-ASH2* level was severely reduced in *sl* compared to wild type (ZHOU *et al.* 2006; data not shown).

We further used *in situ* hybridization to confirm these findings and to investigate the pattern of *Bm-ASH2* expression. In the wild type, *Bm-ASH2* was robustly expressed across 1-day-old pupal wings in regularly spaced clusters of scale mother cells (Figure 1, A and C). Consistent with the reduction in mRNA levels detected by RT–PCR, only a few cells expressing *Bm-ASH2* were detected on the *sl* wing surface (Figure 1, B and D). By contrast, and in agreement with the RT–PCR data, clusters of *Bm-ASH1*-expressing cells were present across the mutant wings in a pattern essentially indistinguishable from that of wild type (Figure 2, A–D).

The *scaleless* wings phenotype is caused by a single locus and correlates with the presence of a 26-bp deletion in the *Bm-ASH2* genomic region: To understand the defect leading to loss of *Bm-ASH2* expression in the *sl*, we decided to characterize the locus at the molecular level. Although *in situ* analysis (Figure 1)

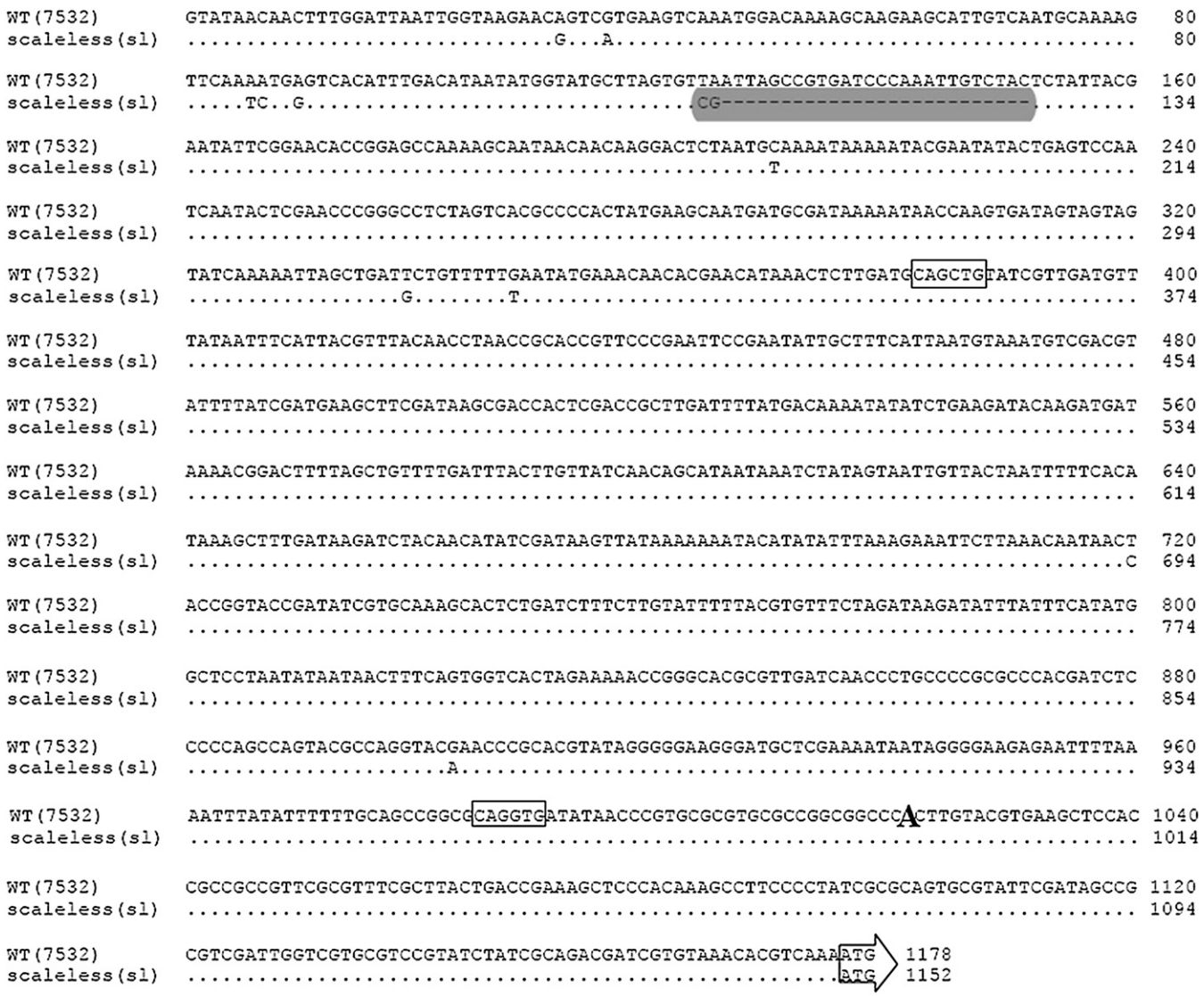

Figure 3.—Comparison of *Bm-ASH2* promoter sequences between *scaleless* and wild type. There are 11 transversions and one transition in the *sl Bm-ASH2* promoter sequence compared to the wild-type one. More importantly, a 26-bp fragment is absent in *sl* (shaded area). Two deduced E-boxes are boxed. The enlarged "A" at 1021 is the predicted transcription start site.

suggested a defect in transcriptional regulation, we nonetheless sequenced the entire *Bm-ASH2* coding region. Unsurprisingly, sequences from the mutant and several wild-type strains had no significant differences (data not shown). Since transcription is often controlled through the chromosomal region flanking a gene on the 5′ side, we decided to analyze 1175 bp of genomic DNA just upstream of the *Bm-ASH2* translation start site. On the basis of *in silico* prediction, this fragment spans ~1010 bp upstream of the start of transcription (Figure 3). A number of isolated single-base-pair changes were identified throughout the region. However, the most striking sequence alteration occurred ~850 bp upstream of the putative transcription start site, where a 28-bp sequence present in the wild type was replaced by only 2 bp in the *sl* (Figure 3).

We used this deletion as a molecular marker to investigate whether the *sl* phenotype segregated with this *Bm-ASH2* mutant allele. Two primers flanking the region deleted in *sl* (Table S1) were used to amplify a 242-bp fragment from the wild-type genomic DNA (Figure 4, lane 1; Figure S1, box 1) and a 216-bp fragment from *sl* (Figure 4, lane 2; Figure S1, box 1). To ensure that our findings are reasonable, we studied individuals with multiple different genetic backgrounds. In the series of crosses shown in Figure S1, individuals were scored for their wing phenotype and then selected for PCR testing. In all cases, phenotypically *sl* individuals carried the deletion allele but not the wild-type allele (Figure 4, lanes 3 and 4; Figure S1, boxes 2 and 3). On the contrary, phenotypically wild-type individuals carried either two copies of the wild-type DNA or one copy of the wild type and one copy of the deletion alleles (Figure 4, lane 5; Figure S1, box 3; data not shown).

Because the original strain in which the mutant was discovered was unknown, a more extensive analysis of

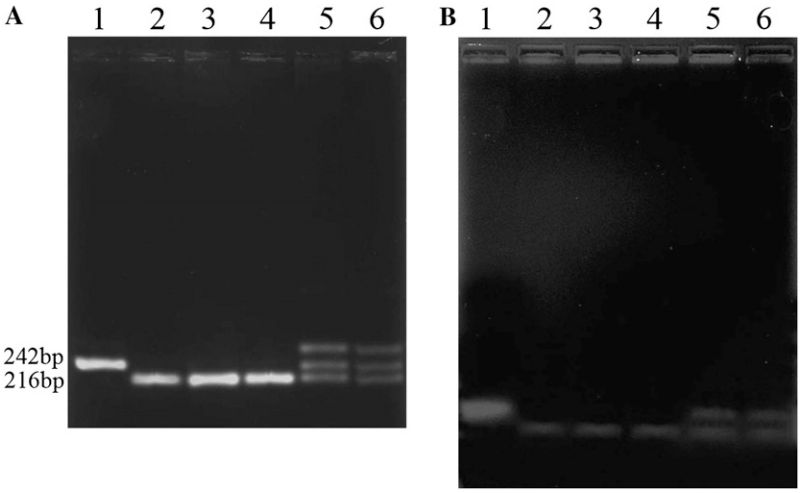

FIGURE 4.—The *scaleless* wings mutant phenotype is tightly linked to the absence of the 26-bp element in the *Bm-ASH2* promoter. PCR was carried out with primers Cash2pF and Cash2pR (Table S1). (A) Native agarose gel electrophoresis. (B) Denaturing alkaline agarose gel electrophoresis. DNA in each lane was from individuals with a specific genotype, and the cross strategies are shown in Figure S1. As expected, a 242-bp fragment was amplified from wild type (7532, lane 1), and a 216-bp fragment was amplified from *sl* (lane 2). All of the individuals with a mutant phenotype have a *sl* genetype (lanes 3 and 4). Both 242- and 216-bp fragments were amplified from heterozygous genotype individuals (lanes 5 and 6). In lanes 5 and 6 in A, an extra band moved more slowly than 242 bp. We supposed that this band was produced by the annealing of a 242- and a 216-nt single-stranded DNA. These fragments contained a small loop and had slower migrating speeds in the natural electrophoresis conditions. Consistent with the supposition, there were only two bands under denaturing electrophoresis (B, lanes 5 and 6).

linkage was carried out from a cross of *sl* to another wild-type strain, Furong (Figure 4, lane 6; Figure S1, box 4). Heterozygous F_1 progeny were intercrossed to generate a mix of phenotypically *sl* and wild-type F_2 progeny. Among 581 F_2 adults, the proportion of phenotypically *sl* to wild-type individuals was ~1:3 (Table 1, $\chi^2 = 0.014$, $\chi^2_{0.05,\,1} = 3.84$). We randomly picked some of the moths for molecular analysis. All 40 F_2 silkmoths with *sl* wings carried the *Bm-ASH2* deletion allele exclusively. Among 112 F_2 individuals with a wild-type phenotype, 34 carried the homozygous wild-type genotype, and the other 78 individuals were heterozygous (Table 1). The ratio of homozygous to heterozygous individuals approximated the predicted 1:2 ratio expected for recessive inheritance at a single locus in a hybrid cross ($\chi^2 = 0.322$, $\chi^2_{0.05,\,1} = 3.84$). The results are consistent with a single gene hypothesis, *i.e.*, that the *sl* phenotype is caused by a recessive allele at a single locus.

These data are consistent with the simple inheritance of *sl* as a recessive trait and support a close linkage between the 26-bp deletion and the mutant locus. However, this evidence is not sufficient to establish that *Bm-ASH2* is the locus responsible for the *sl* phenotype. Therefore, we proceeded to investigate: (1) whether the 26-bp sequence could be involved in regulating *Bm-ASH2* transcription and (2) whether restoring the expression of *Bm-ASH2* in the developing wing could rescue the mutant defect.

The 26-bp region contains sequences that contribute to normal transcriptional regulation of *Bm-ASH2*: To test whether the 26-bp region was involved in transcriptional regulation, we relied on transient expression assays in cultured cells. We had previously shown that the 1175-bp DNA fragment upstream of the *Bm-ASH2* translation start site (Figure 5) is sufficient to drive robust luciferase expression in the presence of the putative upstream regulators Bm-ASH1 and Daughterless and that two E-boxes at positions 194 and 797 likely mediate this regulation (ZHOU *et al.* 2008). As expected, reporters bearing 5′ truncations that eliminate either the 797 E-box or both 797 and 194 E-boxes displayed much lower transcriptional activity, <20% of the full-length ASH2P. However, expression did not rely solely on these regulatory elements because a 979-bp reporter that retained both E-boxes and lacked only 169 bp at the 5′-end was also severely downregulated and expressed at only ~20% of the ASH2P. Interestingly, this truncated 979 bp lacked the 26-bp region deleted in the *sl*.

To investigate whether the lower activity of the 979 bp was due to loss of the 26-bp region (Figure 3, shaded), we assayed the expression of a "full-length ASH2P" reporter generated from the *sl* genomic DNA (sl-ASH2P) and thus lacking the 26-bp sequence. Notably, the activity of sl-ASH2P was significantly lower than that of wild-type ASH2P and similar to the 979 bp.

On the basis of these findings, we hypothesized that the 26-bp region contains one or more *cis*-regulatory elements required for normal expression of *Bm-ASH2*. Consistent with this proposal, an activator present in

TABLE 1

Genetic analysis of *scaleless* phenotype and genotype

	Wild type			
	SS	Ss	*sl* (ss)	Rate (modified)
Phenotype	434		147	3:1
Genotype	34	78	40	1:2 (SS:Ss)

By chi-square test, 434:147 = 3:1 (χ^2= 0.014, $\chi^2_{0.05,1} = 3.84$), 34:78 = 1:2 ($\chi^2 = 0.322$, $\chi^2_{0.05,1} = 3.84$). "SS" and "Ss" separately indicate the homozygous and heterozygous wild-type genotype, and "ss" indicates the homozygous mutant genotype. The data are from the F_2 intercross between F_1's produced with the Furong (wild type) and *sl* strains.

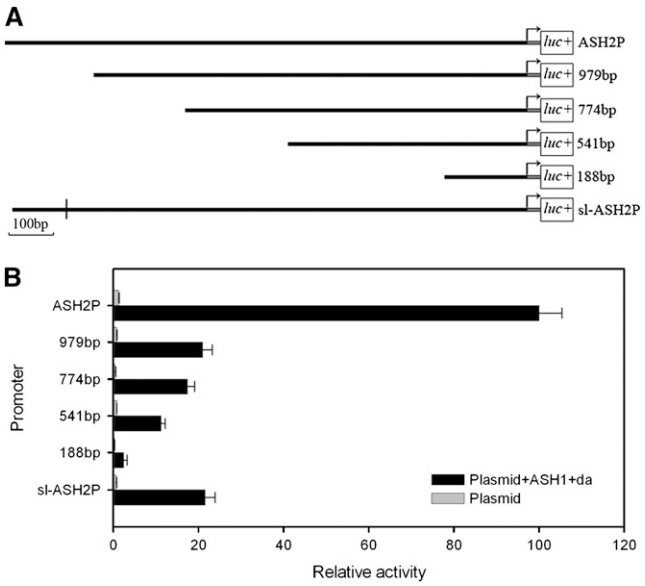

FIGURE 5.—Comparison of *Bm-ASH2* promoter activities between *scaleless* and wild type. The transcriptional activity of sl-ASH2P was significantly lower than that of ASH2P ($P < 0.01$) and similar to that of 979 bp ($P > 0.05$). ASH2P, the full-length fragment of wild-type *Bm-ASH2* promoter; 979 bp, +3 to −976 fragment; 774 bp, +3 to −771 fragment; 541 bp, +3 to −538 fragment; 188 bp, +3 to −185 fragment; sl-ASH2P, the full-length fragment of the *Bm-ASH2* promoter in *sl*. All of the promoter fragments were cloned into the luciferase reporter vector pGL3-Basic. We designated the first base of the start codon as +1, and all ATGs were changed to ATTs for these experiments. "|" in sl-ASH2P shows the missing 26-bp element (A). (B) "Plasmid" shows that only the recombined pGL3-Basic vector containing a particular promoter fragment was transfected into BmN cells, and "Plasmid+ASH1+da" indicates cotransfection of the recombined plasmid together with the transient expression plasmids containing *Bm-ASH1* and *Dm-da*. At least three independent repeats were carried out for each treatment.

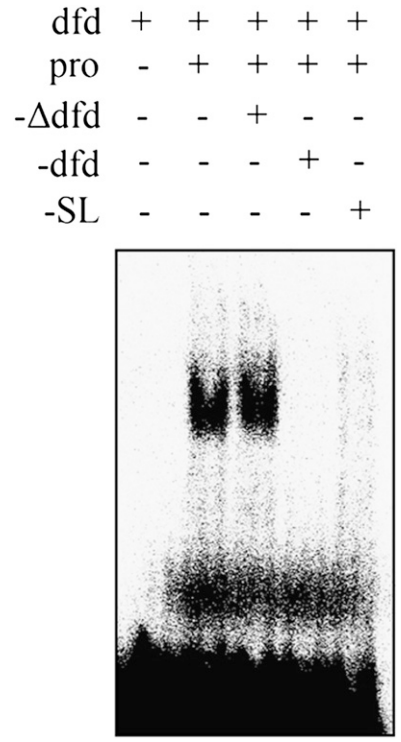

FIGURE 6.—Fragments containing the 26-bp element can be bound by nuclear protein extracts from a 1-day-old pupal wing *in vitro*. "+" indicates that the component was included, and "−"indicates that the component was not included in binding reactions. pro, nuclear proteins; dfd, a wild-type 49-bp double-strand DNA containing the 26-bp fragment ("SL"); and Δdfd, the *sl* 23-bp fragment corresponding to dfd. Minus signs in these terms indicate unlabeled probes.

nuclear protein extracts from 1-day-old pupal wings bound 49-bp double-strand oligos that contained the 26-bp sequence in EMSA. Binding was competed by either cold 49-bp DNA or a smaller DNA spanning the 26-bp sequence, whereas it was unaffected by a cold 23-bp DNA that excluded the 26 bp (Figure 6). These findings, together with the loss of *Bm-ASH2* expression in *sl* tissue, strongly support a role for the 26-bp region in transcriptional regulation of *Bm-ASH2*.

Ectopic expression of *Bm-ASH2* in the pupal wing can rescue the *scaleless* wing phenotype: The findings above strongly support a model whereby decreased expression of *Bm-ASH2* results in loss of scales in the *sl*. To test this hypothesis, we attempted to rescue the mutant phenotype by providing exogenous Bm-ASH2 in the wing.

To achieve this goal, we developed a novel method for transient gene expression *in vivo*. Previous studies have shown that *B. mori* baculovirus promoters are functional in expression vectors and display expected transcriptional activity when injected into silkworm larvae (ZHOU *et al.* 2002; CHEN *et al.* 2004; TANG *et al.* 2005). To assess the efficiency of this method in the pupal wing, we generated a constitutive reporter encoding the luciferase gene under the control of the previously described *ie-1* promoter and *hr3* enhancer (pBacPAK8-ie-1-luc-hr3; CHEN *et al.* 2004). The reporter plasmid was micro-injected into the left forewings of 12-hr-old pupae, and the wings were dissected 36 hr later, lysed, and tested for luciferase activity. Among a total of 14 individuals tested, only 2 (14%) lacked luciferase activity. In the positive samples ($n = 12$), luciferase activity ranged from 1500 relative light units (RLU; 10 or 71.4%) to >10,000 RLU (3 or 21.4%), the highest reaching 248,064 RLU (Table 2).

Having validated this method for the transient induction of exogenous gene expression in the wing, an expression plasmid encoding the Bm-ASH2 was microinjected into the left forewing of 12-hr *sl* pupae. Of 50 pupae treated in this fashion, 37 developed into moths, but 16 were excluded because their left forewings were folded and difficult to score. The remaining 21 moths were evaluated for presence/absence of scales. Nine (42.9%) displayed the *sl* phenotype, whereas 12 (57.1%) displayed significant rescue. In these 12 individuals, the

TABLE 2

Luciferase activity after injection of the pBacPAK8-ie-1-luc-hr3 plasmid into the pupal wing

No.	Luminescence (RLU)		
	Left wing	Right wing	Remainder of the right subtracted from the left wings
1	248,145	81	248,064
2	12,017	90	11,927
3	10,883	89	10,794
4	8,896	93	8,803
5	8,439	96	8,343
6	7,120	80	7,040
7	4,042	76	3,966
8	3,054	84	2,970
9	2,102	79	2,023
10	1,851	88	1,763
11	517	78	439
12	100	78	22
13	89	94	−5
14	79	72	7

Ten microliters of pBacPAK8-ie-1-luc-hr3 plasmid (5 μg) containing the *luc*+ gene was micro-injected into the left forewing of 12-hr-old pupa. Thirty-six hours later, the pupal wing was dissected out and lysed with 50 μl of the passive lysis buffer, and then 10 μg of lysate protein was used for the dual-luciferase reporter assay. The right forewing was injected with 10 μl of same dosage lipofectin in double-distilled water as the control.

TABLE 3

Wing-socket cells produced in silkmoths injected with a plasmid (pBacPAK8-ie-1-ASH2-hr3) containing *Bm-ASH2*

Sort	Mean of socket cell no./mm^2	Standard deviation	T grouping
Wild type	462	31	A
wild type-left	464	22	A
wild type-right	464	31	A
scaleless	51	8	B
scaleless-CK	49	17	B
scaleless-left	374	134	C
scaleless-right	53	12	B

"Wild type" is a wild-type silkworm breed (7532); "wild type-left," refers to the left wing, and "wild type-right" to the right wing of the wild-type silkmoth whose left wing was injected with pBacPAK8-ie-1-ASH2-hr3; "*scaleless*," the *scaleless* wing mutant; "*scaleless*-CK," the *scaleless* wing injected with the empty vector pBacPAK8-ie-1-hr3; "*scaleless*-left," the left wing; and "*scaleless*-right," the right wing of the *scaleless* silkmoth whose left wing was injected with the pBacPAK8-ie-1-ASH2-hr3 plasmid. There are no significant differences among wild type, wild type-left, and wild type-right, and also no significant differences among *scaleless*, *scaleless*-CK, and *scaleless*-right. However, the number of cells per square millimeter of wing portion of *scaleless*-left was significantly greater than those of *scaleless*, *scaleless*-CK, and *scaleless*-right, although the standard deviation was much larger for *scaleless*-left. Different letters in the "T grouping" column indicate a statistically significant difference (ANOVA, $P < 0.01$). At least 10 wings were used for each treatment.

number of scales (as assessed by the presence of scale sockets) on the left forewing was significantly higher than the number of scales on the untreated right one (*t*-test, $P < 0.01$; Table 3; Figure 7). Controls subjected to mock injections (33 *sl* moths) had wings with scale socket totals indistinguishable between treated and untreated sides ($P > 0.05$; Table 3).

Interestingly, expression of exogenous *Bm-ASH1* did not rescue the mutant phenotype, indicating that loss of scales in the mutant was not due to a reduction of AS-C-type factors in general, but to the specific loss of Bm-ASH2 protein. We cannot exclude, however, the possibility that *Bm-ASH2* cooperates with *Bm-ASH1* in the selection of scale mother cells. Moreover, we did not observe formation of supernumerary scales in wild-type wings injected with either Bm-ASH2 or Bm-ASH1 expression vectors (Table 3 and Table 4). This is in contrast to the induction of ectopic bristles in Drosophila by targeted expression of *ac/sc* (Rodriguez *et al.* 1990). This may be due to methodological differences in the introduction of exogenous gene expression, or, alternatively, it may reflect a significant difference in the genetic control of scale *vs.* bristle development. Nonetheless, that transient expression of exogenous Bm-ASH2 was sufficient to rescue the loss of wing scales in the mutant supports the identity of the *sl* locus and the *Bm-ASH2* gene.

Conclusions

The proneural gene *Bm-ASH2* plays a key role in the formation of silkmoth wing scales: Formation of dipteran bristles and lepidopteran scales is controlled mainly by *achaete-scute* genes and homologs (Galant *et al.* 1998). Independent duplication of *achaete-scute* homologs between different insect lineages may have contributed to morphological diversity in insects (Negre and Simpson 2009). During evolution, the emergence of new phenotypes may have been due to changes in the spatial/temporal expression of particular genes, usually caused by mutations in relative *cis*-regulatory elements and/or in *trans*-acting factors (Mannervik *et al.* 1999; Bonifer 2000). Generally, mutations in *cis*-regulatory elements occur more frequently (Stern 2000), and have been shown at genetic and molecular levels (Belting *et al.* 1998; Stern 1998; Sucena and Stern 2000; Gompel *et al.* 2005; Wittkopp 2006).

The silkworm *scaleless* mutant exemplifies a potential molecular mechanism for "morphological divergence" resulting from an alteration of gene expression (Figure 1, B and D). As shown by analysis of cultured cells and gel shift assays, the change in wing phenotype is most likely due to the loss of an essential *cis*-regulatory site in the control region of the proneural gene *Bm-ASH2* (Figures 5 and 6). This hypothesis is strengthened by the

FIGURE 7.—Wing scales on the *scaleless* left forewing injected with the *Bm-ASH2* expression plasmid are increased significantly. An expression plasmid containing the *Bm-ASH2* gene was injected into the left forewing at the early pupal stage. (A) The enlargement of a partial left wing of the moth. (C) The enlargement of the same area of the right wing. The presence of more wing scales on the left forewing than on the right forewing is evident (B). Bars, 100 μm in A and C.

genetic analysis of the mutant (Table 1; Figure 4). Indeed, the *sl* trait displays a "single-gene recessive" inheritance pattern and shows genetic linkage to chromosome 13 (J. QIN, personal communication) on which the *Bm-ASH2* gene is located (SilkDB).

To test this hypothesis, we chose a gain-of-function approach. Foreign gene expression in *B. mori* has been achieved by transgenesis (NIKOLAEV *et al.* 1993; ZHANG *et al.* 1999; UHLIROVA *et al.* 2002; IMAMURA *et al.* 2003; UCHINO *et al.* 2007) or by using modified baculovirus (MORI *et al.* 1995; GUO *et al.* 2005; XIA *et al.* 2006). Here, we have extended our earlier method to induce transient expression of a foreign gene in silkworm tissues. Using this method, we were able to drive *Bm-ASH2* gene expression in cells of the *sl* pupal wing and significantly rescue the mutant phenotype (Figure 7; Table 3). These findings support our hypothesis that loss of expression of the *Bm-ASH2* underlies the *sl* phenotype and that this bHLH factor plays a critical role in the formation of silkworm wing scales.

Developmental mechanisms for the formation of insect wing scales and bristles are most likely conserved: The formation of fly notum bristles and butterfly wing scales is controlled by the expression of *AS-C* family genes (SKEATH and CARROLL 1991; GALANT *et al.* 1998). There are two types of bristles on the thorax of Drosophila: macrochaetes and microchaetes. Development of both kinds of bristles is controlled mainly by the proneural genes *ac* and *sc*. Lower Diptera have only one or two *AS-C*-type proneural genes, with the single one or the *scute*-like one being responsible for bristle formation (WÜLBECK and SIMPSON 2000, 2002; PISTILLO *et al.* 2002). In the butterfly *Precis coenia*, only one *ac/sc* homolog, *B-ASH1*, has been identified, and it is indeed expressed in a pattern that is consistent with a role in wing-scale formation (GALANT *et al.* 1998). Largely on the basis of this observation, it has been suggested that lepidopteran wing scales and dipteran sensory bristles are homologous structures. We have recently reported that four *AS-C* homologs are found in another lepidopteran insect, the silkworm *B. mori*, and that these genes were all broadly expressed in different tissues (ZHOU *et al.* 2008). In particular, all four genes are expressed in early pupal wings. Notably, Bm-ASH2 protein, which in this study is found to be important in the formation of silkworm wing scales, is highly conserved with the B-ASH1 protein of *P. coenia*, and the two proteins bear identical bHLH domains (ZHOU *et al.* 2008). In this work, we show that loss of *Bm-ASH2* gene expression results in the *scaleless* mutant defect. This finding directly supports the proposed evolutionary relationship between the scales of Lepidoptera and the bristles of Diptera.

TABLE 4

Wing-socket cells produced in silkmoths injected with a plasmid (pBacPAK8-ie-1-ASH1-hr3) containing *Bm-ASH1*

Sort	Mean of socket cell no./mm^2	Standard deviation	T grouping
Wild type	462	31	A
Wild type-left	457	27	A
Wild type-right	460	41	A
scaleless	51	8	B
scaleless-left	55	19	B
scaleless-right	50	39	B

Column headings are the same as described in Table 3. There are no significant differences among wild type, wild type-left, and wild type-right, and no significant differences among *scaleless*, *scaleless*-left, and *scaleless*-right. Different letters in the "T grouping" column indicate a statistically significant difference (ANOVA, $P < 0.01$). At least 10 wings were used for each treatment.

We thank F. Pignoni, T. Zhang, and D. Yang-Zhou for helpful discussion of the data and critical reading of the manuscript and H. Bellen for the Drosophila *daughterless* cDNA. This work was supported by grants from the National Natural Sciences Foundation of China (30770279) and the National High Technology Research and Development Program of China ("863" Project no. 2006AA10A119).

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Communicating editor: J. Tamkun

GENETICS

Supporting Information
http://www.genetics.org/cgi/content/full/genetics.109.102848/DC1

Homology of Dipteran Bristles and Lepidopteran Scales: Requirement for the *Bombyx mori achaete-scute* Homologue *ASH2*

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DOI: 10.1534/genetics.109.102848

TABLE S1

Primers used in the paper

Primer Name	Primer Sequence	Primer Use
BmASH1-F	5'-A*GGATCC*CGATACGCGATGCCGATGG-3'	*Bm-ASH1* ORF amplification and cloning
BmASH1-R	5'-T*CTCGAG*CCTTTGTCACTGTTGTTGCCACC-3'	into pBacPAK6-ie-1-hr3 vector
Dmda-F	5'-A*GGATCC*GAAATGGCGACCAGTGACG-3'	*Dm-da* ORF amplification and cloning into
Dmda-R	5'-T*GTCGAC*CTATTGCGGAAGCTGGGC-3'	pBacPAK6-ie-1-hr3 vector
Luc-F	5'-A*GGATCC*ATGGAAGACGCCAAAAACA-3'	*Luc*+ ORF amplification and cloning into
Luc-R	5'-T*CTCGAG*TTACACGGCGATCTTTCCG-3'	pBacPAK6-ie-1-hr3 vector
BmASH2P-F	5'-A*GAGCTC*GTATAACAACTTTGGATTAATTGG-3'	*Bm-ASH2* promoter fragments amplification and cloning into pGL3-Basic vector
BmASH2P-979	5'-A*GAGCTC*GACTCTAATGCAAAATAAAAATACG-3'	
BmASH2P-774	5'-A*GAGCTC*ATTTCATTACGTTTACAACCTAAC-3'	
BmASH2P-541	5'-A*GAGCTC*ACATAAAGCTTTGATAAGATCTAC-3'	
BmASH2P-188	5'-A*GAGCTC*ATATAACCCGTGCGCGT-3'	
BmASH2P-R	5'-G*CTCGAG*AATTTTGACGTGTTTACACGATC-3'	
Cash2pF	5'-GTATAACAACTTTGGATTAATTGG-3'	*Bm-ASH2* promoter size comparison between
Cash2pR	5'-GATTGGACTCAGTATATTCG-3'	*scaleless* and WT
dfdF	5'-TATGCTTAGTGTTAATTAGCCGTGATCCCAAA TTGTCTACTCTATTACG-3'	Oligonucleotides sequences for probe synthesizing in EMSA experiment
dfdR	5'-TCGTAATAGAGTAGACAATTTGGGATCACGGC TAATTAACACTAAGCAT-3'	
ΔdfdF	5'-TATGCTTAGTGTCGTCTATTACG-3'	
ΔdfdR	5'-TCGTAATAGACGACACTAAGCAT-3'	
SLF	5'-TAATTAGCCGTGATCCCAAATTGTCTAC-3'	
SLR	5'-TGTAGACAATTTGGGATCACGGCTAATT-3'	

Restriction sites in the primers are printed in italics.

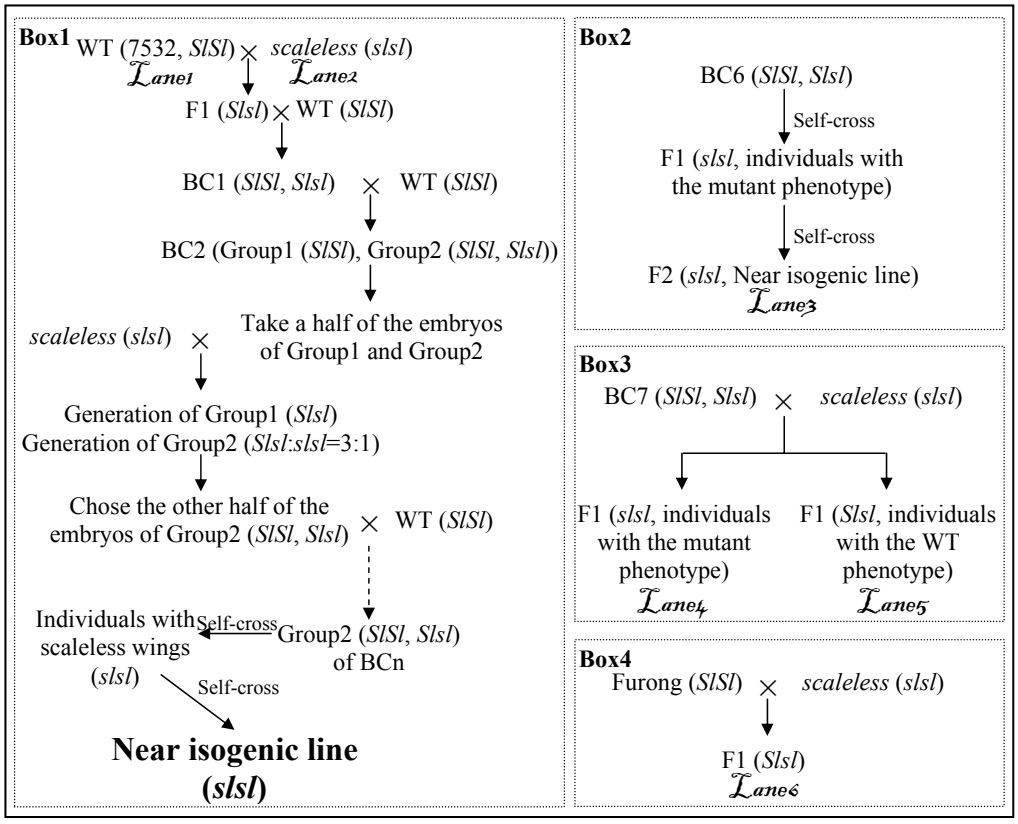

FIGURE S1.—Cross strategies used in the paper. This figure shows the genetic crosses used in the experiments. DNA of Lane 1-6 in Figure 4 was extracted from individuals with particular genetic background signed as Lane 1-6 in this figure. We named the recessive gene controlling *scaleless* phenotype as *sl*, and its dominant allele as *Sl*. Box1 is the process of making a near isogenic line with the *sl* phenotype under most WT (7532) genetic background. Theoretically, when the "n" in BCn equals to 6, the generation will contain about 99% of the WT genetic background ($1/2^{6+1}$=99.2%). Box2 shows a generation of the near isogenic line with 6 times backcross to WT. Box3 shows the Group2 of BC7 cross to the mutant *sl*, generating F1 with two different genotypes. Box4 shows a cross of another WT strain Furong to *sl*, which gave F1 individuals with heterozygous genotype.