Toll ligand Spätzle3 controls melanization in the stripe pattern formation in caterpillars

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A stripe pattern is an aposematic or camouflage coloration often observed among various caterpillars. However, how this ecologically important pattern is formed is largely unknown. The silkworm dominant mutant *Zebra* (*Ze*) has a black stripe in the anterior margin of each dorsal segment. Here, fine linkage mapping of 3,135 larvae revealed a 63-kbp region responsible for the *Ze* locus, which contained three candidate genes, including the Toll ligand gene *spätzle3* (*spz-3*). Both electroporation-mediated ectopic expression and RNAi analyses showed that, among candidate genes, only processed *spz-3* induced melanin pigmentation and that *Toll-8* was the candidate receptor gene of *spz-3*. This Toll ligand/receptor set is also involved in melanization of other mutant *Striped* (p^S), which has broader stripes. Additional knockdown of 5 other *spz* family and 10 *Toll*-related genes caused no drastic change in the pigmentation of either mutant, suggesting that only *spz-3/Toll-8* is mainly involved in the melanization process rather than pattern formation. The downstream pigmentation gene *yellow* was specifically upregulated in the striped region of the *Ze* mutant, but *spz-3* showed no such region-specific expression. Toll signaling pathways are known to be involved in innate immunity, dorsoventral axis formation, and neurotrophic functions. This study provides direct evidence that a Toll signaling pathway is co-opted to control the melanization process and adaptive striped pattern formation in caterpillars.

Spätzle3 | Toll signaling pathway | melanization | striped pattern | *Bombyx mori*

Animals have various color patterns: spot and stripe patterns are frequently observed. These body patterns are often used for camouflage or aposematic coloration to avoid predators (1, 2). In aposematism, the contrast created by the combination of bright and dark colors, such as yellow/red and black (Fig. 1*A*), is important to facilitate detection by predators and hasten avoidance learning (3). The molecular mechanism underlying spot formation in adult and larval insects has become more apparent via many studies in *Drosophila* (4, 5), *Bombyx mori* (6, 7), and several butterflies (8–10). Although stripe pattern formation is well-studied in vertebrates, such as the zebra (11), rodents (12), and zebrafish (13), the molecular backgrounds of this pattern in insects are largely unknown. Because the stripe pattern is often observed in lepidopteran larva and its biological roles are more evident than in other animals (14, 15), it is intriguing to study the mechanism and evolutionary origin of caterpillar stripe formation.

The silkworm *B. mori* is a suitable model organism to study the genetic and molecular mechanisms of color pattern formation. There are extensive stocks of larval marking mutants, whole-genome information, and readily available functional analysis tools (16, 17). The silkworm mutant *Zebra* (*Ze*), which occurred spontaneously in ancient China, has a black stripe on one-fourth of the anterior part of the dorsal side of each larval segment (Fig. 1*B*, *Left*) and provides a good object to study to understand the mechanism of striped pattern formation. Previous genetic studies revealed that the *Ze* allele is dominant over the WT and mapped to 20.8 cM of linkage group 3 (18) (Fig. 1*C*).

In this study, a 63-kbp region responsible for *Ze*, including three predicted genes, was identified by linkage analysis. Additional observation of expression profiles and functional analysis of these three genes showed that the *Ze* allele is caused by a *spätzle* (*spz*) family gene *Bmspätzle3* (*Bmspz-3*). A cytokine-like ligand for the Toll receptor, Spz, is a key molecule in the activation of the Toll signaling pathway, which is involved in both dorsoventral axis formation during embryogenesis (19) and innate immunity (20). In addition, among *spz* paralogous genes in *Drosophila melanogaster*, some have been suggested to be involved in innate immunity and neurotrophic functions (20). However, to date, no evidence has been provided to show that this *spz* family gene regulates the melanin synthesis pathway directly. Therefore, the findings in this study shed light on not only the unique function of this *spz* family gene but also, the molecular mechanism of color pattern formation, which furthers our understanding of the whole melanin synthesis pathway involved in body marking and innate immunity.

Results

Fine Linkage Mapping of the *Ze* Locus in *B. mori*. Each larval segment of the *Ze* mutant strain f40 [*Ze*/*Ze*; *plain* (*p*)/*p*] has a black striped marking in the anterior margin on the dorsal side and a twin spot marking on the ventral side (Fig. 1*B*, *Left*), in contrast to the WT strain N4 ($+^{Ze}/+^{Ze}$; *p*/*p*) without markings (Fig. 1*B*, *Right*). Chemical analysis suggested that the pigments accumulated in the black stripes on the cuticle of the *Ze* mutant

Significance

A stripe pattern is widely observed among animals and often used for warning or camouflage in caterpillars. However, its genetic background is largely unknown. This study showed that the Toll ligand Spätzle3 (Spz-3) is responsible for the silkworm *Zebra* locus, which causes black stripes on the anterior margin of each segment. Exhaustive knockdown experiments of *spz*- and *Toll*-related genes clarified that *spz-3* and *Toll-8* are involved in the melanin pigmentation of *Zebra* and another mutant. The Spz-3/Toll-8 signaling pathway is suggested to induce *Zebra* stripe-specific expression of the pigmentation gene *yellow*. This study sheds light on not only the unique aspect of Spz/Toll functions but also, stripe pattern pigmentation in caterpillars through co-option of a Toll signaling pathway.

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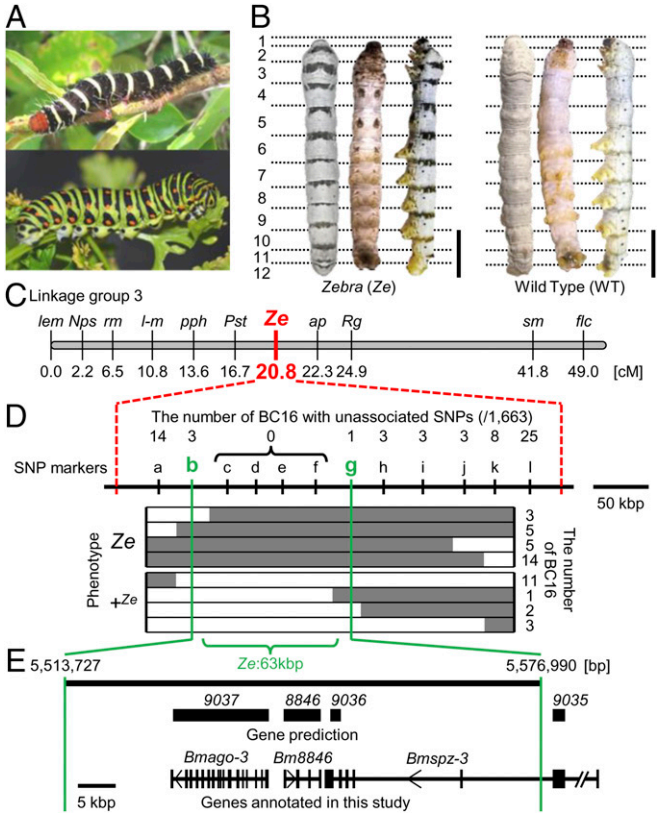

Fig. 1. Striped pigmentation in lepidopteran larvae and fine mapping of the *B. mori* mutant *Ze*. (*A*) Lepidoptera larvae in nature have striped color patterns that function in aposematic coloration. (*Upper*) The frangipani hornworm (*Pseudosphinx tetrio*). (*Lower*) The common yellow swallowtail (*Papilio machaon*). (*B*) Dorsal, ventral, and lateral views of the f40 strain, the *Ze* mutant (*Left*) and the WT N4 strain (*Right*) of *B. mori*. *Ze* has a black stripe on one-fourth of the anterior part of the dorsal side of each larval segment in contrast to N4 without markings. (Scale bars: 1 cm.) (*C*) Genetic maps of *B. mori* linkage group 3. (*D* and *E*) The fine mapping results of the *Ze* region. Regions separated by broken red lines indicate the responsible 400 kbp by standard SNP markers (25). Gray and white bars indicate the genotypes of the heterozygote (*Ze*/+^{Ze}) and homozygote (+^{Ze}/+^{Ze}), respectively. The 63-kbp region responsible for the *Ze* phenotype is denoted by Ze:63kbp. The numbers above the SNP markers indicate recombination events. Gene prediction in the Ze:63kbp was derived from KAIKObase and SilkBase databases.

contained melanin (Fig. S1*A*). Detailed observation revealed that each stripe is composed of seven blocks of pigmented areas (Fig. S1*B*, *Center*). In the dense spots (Fig. S1*B*, red arrowheads), the melanin pigments accumulated on the cuticle just around the joint region between the tendon cell and muscle (Fig. S1*B*, *Right*).

Previous genetic studies have mapped the *Ze* locus to linkage group 3 (18) (20.8 cM) (Fig. 1*C*). Using standard SNP markers (21), low-resolution mapping was first performed with 1,472 progeny [733 *Ze* individuals (*Ze*/+^{Ze}; *p*/*p*) and 739 *p* individuals (+^{Ze}/+^{Ze}; *p*/*p*)] obtained from 10 pairs of parents [backcrossed F1 progeny (BC1) males and *p* females]. Using newly designed SNP markers (Table S1), we further performed fine mapping of 1,663 progeny (822 *Ze* individuals and 841 *p* individuals) obtained from 11 pairs of parents (BC16 males and *p* females). Among 12 additional genetic markers (a–l), the genotypes of four markers (c–f) were completely linked to the *Ze* phenotype (Fig. 1*D* and Fig. S2). These analyses showed that the *Ze* locus was located within an ∼63-kbp region, hereafter referred to as Ze:63kbp.

Ze:63kbp Includes Three Candidate Genes for the *Ze* Locus. The Ze:63kbp region includes three predicted genes (*BGIBMGA008846*, *BGIBMGA009036*, and *BGIBMGA009037*) according to KAIKOBase (sgp.dna.affrc.go.jp/KAIKObase/) and SilkBase (silkbase.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi) databases (Fig. 1*E*). *BGIBMGA009037* encodes an *argonaute3* (accession no. AB332312) homolog, which was named *Bmago-3*. The amino acid sequence of *BGIBMGA009036* contained an *spz*-like cystine knot domain. Furthermore, a BLASTp search and phylogenetic analysis indicated that this is a homolog of *spz-3* in other insects [e.g., *D. melanogaster* (NP_609160.2) and *Tribolium castaneum* (NP_001153625.1)] (Fig. S3*A*). *BGIBMGA009035*, which is located outside of Ze:63kbp, was found to be a part of the *spz-3* gene, and thus, the combined sequences of *9035* and *9036* were characterized as *Bmspz-3* (Fig. 1*E*). *BGIBMGA008846* encodes an NifU-like domain, which has been suggested to be involved in the assembly of iron–sulfur clusters and binds one iron ion at its N terminus (22). However, the homolog and function of this gene are not clear, and thus, we henceforth refer to as *Bm8846*.

To determine whether the *Ze* candidate genes had structural defects in an ORF, the amino acid sequences of the three predicted genes in Ze:63kbp from three strains [*Ze* (f40), *p* (WT; N4), and +^{p} (WT; p50)] were compared. Among the *Ze* and the two WT strains, there were no frameshifts and/or indels for all three genes (Figs. S4–S6). *Bmspz-3* had two nonsynonymous substitutions within a signal peptide (P in the *Ze*, A in the two WT strains) and cystine knot domain regions (S in the *Ze*, N in the two WT strains) (see Fig. 3*A*; Fig. S5). The region encoding the signal peptide for *Bmspz-3*, however, was located outside of Ze:63kbp; thus, the amino acid change in this region should not be involved in the phenotypic change. In contrast, no nonsynonymous substitutions were observed within the functional domains of *Bm8846* and *Bmago-3* (Figs. S4 and S6, respectively).

Expression Profiles of Candidate Genes in Larval Epidermis. Next, the expression profiles of the above candidate genes within Ze:63kbp in the epidermis were analyzed during the fourth instar stage. We prepared mRNA from pigmented and nonpigmented areas of the abdominal third segment in the *Ze* (f40; *Ze*/+^{Ze}) strain and their corresponding areas in the WT (N4; +^{Ze}/+^{Ze}) strain. By quantitative RT-PCR (qRT-PCR), the expression levels of the three genes, which were standardized by the expression of the ribosomal protein gene *ribosomal protein L3* (*BmrpL3*), were compared. In all candidate genes, the expression profiles during the fourth instar stage were almost identical between the pigmented (black circle in Fig. 2*A*) and nonpigmented (white diamond in Fig. 2*A*) areas in the *Ze* epidermis (Fig. 2*A*, *Upper*: *Bm8846*, *Bmspz-3*, and *Bmago-3*). This result suggests the possibility that the *Ze* candidate gene itself does not determine the region specificity of pigmentation. Similar expression patterns of the three genes were also observed in the corresponding area of the WT strain (Fig. 2*A*, *Lower*: *Bm8846*, *Bmspz-3*, and *Bmago-3*). The expression levels of *Bm8846* and *Bmago-3* in the *Ze* strain were higher than those in the WT strain throughout the fourth instar. However, *Bmspz-3* expression was lower and peaked at D3 and E2 during the molting period in the *Ze* strain but only at D3 in the WT strain (Fig. 2*A*, *Center*), indicating some differences in expressional control of the gene between the *Ze* and WT strains. The peak expression during the molting stage suggests that the molting hormone ecdysteroid induces *Bmspz-3* expression.

***Bmspz-3* Knockdown Causes Stripe Pigmentation Loss.** To identify the candidate gene responsible for the black stripe pigmentation in *Ze*, an in vivo electroporation technique (23, 24) was used to knockdown each candidate. Then, the resulting phenotypic changes were observed. Although RNAi is not generally effective in Lepidoptera, this technique enables the introduction of dsRNA or plasmid DNA into the target tissues, such as the epidermis, both easily and effectively. After siRNA for each gene was introduced into the epidermis of the abdominal third and fourth segments of the third instar larvae, the phenotypic change was observed in the fifth instar stage (Fig. 2*B*). In this method, RNAi-induced changes should occur in the left side of the

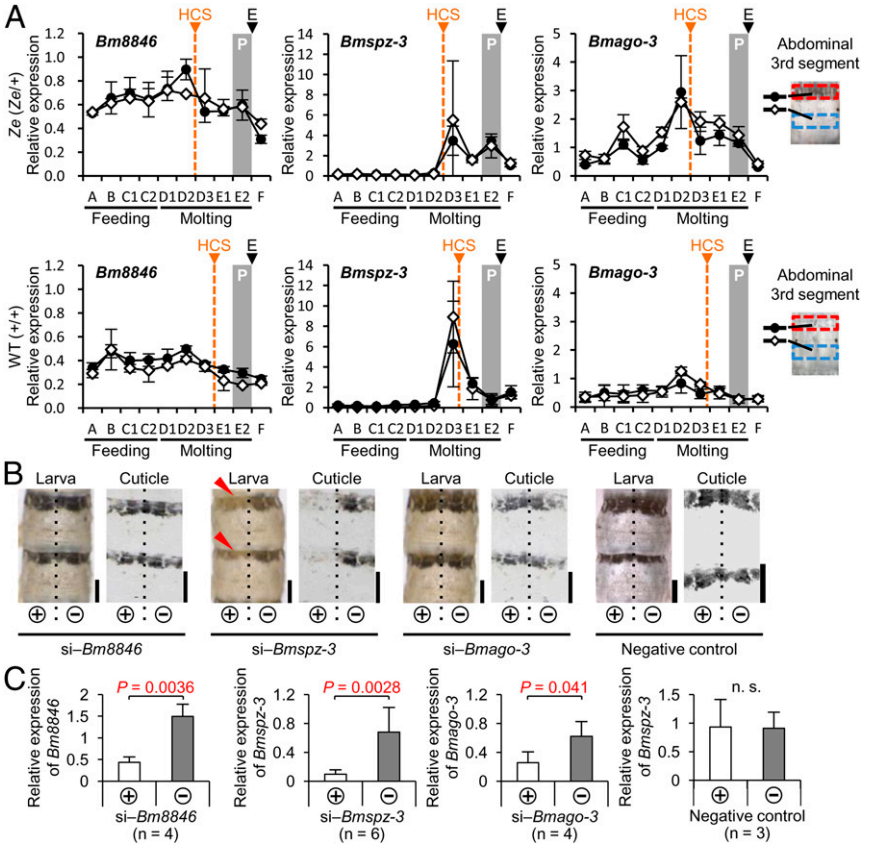

Fig. 2. Expression profiles and effects of siRNA of *Bm8846*, *Bmspz-3*, and *Bmago-3* in the larval epidermis. (*A*) Temporal expression patterns of three genes in the epidermis from the third to fourth ecdysis ($n = 3$ in each period and strain) are shown for the *Ze* mutant (*Left*: *Bm8846*, *Center*: *Bmspz-3*, *Right*: *Bmago-3* in *Upper*) and the WT strain (*Left*: *Bm8846*, *Center*: *Bmspz-3*, *Right*: *Bmago-3* in *Lower*). Expression of the *BmrpL3* gene was used as an internal control. The black circles and white diamonds indicate the pigmented and nonpigmented areas of the *Ze* mutant (or the corresponding areas of the WT strain), respectively, which are shown as red and blue boxes in the abdominal third segment, respectively. E, ecdysis; HCS, head capsule slippage; P, pigmentation period. (*B*) Phenotypic changes by knockdown of the candidate genes in the larva and cuticle specimen on the abdominal third and fourth segments in the *Ze* mutant are shown. Dashed lines illustrate the position of the dorsal midline. (Scale bars: 2 mm.) (*C*) Relative expression levels of genes targeted by siRNA were compared between the introduced (+) and nonintroduced (–) sides. Statistical comparisons were conducted using the one-sided paired Student's *t* test. Error bars indicate the SD.

segment where the plus electrode (+) was touched, because the negatively charged RNA was introduced effectively into the epidermal cells.

Bm8846 and *Bmago-3* siRNA had no effect on the larval phenotypes in the *Ze* strains (Fig. 2*B*). However, when the larvae were treated with siRNA of *Bmspz-3* (*si–Bmspz-3*), the black stripes disappeared slightly in the introduced (left) side (Fig. 2*B*, red arrowheads), although the change was not very clear. To observe the striped pigmentation pattern more clearly, we dissected and detached the cuticle sheet where the melanin accumulates from the epidermis. The black stripes on the cuticle disappeared only in the introduced (left) side of the si–*Bmspz-3*–treated sample (Fig. 2*B*, cuticle), whereas no change was observed in the right side, where no siRNA had been introduced. We confirmed by qRT-PCR that the RNA level of each target gene was reduced significantly in the left side, where the siRNA knockdown occurred (Fig. 2*C*). These results suggest that *Bmspz-3* is involved in striped pigmentation formation in the larval epidermis and is a possible gene responsible for the *Ze* phenotype.

Ectopic Expression of *Bmspz-3* Induces the Black Pigmentation. To further understand how *Bmspz-3* controls melanin pigmentation, we next performed overexpression of the gene by electroporation-mediated somatic transgenesis (23, 24). In this method, after plasmid DNAs were incorporated into the epidermal cells by in vivo electroporation, target genes in the donor plasmid were integrated into the host genome by *piggyBac* transposase in the helper plasmid (Fig. 3*B*). Because there were two amino acid changes between the WT and *Ze* strains (Fig. 3*A*, green letters and asterisks), we made the donor plasmids including various parts of the *Bmspz-3* gene from the WT and *Ze* strains (Fig. 3*B*).

Next, we performed the overexpression of full-length constructs of *spz-3* from the WT and *Ze* strains (Fig. 3*B*, *Spz-3^{WT}FL* and *Spz-3^{Ze}FL*), but unexpectedly, this treatment had no effect on larval pigmentation (Fig. 3*C*). Because Spz proteins should be processed before activation, this result may be caused by the inefficient processing of ectopically expressed Spz-3. To validate this possibility, we next made constructs of processed Spz-3, which included only the CK domain and digested just after Arg-His-Ala-Arg (RHAR) sequence (25) (Fig. 3*A*, red triangle). The WT and *Ze Bmspz-3* CK domain regions were connected to the fibroin signal peptide (26), which is expected to work equally in both plasmids (Fig. 3*B*, *Spz-3^{WT}CK$_{SP}$* and *Spz-3^{Ze}CK$_{SP}$*). The ectopic expression of these plasmids in the WT larva and pupa showed melanin formation among the regions expressing EGFP (Fig. 3*D*). The pigmentation seemed stronger in the anterior part of the segment, suggesting that the *Ze* stripe region is prepatterned by molecules other than *Bmspz-3*.

Spz-3 Is Also Involved in Melanization of the *Striped* Pattern. Phylogenetic analysis showed a highly conserved monophyly for each *spz* family gene among insect species, suggesting their conserved functional roles in some cellular or developmental processes

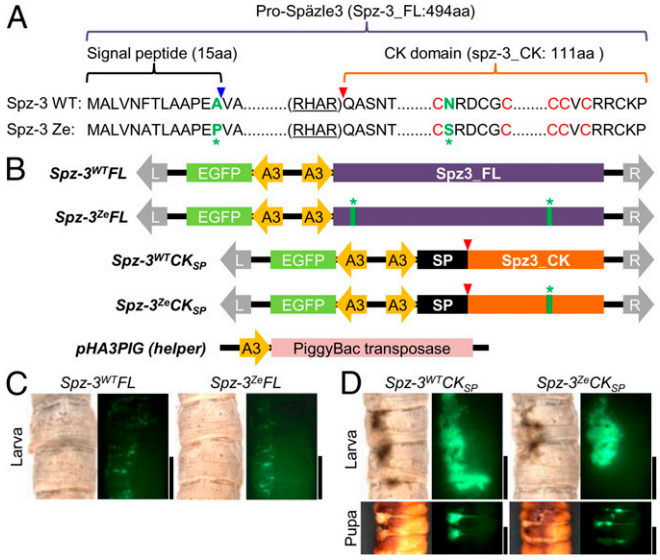

Fig. 3. Ectopic expression of transgene *Bmspz-3* causes melanin pigmentation of the larval and pupal cuticle. (*A*) Alignment of the amino acid sequences of Spz-3 of the *Ze* and WT strains. Green letters indicate nonsynonymous substitutions, and red letters C indicate highly conserved cysteine motifs among the *spz* family genes. (*B*) Scheme of plasmids for transgenic ectopic expression of *Bmspz-3* and the helper plasmid *pHA3PIG*, which encodes *piggyBac* transposase. All plasmids contained the A3 promoter of *Bmactin3*, EGFP as a reporter, and recognition sequences of *piggyBac* (L and R with gray arrows). We prepared plasmids, which encoded the full-length ORF of *Bmspz-3* ($Spz\text{-}3^{WT}FL$ and $Spz\text{-}3^{Ze}FL$ for WT and *Ze*, respectively), and the CK domain region, where the processed form functioned with the signal peptide (26) ($Spz\text{-}3^{WT}CK_{SP}$ and $Spz\text{-}3^{Ze}CK_{SP}$) of both of the *Ze* and WT strains, respectively, to test whether melanin pigmentation was caused by *Bmspz-3*. The blue and red arrowheads in *A* and *B* indicate processing sites for the signal peptide and the processed form of *Bmspz-3*, respectively. The green asterisks indicate the sites of nonsynonymous substitutions as in *A*. (*C* and *D*) The phenotypes of ectopic expression of the full-length (*C*) and processed (*D*) forms of *Bmspz-3* in larvae and pupa are shown, respectively. (Scale bars: 5 mm.)

(Fig. S3*A*). To clarify whether other *spz* family genes in addition to *spz-3* are involved in melanin pigmentation, electroporation-mediated siRNA knockdown was performed. After siRNA treatment of *Bmspz-1* (so-called *spz*), *-2*, *-4*, *-5*, and *-6* in the *Ze* larva, a weak phenotypic change of black pigmentation was detected in si–*Bmspz-2* and si–*Bmspz-5* in *Ze* (Fig. S3*B*, red arrowheads). However, these effects were not as clear as that shown after *Bmspz-3* knockdown (Fig. 2*B*). This result suggests that, although *Bmspz-2* and *Bmspz-5* have some redundant roles in causing pigmentation, *Bmspz-3* is one of the main genes involved in melanization among the *spz* family genes.

Next, we investigated whether the *spz-3* and *spz* family genes are involved in melanin formation in the pigmentation patterns of mutant strains other than *Ze*. *Striped* (p^S), which has a broader black stripe in each segment (Fig. 4*A*), has one allele in the *p* locus (linkage group 2, 3.0 cM), which encodes more than 15 different alleles and is controlled by the transcription factor, Apontic-like (Apt-like) (7). In the p^S larva, knockdown with si–*Bmspz-3* by in vivo electroporation caused drastic loss of black pigmentation in the introduced (left) side (Fig. 4*B*, red arrowheads), similar to that observed in the *Ze* strain (Fig. 2*B*), suggesting that *Bmspz-3* is also involved in the melanization process of the p^S pigmentation pattern. In addition, electroporation-mediated knockdown with si–*Bmspz-2* and si–*Bmspz-5* had a weak effect on the p^S melanization pattern, as shown in the *Ze* mutant (Fig. S3*B*, red arrowheads).

Spz-3 Works as a Putative Ligand of Toll-8 in Melanization. An important question is whether the *Bmspz-3* gene works on melanization through the Toll signaling pathway. Because Spz-3 works as a ligand of Toll-8 in neuromuscle joint formation in *D. melanogaster* (27), siRNA of *BmToll-8* was injected into *Ze* and p^S larva, which resulted in the loss of pigmentation patterns in both strains (red arrowheads in Fig. 4 *C* and *D*, respectively), similar to the phenotypic change observed in the *Bmspz-3*-knockdown larva (Figs. 2*B* and 4*B*). This result indicates that Toll-8 is involved in the melanization process in the pigment pattern formation in silkworms.

A previous report suggested that, in addition to *Toll-8*, 10 genes (*Toll-1*–*Toll-7* and *Toll-9*–*Toll-11*) are encoded in the silkworm genome (28). To determine the possibility of involvement of these genes in larval pigmentation, we knocked down each gene's expression by siRNA with electroporation in the *Ze* and p^S larva. However, there was no notable change to the black pigmentation in any case (Fig. S7), suggesting that *BmToll-8* is the key receptor in the Toll signaling pathway for larval pigmentation.

The *yellow* mRNA Is Specifically Induced in the *Ze* Stripe Region. Detailed observation suggested that the black stripes in the *Ze* strain were composed of polymerized melanin pigments (Fig. S1*A*). Thus, to understand how melanin is synthesized in the striped formation in *Ze*, the expression profiles of genes in the melanin synthesis pathway, including *tyrosine hydroxylase* (*BmTH*), *dopa decarboxylase* (*BmDDC*), *laccase-2* (*Bmlac-2*), and *yellow* (*Bmyellow*), were further analyzed and compared (Fig. S8). The expression of *Bmlac-2*, which peaked during the D3–E1 stages during the molting stage, seemed similar between the WT and *Ze* strains and between the pigmented and nonpigmented regions (Fig. S8 *E* and *F*). The expression levels of *BmTH* and *BmDDC*, which increased in the E2 stage just before the fourth larval ecdysis, were similar between strains and regions, although these expression levels seemed higher in the *Ze* mutant than in the WT strain (Fig. S8 *A*–*D*). The increase in expression of these genes in the nonpigmented regions can be explained by the fact that both are also involved in the sclerotization of the new cuticle (29).

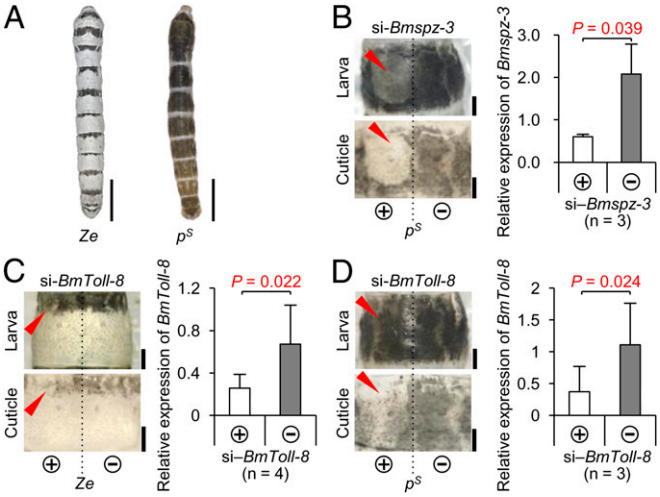

Fig. 4. Spz-3 and Toll-8 signaling pathway is involved in melanin pigmentation in *Ze* and p^S larvae. (*A*) Color pattern of the *Ze* mutant (*Left*) and another striped mutant p^S (*Right*). The p^S mutant has a broader black stripe in each segment than *Ze*. (Scale bars: 1 cm.) (*B*) Phenotypic changes of larva and cuticle introduced by siRNA of *Bmspz-3* and relative expression levels of *Bmspz-3* between the introduced (+) and nonintroduced (–) sides of the p^S mutant. (Scale bars: 1 mm.) (*C* and *D*) Phenotypic changes of larva and cuticle introduced by siRNA of *BmToll-8* and relative expression levels of *BmToll-8* between the introduced (+) and nonintroduced (–) sides of the *Ze* (*C*) and p^S (*D*) mutants. Error bars indicate the SD. Statistical comparisons were conducted using the one-sided paired Student's *t* test. (Scale bars: 1 mm.)

It is remarkable that *Bmyellow* expression was induced specifically in the striped pigmented area of the *Ze* mutant from D3 to E1 during the molting stage (Fig. S8*G*). In the WT strain and the nonpigmented area of the *Ze* strain, no induction of this gene was observed (Fig. S8*H*). Yellow is known to be a key dopa-converting enzyme for the production of melanin in the final process (30, 31). The above results suggest that *Bmspz-3* and its downstream gene network target the *Bmyellow* gene, which enables region-specific pigmentation in the anterior region of each segment in the *Ze* mutant (Fig. 5*A*).

Discussion

Here, linkage mapping and functional analysis showed that the *spz* family gene *Bmspz-3* was responsible for the silkworm mutant *Ze*. The knockdown experiments showed that, among the *spz* family genes, *Bmspz-3* is mainly involved in the melanin formation of the striped pigmentation in the *Ze* mutant (Fig. 2*B* and Fig. S3*B*). Importantly, *Bmspz-3* knockdown also resulted in the loss of pigmentation in the p^S mutant (Fig. 4*B*), indicating that *Bmspz-3* regulates a common pathway in the melanin formation of larval pigmentation (red letters in Fig. 5*A*). The Spz-3 signal seems to be received by the Toll-8 receptor at least in the pigmented area in the *Ze* and p^S mutant strains. The same ligand/receptor relationship has been reported in the neuromuscular junction of *D. melanogaster* (27). Because our recent study has shown that the p^S phenotype is controlled by the *apt-like*–encoding transcription factor (7), this Spz-3/Toll-8 signaling pathway likely interacts with the genetic circuit of *apt-like* and contributes to the phenotypic diversity of caterpillars. Although there are at least six gene classes in the *spz* family in insects (Fig. S3*A*), this report shows that an *spz* gene is involved in the melanization process. It has been reported that Spz-1 works as a ligand of Toll-1 and that the Toll signaling pathway is involved in dorsoventral axis formation (19), muscle development during embryogenesis (32), and the induction of antimicrobial peptide expression in innate immunity (33, 34) (Fig. 5*B*). In addition, Toll-6 and Toll-7 are suggested to function in the synaptogenesis of locomotor neuron networks, with two ligands, Spz-2 and Spz-5, in *Drosophila* (35). Although there is no direct evidence for other functions of *spz* family genes at present, highly conserved structures indicate some functional role of each class of *spz* family genes.

The infection of bacteria or fungi into the insect body induces not only the expression of antimicrobial peptide through the Spz and Toll signaling pathway but also, melanin formation through the prophenol oxidase pathway (36) (Fig. 5*B*). The initial step in both pathways is to recognize the lysine-containing peptidoglycan and β-1,3-glucan from the cell wall of Gram-positive bacteria and fungi by peptidoglycan recognition proteins and β-1,3-glucan recognition proteins, respectively, which are followed by independent serine–protease cascades in the two pathways (33, 34) (Fig. 5*B*). In *Manduca sexta*, it has been suggested that the protease hemolymph protease 6 (HP6) activates both pathways (34, 36), but it is not clear whether this regulation is conserved among insects. At the end of the protease cascade, Spz is processed from pro-Spz by spz-processing enzyme, and Spz and Toll binding activates NF-κB–dependent transcription (20). Although we have not yet studied the roles of NF-κB factors in larval pigmentation, we first show here the relationship between the Toll-like pathway and melanin formation. It is noteworthy that Lac-2, used for larval pigmentation, and phenol oxidase, used for melanization in innate immunity, are different enzymes structurally but show the same enzymatic activity (Fig. 5). Thus, additional comparative studies on larval pigmentation and innate immunity may reveal a more accurate pathway and overall picture of melanin synthesis.

Overexpression experiments showed that ectopic pigmentation was caused by processed CK domain constructs of Spz-3 but not caused by the full-length constructs (Fig. 3 *C* and *D*). This result indicates that the full-length constructs of Spz-3 could not be processed appropriately under the experimental conditions. A recent report on *M. sexta* surmised that Spz-3 processing is mediated by furin-like enzymes based on the recognition sequence RHAR (25). Thus, the full-length construct integrated by somatic transgenesis into the random site of the host genome may not be expressed appropriately when the furin-like processing enzyme is expressed. However, the result that the CK plasmids caused pigmentation of both the *Ze* and WT strains (Fig. 3*D*) indicates no functional difference caused by the amino acid change (N in WT; S in *Ze*) in the CK domain of Spz-3. The two amino acid difference between WT and *Ze* Spz-3 may not contribute to functional

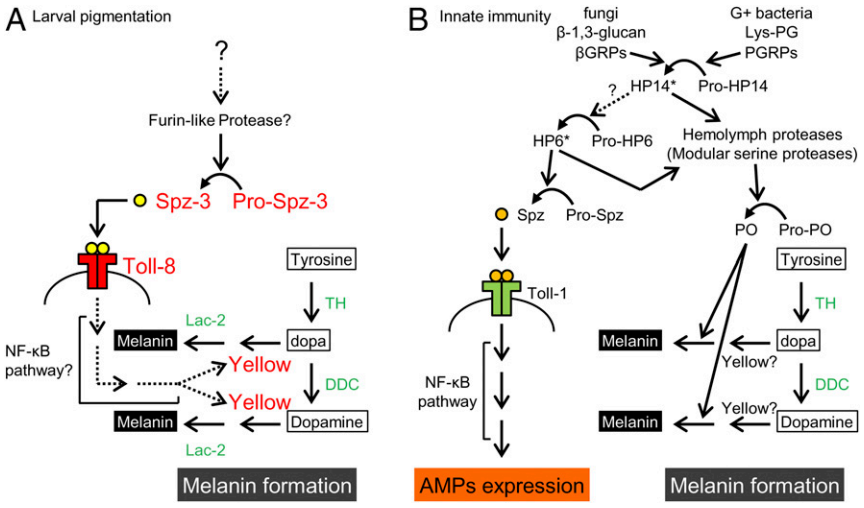

Fig. 5. A model of the pathway involved in melanin formation and larval pigmentation. (*A*) A model of melanin formation through the Spz-3/Toll-8 pathway of the *B. mori* larval epidermis. Spz-3 may activate Toll-8 and up-regulate the *yellow* expression in the pigmented area (red letters). (*B*) Antimicrobial peptide expression through the Spz/Toll signaling pathway and melanin formation through the prophenol oxidase (pro-PO) pathway in the innate immunity in insects (based on refs. 33, 34, and 36). Factors with asterisks (HP14 and HP6) are known to be involved in these two pathways in *M. sexta*, but it is not clear whether these factors are conserved among insects. Broken lines indicate uncertified pathways. Green letters indicate enzymes involved in melanin synthesis. AMP, antimicrobial peptide; βGRP, β-1,3-glucan recognition protein; Lys-PG, lysine-containing peptidoglycan; PGRP, peptidoglycan recognition proteins; PO, phenol oxidase; Pro-HP, precursor of hemolymph protease; Pro-PO, precursor of phenol oxidase.

differences. It is possible that the phenotypic change between the *Ze* and WT strains is caused by different expression patterns between *Ze* and WT *Bmspz-3* (Fig. 2*A*, *Center*), although it remains unclear whether a *cis*-regulatory sequence controls the temporal expression patterns of *Bmspz-3*.

To understand the stripe pattern formation, the molecular mechanism underlying region-specific pigmentation should be clarified. The *Bmyellow* gene, which directs melanization in the nearly final step, was expressed only in the future pigmented area (Fig. S8 *G* and *H*), but unexpectedly, the responsible gene for the *Ze* phenotype, *Bmspz-3*, did not show region-specific expression patterns (Fig. 2*A*, *Center*). Even with innate immunity, localized melanin formation is also triggered by HP14 in *M. sexta* in the most upstream step in the protease cascade by recognizing microorganisms (33). Several possibilities might explain this result. First, it is possible that some factors in the protease cascade upstream of Spz-3 processing are expressed in a stripe region-specific manner, which enables localization of the activated Spz protein only in the striped region. Second, the putative receptor Toll-8 or its downstream target genes are possibly expressed only in the future striped region. However, to date, there is no direct evidence of this possibility. The observation that the *Ze*-pigmented region is structurally distinct from other areas in the same segment (Fig. 1*C*) implies that some specific factors regulate formation of the developmental structure. This type of factor may also regulate expression of the *yellow* gene in a region-specific manner. Similarly, the region-specific expression of the *yellow* gene is observed in the spot patterns of *Drosophila* wings as well as the stripes of the *Drosophila* abdominal segments (4, 37). Some elements reported to be involved in these color patterns may contribute to the up-regulation of *yellow* expression in the *Ze* mutant. In the formation of eyespot patterns of *Papilio xuthus* larva, however, not only *yellow* but also, many other melanin synthesis genes, such as *TH*, *DDC*, and *tan* (38, 39), as well as the transcription factors *spalt* and *E75* (31) are expressed specifically in the future black region. In contrast, the results of the *Ze* mutant showed no expression changes in *TH*, *DDC*, or *lac-2* between the WT and *Ze* strains. The *Ze* strain is a spontaneous mutant isolated 1,000 or 2,000 y ago at most, and thus, the expression change of *spz-3* may affect only a small number of genes. In contrast, the eyespot pattern of *Papilio* larvae has been selected as an adapted trait against predators over a long period, in which many genes are networked to optimize the phenotype.

Materials and Methods

Silkworms were reared on mulberry leaves or an artificial diet at 25 °C under long-day conditions (16 h light:8 h dark). Linkage analysis, qRT-PCR, 5′ and 3′ RACE, and functional analysis are detailed in *SI Materials and Methods*. Chemical treatment and phylogenetic analysis can be found in *SI Materials and Methods*. Primers used in this study are listed in Tables S1 and S2.

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