

The ABCs of Eye Color in *Tribolium castaneum*: Orthologs of the *Drosophila white*, *scarlet*, and *brown* Genes

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ABSTRACT In *Drosophila melanogaster*, each of the three paralogous ABC transporters, White, Scarlet and Brown, is required for normal pigmentation of the compound eye. We have cloned the three orthologous genes from the beetle *Tribolium castaneum*. Conceptual translations of *Tribolium white* (*Tcw*), *scarlet* (*Tcst*), and *brown* (*Tcbw*) are 51, 48, and 32% identical to their respective *Drosophila* counterparts. We have identified loss-of-eye-pigment strains that bear mutations in *Tcw* and *Tcst*: the *Tcw* gene in the *ivory* (*i*) strain carries a single-base transversion, which leads to an E → D amino-acid substitution in the highly conserved Walker B motif, while the *Tcst* gene in the *pearl* (*p*) strain has a deletion resulting in incorporation of a premature stop codon. In light of these findings, the mutant strains *i* and *p* are herein renamed *white^{ivory}* (*wⁱ*) and *scarlet^{pearl}* (*st^p*), respectively. In addition, RNA inhibition of *Tcw* and *Tcst* recapitulates the mutant phenotypes, confirming the roles of these genes in normal eye pigmentation, while RNA interference of *Tcbw* provides further evidence that it has no role in eye pigmentation in *Tribolium*. We also consider the evolutionary implications of our findings.

KEYWORDS ABC transporters; eye pigmentation; evolution of development

OMMOCHROME pigments, derivatives of tryptophan processing, are a broadly used and highly variable source of coloration in the animal kingdom (Linzen 1974). Among insects, ommochromes function as an important source of visible eye pigments (Linzen 1974). Species as diverse as mosquitoes, moths, bugs, and bees use only ommochromes for eye coloration (Dustmann 1968; Beard *et al.* 1995; Quan *et al.* 2002; Moraes *et al.* 2005; Sethuraman and O'Brochta 2005), while in other species, like the grasshopper, *Schistocerca gregaria* (Dong and Friedrich 2005), or the fruit fly, *Drosophila melanogaster* (Summers *et al.* 1982), ommochromes, together with the guanine-derived pteridines, create wild-type eye color.

In *Drosophila*, eye coloration has attracted a great deal of study (Linzen 1974; Summers *et al.* 1982), which has revealed that proper allocation of pigments depends on

three half-type ATP-binding cassette (ABC) transmembrane transporters, which import pigment precursors into the appropriate cells for final processing (Mount 1987; Dreesen *et al.* 1988; Tearle *et al.* 1989; Mackenzie *et al.* 1999). The transport protein, White (W), works with its paralog, Scarlet (St), to import precursors of ommochrome pigments (Sullivan and Sullivan 1975; Tearle *et al.* 1989). Because these pigments provide brown coloration in *Drosophila*, failure of St function results in bright red eyes. Pteridines are the source of red pigmentation in this species and require the function of the transporter, Brown (Bw), along with W, to be properly allocated (Sullivan *et al.* 1979; Dreesen *et al.* 1988). When Bw function is impaired, only the brown ommochromes are properly imported into eye tissue. However, complete loss of W function means neither pigment source can be transported, so the naturally whitish hue of the eye tissue remains visible.

Unlike in *Drosophila*, mutant *white* orthologs are not the only source of the white-eyed phenotype in species that use only ommochromes as eye pigments. In the silkworm, *Bombyx mori*, mutations in the *st* ortholog, *Bmw-2* (Tatematsu *et al.* 2011), result in white eyes, as do mutations in the enzymes that convert tryptophan to ommochrome pigments (Quan *et al.* 2002). The same is true for the mosquitoes, *Anopheles gambia* (Benedict *et al.* 1996a; Mukabayire *et al.* 1996) and *Aedes*

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aegypti (Cornel *et al.* 1997), as well as the honey bee, *Apis mellifera* (Dustmann 1968).

The red flour beetle, *Tribolium castaneum*, has attracted a great deal of study as a stored-grain pest and has become established as a useful model for genetic and evolutionary research (Shippy *et al.* 2000; Patel *et al.* 2007; Tribolium Genome Sequencing Consortium 2008). Eye-color genes are attractive as markers for genetic transformation due to the ease of scoring, and naturally occurring *Tribolium* eye-color mutants have facilitated these efforts and encouraged further study into this process (Lorenzen *et al.* 2002a,b, 2003, 2007). Although this species is characterized with almost black eyes, evidence suggests that this coloration is due exclusively to ommochrome pigments. The earliest work to establish this found that reduced function of *Tribolium* orthologs of *vermillion* (*Tcv*) and *cinnabar* (*Tcn*), both important enzymes in the tryptophan-to-ommochrome pathway, resulted in beetles with white eyes (Lorenzen *et al.* 2002a). More recently, analysis of the *Tribolium* genome for ABC transporters revealed predicted orthologs for *w* (*Tcw*) and *st* (*Tcst*) (Broehan *et al.* 2013). This study also used RNA interference (RNAi) to diminish the function of these genes. As expected, RNAi of the putative *Tcw* resulted in white eyes. More interestingly, knockdown of the putative *Tcst* also caused a white-eye phenocopy, while control experiments with pteridine biosynthesis enzymes did not alter eye color or pigmentation of any other examined tissue (Broehan *et al.* 2013). Furthermore, to date, no study has identified a *Tribolium* ortholog of *bw* (*Tcbw*) (Tatematsu *et al.* 2011; Broehan *et al.* 2013; Wang *et al.* 2013). Altogether, these results suggest that the visible pigments of *Tribolium* eyes are composed only of ommochromes.

Here, we report, for the first time, the identification of a potential *Tcbw* and show that it does not play a role in transporting visible eye pigments. We also expand on the existing knowledge of *Tcw* and *Tcst* by cloning and analyzing their transcripts to determine all intron/exon boundaries and by identifying potential promoters. Furthermore, we examine existing eye-color mutants and identify lines that possess lesions in these genes. Finally, we offer some evolutionary considerations for the varied repertoire of these ABC transporters among several species.

Materials and Methods

Linkage and complementation analysis of *Tribolium* recessive eye-color strains

Beetles were reared in yeast-fortified wheat flour under standard conditions (Beeman *et al.* 1986). The wild-type *Tribolium castaneum* strains used in this work were (1) GA1 (Haliscak and Beeman 1983), (2) GA2 (Lorenzen *et al.* 2002a), and (3) T-1 (Thomson *et al.* 1995). Strains homozygous for the following recessive eye-color mutations were also used in this work: (1) *pearl* (*p*) (spontaneous) (Park 1937); (2) *ivory* (*i*) (X-ray-induced) (Bartlett 1962); and (3) *red²²* (spontaneous) (Lorenzen *et al.* 2002a). The dominant-visible, recessive-lethal marker *Short elytra* (*Se*) (Lorenzen *et al.*

2007) was used for linkage analyses of the three eye-color mutations; this strain has wild-type (black) eye pigmentation. Data presented here strongly suggest that *ivory* and *red²²* are alleles of *Tribolium white* (*Tcw*) and that *pearl* is an allele of *Tribolium scarlet* (*Tcst*) (see below). Therefore, these strains will hereafter be referred to as *white^{ivory}* (*wⁱ*), *white^{red²²}* (*w^{r22}*), and *scarlet^{pearl}* (*st^p*), respectively. The *mas*, *p*, and *au* stock is homozygous for *st^p*, as well as for the incidental markers *missing abdominal sternites* (*mas*) (Hoy and Sokoloff 1965) and *aureate* (*au*) (Hoy *et al.* 1966). For a complete list of known *Tribolium* eye-color mutants, see Lorenzen *et al.* (2002a). The Pig-23 strain is homozygous for a *piggyBac* insertion on the fourth linkage group. This enhancer-trap strain shows enhanced green fluorescent protein expression in the imaginal wing and elytral discs of last-instar larvae, enabling easy discrimination between last-instar and penultimate-instar larvae (Lorenzen *et al.* 2003). Importantly, the Pig-23 strain used in this work is in an otherwise wild-type background (*i.e.*, has black eye pigment).

Linkage of *st^p*, *wⁱ*, and *w^{r22}* was evaluated by two-point linkage tests between each eye-color mutant and the dominant marker *Se*. Each mutant stock was mass-crossed to *Se* males. Selected F₁ progeny, *Se*/(*st^p*, *wⁱ*, or *w^{r22}*), were backcrossed to the appropriate mutant strain in virgin, mass crosses. Complementation among the recessive mutations *st^p*, *wⁱ*, and *w^{r22}* was determined in hybrids.

RNA isolation and complementary DNA synthesis

Total RNA was isolated from 1 g of *Tribolium* pupae (GA1 or *st^p*) or ovaries (GA2) by homogenizing the tissue in 1 ml of Trizol (Life Technologies) following the manufacturer's instructions. Messenger RNA (mRNA) was isolated from 1 mg of total RNA using the MessageMaker RNA Isolation Kit (CellScript) following the manufacturer's directions. Alternatively, total RNA was purified and converted to a complementary DNA (cDNA) template using an oligo(dT) primer (RT-Uni). To improve 5' RACE results, mRNA was given a 5' tag using the FirstChoice RLM-RACE Kit (Ambion) and then converted to cDNA using gene-specific primers (primer sequences can be found in Supporting Information, Table S1).

Total RNA (~1 µg) isolated from *st^p* pupae was reverse-transcribed using AMV reverse transcriptase (Roche) with an oligo(dT) primer. The resulting cDNA was amplified using the *Tcw*-specific primers *Tcw5' UTR* and *Tcw3' UTR*. A fragment of the expected size was ligated into pCRII-TOPO (Invitrogen).

To obtain a cDNA fragment containing the 5' UTR of *Tcw*, nested, gene-specific reverse primers were used in conjunction with nested, vector-specific primers against a 6- to 24-hr embryonic cDNA library (Shippy *et al.* 2000). First-round PCR was performed with 3.6 ng of cDNA as template, using 5'WR1 and M13(R). A second round of PCR was performed with the nested primers 5'WR2 and SP6, using 1 µl of the first-round product as template. The second-round PCR product was ligated into pCR4-TOPO (Invitrogen) and the resulting clone sequenced. Primers from the 5' and 3' UTRs were used to amplify a *Tcw* cDNA (pCw; GenBank accession

no. AF422804) that included the complete coding region. The reverse primer was designed to contain most of the 3' UTR, as well as the poly(A) signal to enable use of the cDNA in transfection constructs.

To obtain a cDNA fragment containing the 5' UTR of *Tcst*, nested, gene-specific reverse primers were used in conjunction with nested, 5' tag primers against early pupal cDNA made using *Tcst* 5' RACE outer primer. First-round PCR was performed with 1 µg of cDNA as template, using *Tcst* 5' RACE inner and the RACE kit's 5' RACE outer primer. A second round of PCR was performed with the nested primers *Tcst* 5' RACE inner2 and 5' RACE inner primer, using 1 µl of the first-round product as template. The second-round PCR product was cloned and sequenced as with *Tcw*. Primers from the 5' and 3' ends of *Tcst* were used to amplify a cDNA (GenBank accession no. KP120763) that included the complete coding region. Due to the low abundance of *Tcst* message, nested, gene-specific forward primers were used in conjunction with a common reverse primer, Uni-linker. First round was performed using *Tcst* FL F₁ on 130 ng of template cDNA made with RT-Uni primer. A second round of PCR was performed with the nested primer St-utr F₂ and 1 µl of first-round product as template. The second-round product was then cloned and sequenced as above.

A section of *Drosophila* Brown (LSGGERKRLSLAELITDPIFLFCDEPTTGLDS) was used as query against the *Tribolium* genome (TBlastN), and a 10-kb region (centering on the nucleotides encoding the Walker B motif) of the identified scaffold was evaluated using BlastX (Altschul *et al.* 1997) and FGENESH (Salamov and Solovyev 2000). The identified prospective coding sequence was used to query a variety of *Tribolium* databases, including in-house databases consisting of transcriptomic data from GA2 ovaries and unfertilized eggs (MiSeq, Illumina), resulting in the identification of an EST contig very similar to our predicted gene. These data were used to modify our gene prediction, which was then used to design primers for cloning the full-length cDNA (GenBank accession no. KP120764) from the same ovary RNA pool. Due to the low abundance of *Tcbw* message, nested, gene-specific forward primers were used in conjunction with a common reverse primer, Uni-linker. First round was performed using *Tcbw* FL F₁ on 280 ng of template cDNA made with RT-Uni primer. A second round of PCR was performed with the nested primer *Tcbw* FL F₂ and 1 µl of first-round product as template. The second-round product was then cloned and sequenced as above.

Sequence data were analyzed using the Vector NTI (Invitrogen) sequence analysis program. Putative promoters were identified using the Neural Network Promoter Prediction Program (Reese 2001; http://www.fruitfly.org/seq_tools/promoter.html) with a cutoff score of 0.70.

RNA interference

Double-stranded RNAs (dsRNAs) were generated using gene-specific, T7-tailed PCR products as templates for *in vitro* transcription. T7-tailed PCR products were purified

using the QIAquick PCR Purification Kit (Qiagen), and 1 µg was used as template for *in vitro* transcription using the MEGAscript T7 *in vitro* Transcription Kit (Ambion) to generate sense and antisense RNA in the same tube. The resulting dsRNAs were purified using the MEGAclean Kit (Ambion) and individually tested for phenotypic effect. Approximately 0.2 µg of each dsRNA (~1 µg/µl) was injected into last-instar Pig-23 larvae (Lorenzen *et al.* 2003). Injected larvae were allowed to pupate, and individuals scored for eye pigmentation as pupae and again as adults.

Recombinational mapping

To map *Tcw*, single-pair crosses were set up between T-1 virgin females and *mas*, *p*, and *au* males. F₁ virgin females were backcrossed to the male parental type in single pairs. The backcross progeny were sorted by eye-color phenotype as late-stage pupae, and single-beetle DNA isolations were performed using the Wizard Genomic DNA Isolation Kit (Promega, Madison, WI) according to the manufacturer's protocol.

A 176-bp PCR product that spans the fifth intron of *Tcw* was generated using the primers WH-11 and WH-12RC. A single-strand conformational polymorphism (SSCP) previously detected in this fragment was used for mapping, as previously described (Lorenzen *et al.* 2005).

Homolog identification, protein alignments, and phylogeny

Potential eye-color transporters homologous to DmW were identified using TBlastN on FlyBase separately for *A. aegypti*, *A. gambia*, *A. mellifera*, and *B. mori*. The maximum-likelihood phylogenetic tree was constructed in the MEGA program, version 6 (Tamura *et al.* 2013), using default parameters in all categories except the following: bootstrapping with 500 replicates, LG model of amino-acid substitution with Gamma distributed substitution rates with Invariant sites (based on Best Model determination within the MEGA program), and Partial Deletion treatment of gaps/missing data (Hall 2013).

The sequence logo (Schneider and Stephens 1990) was constructed using the tool at <http://weblogo.berkeley.edu> (Crooks *et al.* 2004), using an alignment constructed by the PSI-Coffee tool at <http://tcoffee.crg.cat> (Notredame *et al.* 2000; Tommaso *et al.* 2011).

Results

Structure of *Tcw* and *Tcst*

Broehan *et al.* (2013) previously described two *Tribolium* ABC transporters (NP_001034521.1 and XP_968696.1) as orthologs of the *Drosophila* genes *white* and *scarlet*, respectively. To confirm the structure of these computed-gene models, 5' RACE was performed to identify the full-length 5' UTR, followed by amplification of nearly full-length cDNAs (5' UTR to 3' UTR, Figure S1) to confirm that all exons were identified. The structure of *Tcw* (Figure S2A) is in good agreement with the previously published prediction; the longest 5' UTR is

173 bp in length; the CDS is composed of 2010 bp on 10 exons, encoding a protein 669 amino acids in length; and the 3' UTR is 37 bp long. The structure of *Tcst* (Figure S2A), on the other hand, differs significantly at the 5' end, better resembling GLEAN model 11998 (<http://beetlebase.org>). The start of the *Tcst* transcript was previously predicted to be ~4 kb downstream from *Tcw*. However, our RACE results suggest that the initiation of *Tcst* transcription lies only ~160 bp downstream of the *Tcw* stop codon (Figure S2A); the longest *Tcst* cDNA has 109 bp of 5' UTR with a CDS consisting of 1959 bp on 14 exons encoding a protein 652 amino acids in length and a 3' UTR of 24 bp.

To identify promoter elements that may drive the expression of *Tcw*, we analyzed sequence upstream of its 5' UTR. A single putative promoter (score = 0.97, Neural Network Promoter Prediction) was identified within a 1-kb region (−827 to +173, where +1 corresponds to the 5'-most nucleotide of the longest *Tcw* cDNA). This element is located from −46 to +4 (Figure 1). Although it lacks the canonical TATA box structure, several arthropod initiators (Inrs) (TCAGT, GCAGT, ACAGT, and TCATT) (Cherbas and Cherbas 1993) were identified nearby. Specifically, there are four Inrs upstream of the putative promoter element, one within and two downstream. The Inrs are clustered in this region and appear infrequently in the flanking DNA. Arkhipova (1995) identified a number of “downstream elements,” the triplets ACA, AAC, TCG, and GTG, which she noted are generally found in the interval of +20 to +35 and are significantly overrepresented in TATA-less promoters. *Tcw* has numerous corresponding triplets in the interval −49 to +74 (Figure 1). Moreover, these triplets are sparsely distributed upstream until reaching a cluster of six elements within the region +10 to +57. Furthermore, the presence of a downstream promoter element (DPE) motif, GA/TCG (Smale 1997), at +51 suggests that transcription

of *Tcw* indeed starts at, or near, the putative promoter sequence. Therefore, we conclude that *Tcw*, like *Drosophila white*, is transcribed from a TATA-less promoter.

A region starting at the ninth exon of *Tcw* and extending through the first exon of *Tcst* was also analyzed for promoter motifs, Inrs, downstream elements, and DPEs that may drive *Tcst* expression. As with *Tcw*, there are numerous downstream elements in the sequence near the *Tcst* start codon (Figure 1), with a cluster of six triplets in the region +6 to +31. Initially, no putative promoter sequences were identified, but relaxing the parameters (0.80–0.70) revealed two possible promoter sequences, including one within the 3' UTR of *Tcw*. Because there are no clear TATA box sequences, we conclude that, like *Tcw*, *Tcst* is transcribed from a TATA-less promoter. However, we cannot rule out the possibility that *Tcst* transcription is driven by a promoter upstream of the *Tcw* 3' UTR. If this is the case, the promoter could lie either within the *Tcw* gene itself or upstream of *Tcw*.

Identification of *Tcbw*

Previous work has suggested that the *Tribolium* eye lacks pteridine pigments (Lorenzen *et al.* 2002a,b; Broehan *et al.* 2013). While this conclusion has also been supported by an apparent lack of a *Tribolium bw* ortholog, *bw* orthologs have been identified in other species that also lack pteridine eye pigments (Tatematsu *et al.* 2011; Wang *et al.* 2013). Furthermore, *Bw* is known in *Drosophila* to play a role in other tissues (Campbell and Nash 2001; Borycz *et al.* 2008), so it would not be surprising for the *Tribolium* lineage to have maintained an ortholog of this gene. To distinguish a *bw* ortholog from other ABC transporters in the *Tribolium* genome, a motif unique to pigment transporters was required. Comparison of *Drosophila* and *Tribolium* ABC transporters suggested that the presence of a cysteine (C) in the Walker B domain (CDEPT)

>*Tcw* promoter region

tcaatctttatcagttatctgaacaattacttttcggttaaaatctgcttaaaactagcgaaggtgtgaccgttttgtagatatggcctgtattttgtag
tggaaacaaaaagattatcagtttttctaaccgccttacagctccaatatcgaataaaatcttttagaggggttgaaacgcttcaaccgtaaaataagagggc
agtggggaagacatatgatgatCAATGACTACAATGTATCAATTTACAAGGTATTTTCAATTCAGACAGCGTTCGACAGTTAGCCTAAGTAACAAT
CATTGTTTCCAGATTATTTAAATACATAGTACAAGAGATAAATTTTCATGTGTACTACTTTTAAATTTGTGTTATTGTATTAGTGTCAGTGTAAAAATG

>*Tcst* promoter region

cacttgtagtatttgcttttttaggcccatttcgtaattggtatgtggttatgttgccggtccttatcgtgggatttcgactggtggtcttttctggcat
tgtagttaagacttgccgtttcaaatagttgagttgacttttgtaattaataaaattgtgcgagaattcaatctggtgttattaacgcttttgataaat
ttagatcgatcgctgaaaaaaataccaaggggtggggaaccggtgtaaaattacctagggctcataccgacttaataattcagaagcACTGTACAAATA
ACAGTCAAGTGTTTCGGTGTTTGCTTCTAGTAAATATTTGGATACTTTTAAACAATTACTAAATGCTAGGAGCGATAGTGATTTAGTGGTTTGGGATG

>*Tcbw* promoter region

taaaatctatactgttggtttggaagaataaataattacaattacttaataataaaattagaataaaagttaaaaaaccacaaatatttttcatt
tatttccagatcctgttcttaataaccttaatttttatattgttgattaccttttacagcgataggaaacaaacagtgataatctgatggtggacttatc
gtagttttgtcaaggtGTTTTATAGTTTATCCAAAATAGAGAATAGAAAAATAGTAGATATACATAGAAGTTGACCCTGTTTCAACATTTACTTACAC
TAAATTTTAAATATTTAATTAAGCATTTCGGATAATGTGCGCATAAGTTAATTCGTCTGATAAGGTAATAGACGTGTGAACCACTTAGCGAGGAATAATG

Figure 1 Nucleotide sequence of the putative *Tcw*, *Tcst*, and *Tcbw* promoter regions. Capital letters indicate the longest transcript detected (first capital base = +1), with the premature ATGs in boxes, while the boxed and boldfaced ATGs indicate the start of the proper coding sequence. Proposed initiator sequences are underlined, clusters of downstream elements are denoted by dashed underlines, and predicted promoter sequences are double-underlined. Boldface type denotes a consensus DPE in *Tcbw* and a putative DPE in *Tcw*. Overlapping features are italicized.

was diagnostic for eye pigment transporters (see below). This prompted the use of an extended Walker B motif as query for searching the *Tribolium* genome (TBlastN).

Blast analysis indicated the presence of three such genes: two on linkage group 9 (GenBank contig no. AAJJ01001213.1) and a third on the X chromosome (GenBank contig no. AAJJ01000541.1). Since the first linkage group contains *Tcw* and *Tcst*, we focused on the ABC transporter identified in the X chromosome. The CDS was computationally determined using a combination of BlastX (Altschul *et al.* 1997) and FGENESH (Salamov and Solovyev 2000). This sequence was then used in queries of our *Tribolium* RNA-Seq data. Matches were found initially in sequence data obtained from GA2 ovaries and subsequently in data from unfertilized eggs. These matches were then confirmed by cloning and sequencing cDNA from these stages (Figure S1). Interestingly, the deduced amino-acid sequence of the full-length CDS shows nearly the same degree of sequence identity with *Drosophila* White, Scarlet, and Brown (28, 32, and 32%, respectively). However, based on strong support from phylogenetic analysis (see below) we are confident that this gene does indeed represent the *brown* ortholog. Therefore, we name this gene *Tribolium castaneum brown* (*Tcbw*).

The longest *Tcbw* 5' UTR identified is 180 bp in length. The CDS consists of 1746 bp, spread out over 12 exons (Figure S2B), encoding a protein 581 amino acids in length, with a very brief 3' UTR of ~29 bp. We also analyzed the region upstream of this gene's 5' UTR for possible promoter elements. Promoter prediction software failed to detect a promoter within the region -600 to +180; however, *Tcbw* possesses a consensus DPE (Figure 1). Therefore, like *Tcw* and *Tcst*, *Tcbw* is likely driven by a TATA-less promoter. *Tcbw* also has a small cluster of downstream elements, but rather than being within the 5' UTR, these elements are in the interval -50 to -40. This may indicate that our longest transcript is truncated and thus lacking the full 5' UTR.

RNA interference

To confirm the role of *Tcw* and *Tcst* in eye pigmentation, and to ensure that our RNAi-mediated gene knockdowns were

working, we performed RNAi by injecting late-stage larvae with each of the respective dsRNAs. In *Drosophila*, *W* is essential to both pigment pathways (Sullivan and Sullivan 1975; Sullivan *et al.* 1979; Summers *et al.* 1982), so it was not surprising to see that all individuals injected with *Tcw* dsRNA as larvae lacked eye pigmentation as pupae and adults (Figure 2B). Moreover, our results for *Tcst* were also similar to that previously reported (Broehan *et al.* 2013). Specifically, of the 44 larvae injected with *Tcst* dsRNA, all but one had white eyes after eclosion (Figure 2C), and they continued to lack eye pigmentation for at least another 2 weeks. Since, in *Drosophila*, *St* functions to import molecules of the ommochrome pathway, this result further indicates that *Tribolium* eyes are colored only by ommochromes. Last-instar larvae injected with buffer alone had normal eye pigmentation throughout development, indicating that the loss of pigmentation was due to the addition of gene-specific dsRNA in both cases.

Beetles with compromised *Tcst* or *Tcv* activity, but with presumably wild-type function of *Tcbw*, lack all discernible eye pigmentation (Lorenzen *et al.* 2002a), suggesting that pteridines are not used for this purpose in *Tribolium*. To verify that *Tcbw* has no role in eye pigmentation, we injected larvae with *Tcbw* dsRNA. Injected individuals were examined for pigmentation of the eyes (Figure 2D) as well as other tissues known in *Drosophila* to rely on *Bw* for proper pigmentation (data not shown). No abnormal effects were seen among 34 injected individuals. In *Tribolium*, the ocular diaphragm, which appears as a ring of black pigment surrounding each eye, is unaffected by reduced expression or function of *Tcw* or *Tcst*, and one explanation for this phenomenon is that pteridines or other non-ommochrome pigments may contribute to part or all of the black pigmentation of this structure. However, *Tcbw* RNAi also failed to affect the ocular diaphragm. These observations further support the conclusion that *Tcbw* has become dispensable for eye color in the *Tribolium* lineage and that pteridines are not used as visible eye pigments in this insect.

Analysis of mutant lines

Since RNAi of both *Tcw* and *Tcst* resulted in white eyes in pupae and adults, we examined known eye-color mutants

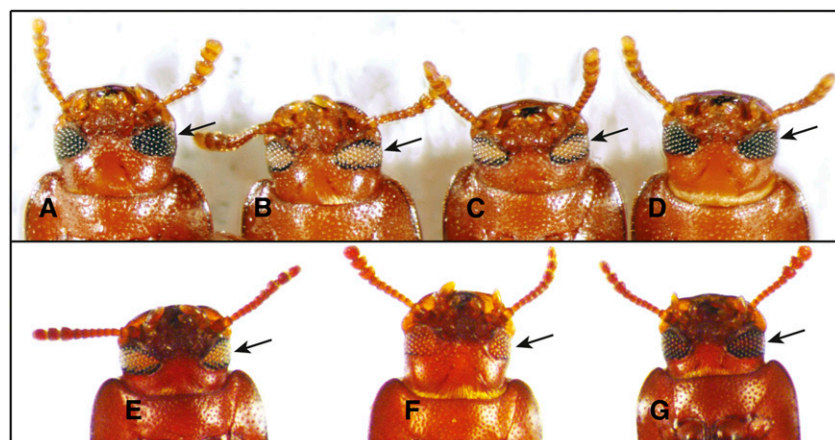


Figure 2 Effect of reduced *Tcw*, *Tcst*, and *Tcbw* activity on eye pigmentation by RNAi (B–D) or mutation (E–G). Dorsal views of similarly aged adult GA-1 beetles that were (A) uninjected to show wild-type eye pigmentation, (B) injected with *Tcw*-specific dsRNA, (C) injected with *Tcst*-specific dsRNA, and (D) injected with *Tcbw*-specific dsRNA. Also shown are similarly aged adult beetles of (E) the *Tcst* mutant, *pearl*; (F) the *Tcw* mutant, *ivory*; and (G) the *Tcw* mutant, *red²²*. Note that *Tcbw*-specific dsRNA has no effect on eye pigmentation (D), while the *Tcw* mutations are not total loss-of-function mutants since both mutants still possess some eye pigment.

to determine if any represented defects in these two genes. Since the white-eyed phenotype seen in *p* beetles had long been thought to be due to a mutation in *Tcw*, we used SSCP to measure recombination between *Tcw* and the *p* mutation (Figure 2F). A dimorphism within a 176-bp region spanning the fifth intron was found between a wild-type strain (T-1) and *p* mutants, and segregation of this marker was followed in backcross progeny. DNA was isolated individually from 100 white-eyed and 100 black-eyed backcross progeny. Of the 180 DNAs yielding PCR products (94 white-eyed and 86 black-eyed), no recombinants were found, suggesting that *p* bears a lesion in *Tcw*. However, *Tcw* cDNA sequence obtained from homozygous *p* mutants exhibited no obvious mutation. Since *Tcw* and *Tcst* are colocalized in the genome, *Tcst* genomic DNA was also analyzed from homozygous *p* mutants and examined for defects, revealing an 85-bp deletion starting in the 11th coding exon (Figure S1) and extending into the adjacent intron (Figure S3), which results in the incorporation of a premature stop codon and truncation of the protein (~25% loss). Therefore, we renamed the *p* mutant *st^p*, an allele of *Tcst*.

Interestingly *Tribolium* has a second, non-allelic eye-color mutation, *ivory* (*i*) (Figure 2E) (Bartlett 1962; Bartlett and Bell 1966), which is tightly linked to *st^p* (Dewees and Bell 1967). We confirmed linkage and found that *st^p* and *i* show very little crossover (0.67 and 1.73%, respectively) with a nearby marker, *Short elytra* (*Se*). Therefore, we examined homozygous *i* beetles to determine if *i* might harbor a lesion in *Tcw*. Sequence analysis revealed a single-base transversion (G → C, Figure S1), resulting in a conservative amino-acid substitution (E → D). This substitution occurs in a region of the Walker B motif (DEPT) that is nearly invariant in ABC proteins known to be associated with pigment transport in arthropods (see below). This motif is important in ATP hydrolysis (Walker *et al.* 1982), which is necessary to energize transport. So this mutation might reduce the efficiency of this process, resulting in severe reduction, but not complete loss, of eye pigmentation as is seen in *i* mutants. We conclude that the *i* phenotype is in all probability a result of this mutation and rename it *wⁱ*.

We have examined some other eye-color mutations for association with *Tcw*, *Tcst*, and *Tcbw*. The recessive *wⁱ* and *red^{Z2}* (renamed *w^{rZ2}*; Figure 2G) mutations fail to complement, and their expressivity is additive. Specifically, the eye color of mature *wⁱ/w^{rZ2}* beetles is lighter than that observed in *w^{rZ2}* homozygotes, but darker than those of *wⁱ* homozygotes (data not shown). Although we searched the complete CDS as well as the region immediately upstream, we were unable to detect a lesion in *w^{rZ2}*. This suggests that the lesion responsible for the hypomorphic *w^{rZ2}* phenotype may involve a regulatory region. Indeed, a number of nucleotide changes are found between the *w^{rZ2}* and GA2 sequences within the upstream region (Figure S4). While it is difficult to conclusively determine if any of these changes are causative, one or some combination of these changes may sufficiently reduce *Tcw* expression to prevent normal pigment accumulation.

There are at least two genes known to be associated with eye pigmentation on the X chromosome, *red-1* and *platinum* (Lorenzen *et al.* 2002a). Interestingly, besides *Tcbw*, the X appears to have only two other orthologs to *Drosophila* eye-color genes (Table 1): *cardinal*, an enzyme of the ommochrome pathway (Ferre *et al.* 1986; Tearle 1991), and *ruby*, which encodes a lysosomal protein important in the formation of pigment granules (Mullins *et al.* 2000), in which the final pigments of both pathways are created and stored (Ferre *et al.* 1986; Lloyd *et al.* 1998). Because of *ruby*'s role in formation of lysosome-related organelles, it seems likely that this gene, and its role in pigment granule formation, would be conserved in *Tribolium*. Similarly, the conservation of the ommochrome pathway in *Tribolium* eye pigment would support the conservation of enzymes important to that pathway, such as *cardinal*. Thus, we conclude that the *Tribolium* X chromosome eye-color mutants are most likely mutations in the *Tribolium* orthologs of *ruby* and *cardinal*, rather than in *Tcbw*.

Phylogenetic analysis of ABC-transporter eye-color homologs

The poor match of the hypothetical TcBw sequence to its counterpart in *Drosophila*, as well as the other *Drosophila* ABC eye-color transporters, raised questions of its evolutionary relationship within this subfamily of proteins. To resolve some of these questions, we searched the genomes of four well-sequenced and extensively studied insect species (*A. aegypti*, *A. gambia*, *A. mellifera*, and *B. mori*) using TblastN to identify proteins homologous to DmW. Surprisingly, each of these four species was found to possess four W-like ABC transporters, unlike the three known in *Drosophila*. However, altogether, these transporters fell into three distinct groups based on their relationships to the three *Drosophila* transporters (Figure 3). Each species analyzed had only a single W ortholog, and this White-related group appears to be the most tightly conserved, probably due to its critical role in multiple processes and its highly conserved role in eye-pigment transport.

TcBw clearly falls into the Brown group, but, in general, the Brown orthologs appear to be the least conserved group of this transporter family. Even among the closely related dipteran species, Brown group members possess long branches, suggesting a higher degree of divergence, likely due to the ambiguity in use of pteridine pigments. In *Bombyx* and *Apis*, our analyses suggested that the fourth White homolog belonged to the Brown group. Furthermore, our annotations showed that, in each of these species, this fourth gene was located next to the *brown* ortholog in a tail-to-tail formation, suggesting a common origin. A characterization of the *Bombyx* gene has since been reported (Wang *et al.* 2013), and it was given the name *Bmok*. This same study also identified the extra *Apis* transporter as an *ok* ortholog (*Amok*) and noted the tandem arrangement in both species. However, our own examinations of the *Tcbw* and *Dmbw* genomic regions, as well as Blast searches of whole genomes, failed to identify any candidates for a second *bw*-like gene in these species, so

Table 1 *Tribolium* orthologs of *Drosophila* eye-color genes

<i>Drosophila</i> gene	FlyBase no.	Associated pigment pathway	Location of <i>Tribolium</i> ortholog	Associated <i>Tribolium</i> gene model
<i>cardinal</i>	FBgn0263986	Ommochrome	LG1 (X)	GLEAN_04579
<i>carmine</i>	FBgn0000330	Both	LG10	GLEAN_11059
<i>carnation</i>	FBgn0000257	Both	LG8	GLEAN_06102
<i>cinnabar</i>	FBgn0000337	Ommochrome	LG2	GLEAN_00876
<i>cinnamon</i>	FBgn0000316	Pteridine	LG4	GLEAN_08136
<i>claret</i>	FBgn0000247	Both	unk	No model ^a
<i>clot</i>	FBgn0000318	Pteridine	unk	No model ^a
<i>deep orange</i>	FBgn0000482	Both	LG8	GLEAN_06604
<i>garnet</i>	FBgn0001087	Both	LG6	XM_966877.2
<i>Henna</i>	FBgn0001208	Pteridine	LG3	GLEAN_00087
<i>karmoisin</i>	FBgn0001296	Ommochrome	LG2	GLEAN_01228
<i>light</i>	FBgn0002566	Both	LG6	GLEAN_15204
<i>lightoid</i>	FBgn0002567	Both	LG5	GLEAN_14165
<i>maroon-like</i>	FBgn0002641	Pteridine	LG2	GLEAN_00626
<i>orange</i>	FBgn0003008	Both	LG7	GLEAN_08912
<i>pink</i>	FBgn00086679	Both	LG4	GLEAN_07512
<i>prune</i>	FBgn0003116	Pteridine	LG3	GLEAN_02956
<i>Punch</i>	FBgn0003162	Pteridine	unk	GLEAN_10564
<i>purple</i>	FBgn0003141	Pteridine	LG6	GLEAN_15575
<i>raspberry</i>	FBgn0003204	Pteridine	unk	GLEAN_05099
<i>rosy</i>	FBgn0003308	Pteridine	LG9	GLEAN_12131
<i>ruby</i>	FBgn0003210	Both	LG1 (X)	GLEAN_13609
<i>sepia</i>	FBgn00086348	Pteridine	LG3	GLEAN_03873
<i>vermillion</i>	FBgn0003965	Ommochrome	LG4	GLEAN_08028

^a Although evidence suggests that orthologs of these genes are present in the *Tribolium* genome, there are currently no gene models that match potentially orthologous sequence.

it is likely that the extra gene has been lost in the *Tribolium* and *Drosophila* lineages.

The Scarlet group seems to be fairly conserved compared to the members of the Brown group, perhaps owing to the ubiquitous use of ommochromes as eye pigments. Interestingly, the “extra” White homologs of *Anopheles* and *Aedes* fall into the Scarlet group (a result also seen in Wang *et al.* 2013), and we call them “Scarlet-like” to distinguish them from the Scarlet orthologs in this group.

In identifying *Tcbw*, it was necessary to specify it from other families of ABC genes. The CDEPT motif in the Walker B functional domain is nearly invariant in all eye-color transporters examined (Figure 4). Only *Apis* Scarlet and Brown possess changes to this motif, with a leucine replacing the cysteine (see Figure S5). Another motif helpful in identifying eye-color transporters from other ABCs is the IHQP motif (Figure 4), which is a part of the ATP-hydrolyzing H-loop domain (Mackenzie *et al.* 1999; Zhou *et al.* 2013). In all White and Scarlet group members, as well as in most of the Brown group, this motif is separated from the CDEPT motif by exactly 28 amino acids. This distance is altered in the Brown orthologs of mosquitos by the addition of a single amino acid, of *Apis* by a single loss, and of *Drosophila* by a 66-amino-acid insertion (see also Dreesen *et al.* 1988). The IHQP motif itself is most variable at the I position; the Q is replaced only in the two mosquito Browns, while the H and P are invariable (Figure S5). The H, after which the domain is named, is conserved even beyond the eye-color transporters (Mackenzie *et al.* 1999) and is necessary to facilitate the proton-transfer step

of hydrolysis (Zhou *et al.* 2013). One final feature of interest is a six-amino-acid deletion, specifically in all members of the Brown group, which lies 25 positions toward the amino terminus from the CDEPT (positions 305–311, Figure 4 and Figure S5). While it is likely that many of these specific features (and any alterations) have functional significance beyond the broad categorizations, what those purposes may be remains to be determined. However, these features should be helpful in identifying ABC eye-color transporters in other species.

Discussion

In this study, we have identified the gene structure and promoter regions of the *Tribolium* orthologs to the *Drosophila* eye-color ABC transporters, *white*, *scarlet*, and *brown*. We have also identified mutants of *Tcw* and *Tcst* and, using RNAi, have shown that *Tcbw* does not contribute to eye color in this beetle. Finally, we have compared several eye-color ABC transporters from different species to identify domains that should prove helpful in identifying orthologs in other insects.

Given the close proximity of *Tcw* and *Tcst*, both physically and in joint function, it seems likely that these two genes might share a common promoter (Deweese and Bell 1967). However, our RNA-Seq data suggest otherwise. Specifically, while we were able to identify *Tcw* and *Tcbw* sequence reads from ovary- and egg-derived sequence data, we were not able to find *Tcst* reads (data not shown). Furthermore, efforts to clone a single, operon-like transcript (Deweese and Bell 1967) containing both *Tcw* and *Tcst* proved to be unsuccessful. Thus,

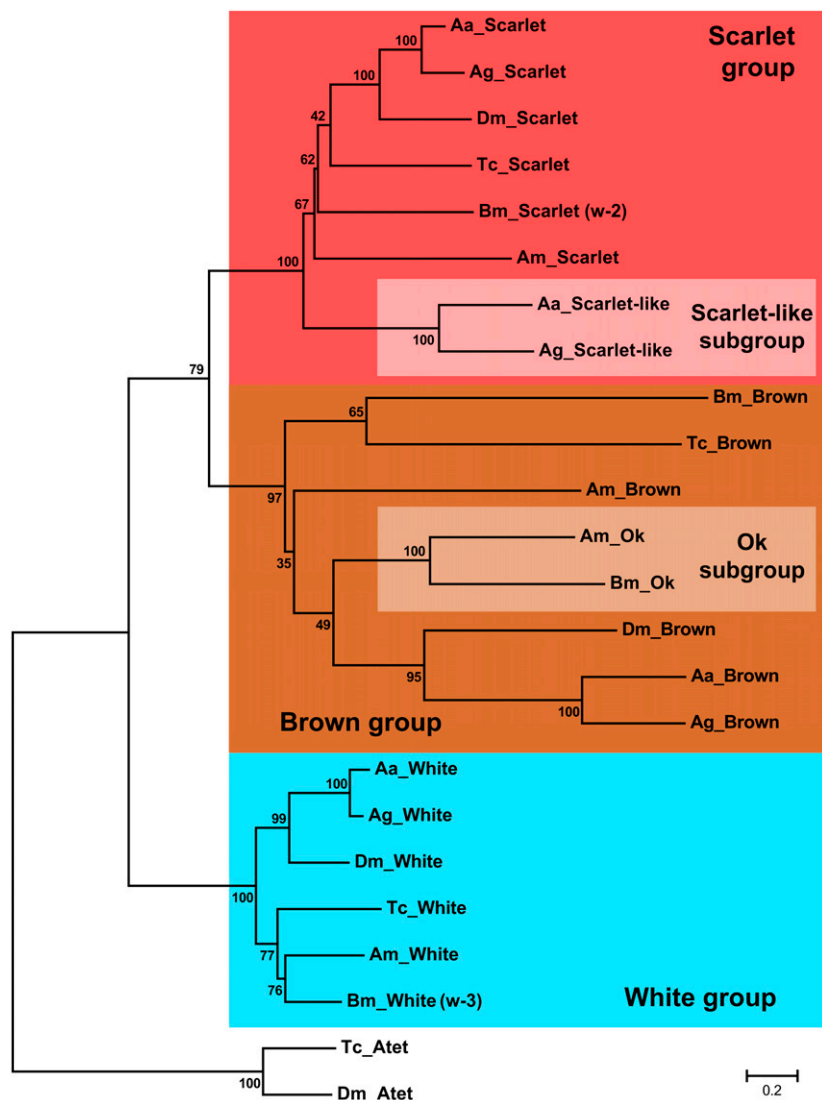


Figure 3 Phylogeny of ABC eye-color transporter orthologs showing relationships between the different orthology groups. White orthologs are in the blue box; Brown orthologs are in the brown box, with the Ok subgroup highlighted in light brown; Scarlet orthologs are in the red box, with the Scarlet-like subgroup highlighted in pink. Bootstrap consensus values are given for each node. Sequences of *Drosophila* (NP_001097079.1) and *Tribolium* (XP_973458.1) Atet proteins were used as an outgroup to root the tree. Aa: *A. aegypti*, White AAEL016999-PA, Scarlet AAEL017106-PA, Scarlet-like XP_001657117.1, Brown modified from AAEL017188-PA; Ag: *A. gambia*, White AGAP000553-PA, Scarlet XP_310585.4, Scarlet-like XP_321812.4, Brown XP_308215.4; Am: *A. melifera*, White modified from XP_001122252.2, Scarlet XP_001122240.1, Brown XP_395665.4, Ok XP_006559105.1; Bm: *B. mori*, White NP_001037034.1, Scarlet NP_001243922.1, Brown XP_004932454.1, Ok XP_004932395.1; Dm: *D. melanogaster*, White NC_004354.3, Scarlet NT_037436.3, Brown NT_033778.3; Tc: *T. castaneum*, White AF422804.1, Scarlet KP120763, Brown KP120764.

we conclude that *Tcw* and *Tcst* expression must be primarily managed independently. One intriguing possibility, given the placement of potential *Tcst* promoter elements within the *Tcw* 3' UTR, is that activation of *Tcst* might interfere with *Tcw* expression and vice versa. This might provide a method of fine-tuning control of these two gene products, so that they are present in low, but sufficient, quantities. The presence of multiple potential translation start sites in the 5' UTRs of each of these genes (Figure 1) also suggests a method of more precisely controlling expression, if translation is initiated as frequently at one of these false starts as it is at the actual start site. Since each of these false start sites is followed fairly quickly by an in-frame stop codon, it is unlikely that they initiate production of usable proteins. Therefore, the likeliest explanation seems to be limiting actual protein production through regular mistranslation.

The RNA-Seq data also raise questions about what function *Tcw* and *Tcbw* are serving in ovaries and embryos. The fact that both of these genes are being expressed suggests that this is not random noise, but that they may be working together for

some important role in early egg or embryo development. We can only speculate about that role, perhaps related to waste or nutrient processing during oogenesis or embryogenesis, based on evidence that these ABC transporters function more broadly than simply in eye-color development through their roles in importing guanine and certain derivatives.

For example, several studies in *Drosophila* have implicated W, and to some extent St and Bw, in normal neurological functions. Expression of *w* has been found in eyeless adult heads, while loss of W function has been associated with altered responses to anesthetics (Campbell and Nash 2001) and other stimuli, diminished learning ability (Diegelmann *et al.* 2006), and increased sexual arousal coupled with decreased mate preference (Anaka *et al.* 2008; Krstic *et al.* 2013). *st* mutants have also been shown to have altered learning capabilities (Diegelmann *et al.* 2006), while both *st* and *bw* mutants show altered anesthetic responses (Campbell and Nash 2001). The most likely explanation for these phenomena is that biogenic amines are altered in amount and subcellular location in all three mutant types (Borycz *et al.* 2008),

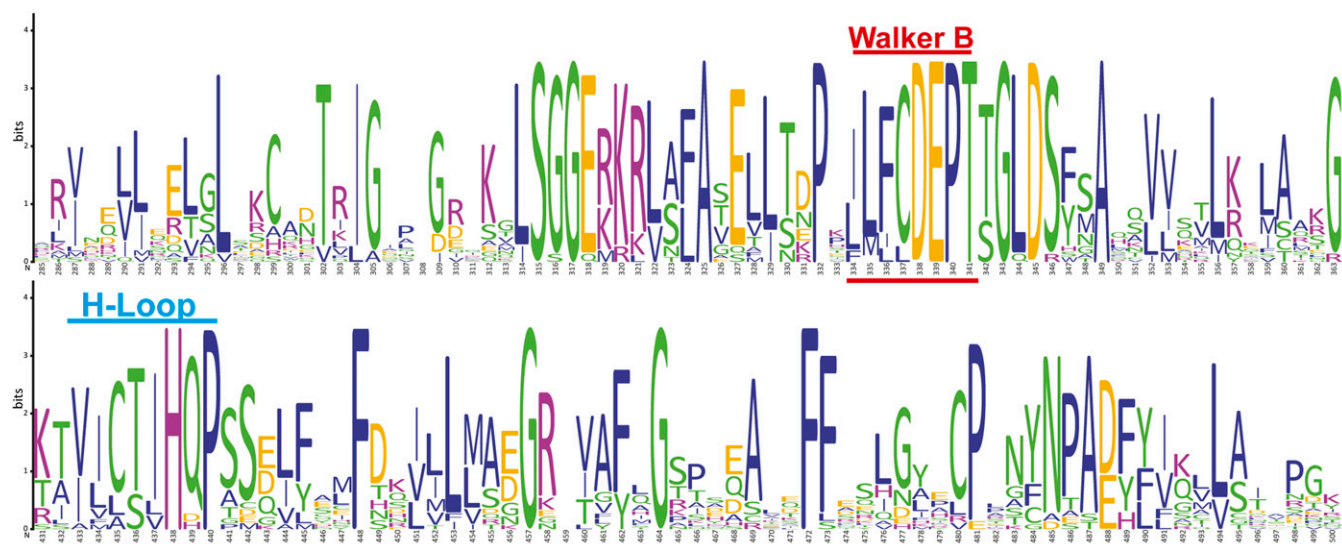


Figure 4 Sequence logo of ABC eye-color transporter homolog alignment. Two important features are highlighted: the CDEPT motif of the Walker B domain (underlined in red) and the IHQP motif of the H-loop (underlined in cyan), both of which are highly conserved in the eye-color transporters. Acidic amino acids are shown in gold, basic in magenta, nonpolar in blue, and polar in green.

probably as a result of altered cellular import of the tryptophan- and guanine-derived precursors of these amines. Thus, the neurological functions of W, St, and Bw are not unlike their pigmentation roles. Given the general conservation of these amines, it also seems likely that these neurological roles are evolutionarily conserved, although it remains to be determined if the orthologs of these genes actually possess such functions in non-drosophilid insects. This question is particularly important to consider with respect to *bw* orthologs. It may be that the lack of pteridine eye pigments in most of the species examined has slackened the evolutionary constraints on members of the Brown group, permitting a greater degree of divergence. However, a conserved function outside of pigment transport would call this conclusion into question. Indeed, such conserved roles could explain why *bw* orthologs have persisted, and even expanded, even though the use of pteridines as eye pigments is not conserved.

It is also interesting to note an expansion of *st* orthologs; the extra White homologs in *Anopheles* and *Aedes* are clearly related to Scarlet and appear to form a distinct subgroup. Their relatedness to Scarlet might explain certain discrepancies in the literature. Tatematsu *et al.* (2011), for example, report a single *st* ortholog in *Anopheles* on chromosome 2R, but evidence from others suggested that both *Agw* and *Agst* were on the X chromosome in a tandem tail-to-head arrangement like that seen in *Tribolium*, *Bombyx*, and *Apis* (Benedict *et al.* 1996a; Zheng *et al.* 1996; Tatematsu *et al.* 2011). A second *st* ortholog explains how *Agst* could have been identified on separate chromosomes, while suggesting that the tandem arrangement is likely ancestral, perhaps resulting from the original duplication that created the *w* and *st* genes (Tatematsu *et al.* 2011).

So far, no function has been identified for *AgSt*-like, but it is possible that it might serve a similar purpose to the role of

BmOk. While not necessary for eye color, BmOk is necessary for the proper importation of uric acid into the larval integument (Wang *et al.* 2013), a role that is also dependent on the proper function of the *Bombyx w* ortholog, *Bmw3* (Abraham *et al.* 2000; Komoto *et al.* 2009; Tatematsu *et al.* 2011). Interestingly, *Anopheles* mosquito larvae also use uric acid to pigment their integuments, a function dependent on mosquito W orthologs, while mutations in a locus called *collarless* prevent proper importation of uric acid without affecting eye color (Benedict *et al.* 1996a,b, 2003). Benedict *et al.* (1996b) determined that the enzymatic activity for converting precursors like guanine and xanthine into uric acid appear to remain intact in *collarless* mutants, suggesting that a malfunction in a transport protein is likely responsible for the phenotype. Indeed, the role of W orthologs in this phenotype is not surprising, given that this protein, along with Bw, is necessary for the transport of uric acid precursors in *Drosophila* (Sullivan *et al.* 1979). Therefore, it has been hypothesized that *collarless* encodes a Bw-like transporter (Benedict *et al.* 1996b). While this hypothesis has been borne out in *Bombyx*, with the discovery that the Bw-related OK is necessary for proper importation of uric acid in larval integuments (Wang *et al.* 2013), it seems unlikely that the same will be proven true for *Anopheles*, since *Agbw* appears to be located on chromosome 2L (Wang *et al.* 2013) while the *collarless* locus is on 2R (Zheng *et al.* 1996) and *Anopheles* appears to lack an *ok* ortholog. However, *Agst*-like is located on 2R (Tatematsu *et al.* 2011; Wang *et al.* 2013), making it a likely candidate for the *collarless* locus. It would be interesting to determine if this is true, since it would mean that an ommochrome-precursor transporter has been modified to transport pteridine and uric acid precursors. It is worth noting that certain allelic combinations of *collarless* exhibit an occasional dorsal red stripe that appears to be the result of ommochrome misallocation (Benedict *et al.* 2003),

perhaps hinting at an ommochrome-transporting ancestry. What is less certain is the role of the fourth W homologs in *Apis* and *Aedes*. *Aedes* species do not have the white body pigment seen in *Anopheles*. They do store high concentrations of uric acid in vacuoles of the fat body (Benedict *et al.* 1996b), but whether AaSt-like contributes to this is unknown. And although the *Apis* homolog is clearly an ortholog of *Bmok*, uric acid storage and larval body color do not appear to have been studied in the honeybee.

The study of eye color has been helpful in identifying useful markers for genetic transformation, but is also well positioned to advance our knowledge in other important areas, from the evolution of development to neurological origins of behavioral traits. Identifying the eye-color ABC transporters in other species will be helpful in determining how evolution has shaped the use of various eye pigments. The additional role of these transporters in *Drosophila* neurobiology seems particularly important, and determining if these functions are conserved beyond this species would be useful in the evolutionary studies of these genes. Such studies, in turn, would contribute to a better understanding of the molecular controls of a variety of complex behaviors, such as mate selection and courtship, as well as responses to physical and chemical stimuli, which themselves could contribute to better pest control strategies.

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The ABCs of Eye Color in *Tribolium castaneum*: Orthologs of the *Drosophila white*, *scarlet*, and *brown* Genes

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>Tcw cDNA
 CAATTGACAGCGCTGCAGCTAGCTAGCTAAGTAAACATCATTGTTTCCGAGATTATTTAAATACATAGTACAAGAGATAAAATTTTCATGTGTACTACTTTT
 TAAATTTTGTGTTATTGTTATTAGTGTGCTAGTGTAAATATGGAATAATGAACTGAACCACTTTTATCCGGAGTTGATACAAATAAACGGGAATAGTGGCG
 ATAGCACAAAGTTCGGCCACTTCTATCGATTTATCAACGTTTCAGAGTACCAACATATGGGACAACCTCTACCCGACCAGCAAATTTGGTACCACCAGATGA
 GCGAATTACCTACAGCTGGACGGAAATTAACGCTTTCGCCAACGTTTACCCCCAAAACAAAATTTTCAATTTGATAAAACGGAAAGATCTCCAGTT
 CAGAAAAAGCACATTTTGAACACGTGTTTGGGGTGGCTTATCCGGGGAGCTTTTGGCGATACTGGCTCCAGCGGTGCCGGAACCAACCCCTCTCA
 ACACATAACCTTCCACAGCTTTCGAATTTGACCGTTTCCGGACTCCGGTGCCTAAACGGAATTCGGGTGTCGTCGAAACCTTGGCCAGTCAATCCGGC
 TTATGTCCCAACAGACGCTTATTATTCGGGACTTTGACGGTTAAGGAGCACTTAATTTTCAAGCTTTGCTACGGATGGACCGTGACATCTCCTACAGC
 CAGAGAATGGCCGAGTCGAGGAAGTTATTTCCGATTGGCTTGTCAAAGTGTCAAACACACCCGATTGGGATTTTGGGGCGGATTAAGGGGATTTTCAG
 GTGGTGAACGAAACGCTCTCTTTTTCAGCCGAAAGTCTTGACAAACCCGAAACATAATGTTTTCGATGAGCCCACTTCTGGACTCGACTCTTTCATGGC
 GCTGACCGTCTACGAGTGTCTAAGGAGATGGCAATGACCGGAAAAACCGTCATTGTACGATCCATCAGCCCTCCAGTGAGGTGATTCCATGTTTGAT
 AAGTTGTTGTTGATGCTGAGGGACGAACGGCGTTTTTGGGAAGTCCGGAAGAGGCCGAAACTTTTTCGGGAGCTTGAAGCCCGGTGTCGCGGAATT
 ACAACCCCTGCCGATTATTTTCATCCAATTTTAGCCATAGTCCCCGAAAGGAGGAGTCGAGTCGACAGCAGTTAACTTAATTTGCGCAAAATTCGAACG
 CTCGAATATCGGGGTTAAATTCGCACTCGAGGCTGCCACTACTGAGAGAGAAGGGGTTATCATGATATTTGGATGAGTGGTGAAGTTTAAAGTCCG
 TATAAAGCCTCGTGTGGGCCCAATTAAGGCTGTTTGTGGCGTCCATTTTGGCAGTTTTTAAAGAACCGCTTTTGATTAAAGTTCGTTTACTTCAA
 CTCATTATCATTTTCGCTCGTTATTGGAGCGATTATTTTCGGAACAAGATTGAACGAGATGAGTAATGAACATAAATGGAGTACTTTTCGTTTTCGAC
 AAATATGACATTTCAAACCGTTTTCGCGTTTATTAATGTGTTTGTGGCGAGCTCCCGCTTCTCTCCAGGAGCACCGCAATGGAATGATCCGCTCCAGT
 ATTTATTTTCATCAGTAAACGCTCGCGAATCTCCCATTTTATAATAATCCCGTACTTTAACATCCGTTTGTATTTTCATGATCGGATTAACCTCAC
 ACGGCTTCGATTTTACATCGCTTGTGGCATCATGATCCTGGTGCACACGATAGCGATCAGTTTGGATACCTGATATCCTGTGTCTCCGCTAGCGTTTC
 TATGGCTCTGTCAATCGGACCTCCTTTAGTTATTCGTTTTTGTGTTTGGAGGCTTCTTCTTAACGTTAGCTCGATACCGGATTTATTTCAAATGGTTG
 TCGTTTCTGCTGGTTTCGCTACGGAATGGGCTCTGATGATCAACGATGGGGAATGTGACCAACATTAGTGGCCCAACGCGCATCCGCTCCGCTCG
 CTAAGACGGGCATGTTATTTTGGAACTTTCCATTTTTCGAGGCGCATTTTCGTAATGGATGTGGTTATGTTGGCGCTCTTATCGTGGGATTTTCGACT
 GGTGGCTTTTCTGCGCATTTGTAGTTAAGACTTGGCGTTTCAAATAGTTGAGTTGTAATTTGTAATTAATAAATTTGTCGAG

>Tcst cDNA
 ACTGTACAAATAACAGTCAAGTGTTCGGTGTGTTGCTCTAGTAAATATTTGGATACTTTTAAACAATTACTAAATGTCTAGGAGCGATAGTGTATTAG
 TGGTTTGGGATGCTTCGGACGAAGAAGGTACACAATATCTCCCTAAATACGAGTTGGCGAAATTTTCGGAATAATGAAAGTTCGGAATTTTCGGCG
 AAAGCGACAGTTCCTCGAAAAATCCGGACTTACAGCAATGTTCCCGATGGAGGAAGAGTAACCTCTCGCTGGAACGACGTTTCCGCTCATACCCA
 AACCAAGAAAAACGGCAAAACCATATGAACGAATCATAAACGAGTCAACCGTGCCGTAAGCTGGAAGTTTATGTTGGCTTTGATGGGAGCAAGTGGT
 GCCGGGAAAGTACATAATGTCAACGTTAGCCACAGAATCACCGGTGGCGCTTTTGTGAGGGAGATGTTTAATTAACGAGCGCTTATGGCAACT
 ATATGAAGTATATCAGCGGGTTTATGACCAAGAGGACATTTTATCGGCTCTTTGACTGTCTCAGAACATATGAATATAATGGCAAGGCTCAAAATGGA
 CCGCAAAACGACACAACAGGAGCGAAATTCGAAATTTTGAAGGTTTAGGACTCACTAAGTGTCTCAATACGAGGATTTGGGATCAATGGA
 GAGAGCAAGTCTTGTGAGGAGCGAAAAAAGACGCTCGCTTTCGCAACGAGACTCCTTACGAGCCCCCAATTTATTTCTGCGACGAACCCACGACCG
 GTCTCGACTCATCTACGCCCCAAAAATTTGAACAATGATGAACAAATGGCGTCAAGTGGCAAAACAATCCTTTGCACAATCCATCAACCCCTCATCTGA
 CATTTTTCGCAATGTTTCAGTCAATTAATTTCTGATGGCTGATGTTGCAATTGCGTTTATTCGGAATGGATGAGTTTCCACAGAAATACCCCTCTGTTTAAAGGGAGTACAATTC
 TACAGGTGCCCCACGTCGTACAATCCGGCCGATTTCCTCATCAAAACGCTAGCAACGACGCCCGGTTTGGAGAAATAGCAACAGTGTATAAAGAGAA
 TTTGTGATTATTTTCGAGTCAGTGATTACAACAAAGAGTGAACGTTGTTGTTTCAGTATGAATTTACATGGGAGAGCAGTGGAGAGCAAGATTTACAA
 ACTGAGGACCAATTTAATGAGATGTTTTTGGCAAAATTTGATTGGTTGAGCTATCGATGTTTTTGGATTGTTGGAGGGATCCTACTCTTCAAGCA
 ACGAAATATCGGAGAAATTTGTTATGGAATTTGATCGGTTTGTGTTATTTGGGCACAGATTTTACGACACAGGTTGGGATTCAAATGTTGAAGGAA
 TTATTTTTTTATGTTTTCGAAATAACATTCACACCGATGATTCGATTTTGGATGAGTTTCCACAGAAATACCCCTCTGTTTAAAGGGAGTACAATTC
 AGGGCTGTATTCATCGTTTCTGTATTTCTATCGCGGATTATGGCAATGCTGCCTGGTTTAAATATCGAGCCGATTTTGTTCGTGATTATCGTCTATTGG
 CTTTCGGGCTCCGTCGCCACCACTTATGCTTCTTAATGACAATTTGGCGGGCATTCTTACCTGAAATTCGGCCGCTGCGTGCAGAAATATCTTCTCCA
 ATGCGTTTCGATTCGTCGCCGACGCTATGGCCTATTTGGTGCCTTTTGAATACGTTGCTTATGCTAAACATCGGAGTTTTTGTAAATTAAGCACTCTTCC
 ACGGGTTTTTCTTGACCAATATCTCTCTGTTGATGTTTCAACCGAATCCATTTCTACAGTGAATGGAACGGAATAAAAAATATCAGTCCGAT
 ATTTCCGACCGGAAATCCCTGCTCACTGCAGACCTCAAGTCTTAGAAAAATACAGTTTTCAGAAGACAATTTGTCACGTGATTATGCGAGCATGC
 TGTCTCTGTATCATTTTCTACTGTTTAAAGTTTCATCTGTTTGTGGCTGAAATCAGGAAGAGATAATTTGAATAAAAACTTACCAAAAA

>Tcbw cDNA
 GTTTTATAGTTTTTATCCAAATAGAGAATAGAAAAATAGTAGATACATAGAAGTTGACCTGTTTCAACATTACTTACACTAAAAATTTAAATATTT
 AATTAAGCATTCGGATAATGTGCGCATAGTTAATTCGTCGTGATAAGGTAATAGACGTTGTAACCACTTAGCGAGGAATAATGCTCCCAACAATCTTTTT
 GTCGTGGCACAATATAATGTTAAAGTATCCGAAAAAAGCACAAATTTTGCAAAACCACTAACCGAAAAAATTTTGGAAAGATGTGTGAGGAAGT
 GTCGAGAGTGGTGCCTTAACGTTATCTGGGCAACGTTGATGCGGCAAACTACCTTTTAACTTCAATTTCTGCTGCGCGTAACGAAAAAGTGGTT
 CTTTAAAAATCAATAACAGGACATTTTCGACGAACTGCAAGAAACGATTCGGGATATTGTACAGGAAGATATTTTACCAACTGCTTAACGTTTT
 CGAGCATTCGAGTTTCAAACTGGGCTCCAATGCAGCGACAAAAACGAAAAAACCCGTAATTTTATTATCAACGGCAGCTCTCGGAGCTTAGTCTGGAA
 CGGCATGCTGACACTTTGATAGAAAAATTTGCAAGTGGTGAGAAACGAAGACTATCACTGGCGGGAGAAATTAATAGTAATCCTTCTATACATGTTTGGC
 ATGAACCCACAACAGGACTCGACAGTTACAACGCGTTCTGTTGCTTGAATAATTAACCAATTTGCAACTTTAGGAAAAATGTTTTAGCAACCATTCAT
 TCAGCAATCTTCAAGTTGTTCCACTATTTTCGATAACATACGCTCATGGCTGAGGGGAAAAATAGTGTTCAGGAAGCAACACGAATCTAAATGTT
 TTTGATAATTTGAATTTGCACTGCCCCAAGGCAATCCCGCCGAATTTTATTAATTTGCTCTCACTAAGGAAGATGTTTCTAAAAATGGAATGTTT
 ATAAACGCAAAAAATCGCAACCGCAAAATCATGTACATTTTCGATAATTTATTCCTAAAAAACAACCAAAAAATGTAATTTTACGACCTGAAGTG
 GCTGTTGTTGAGATGTTACTTAAATACGAAGAGAAACAAATCATTAATTTGGGAATTTACTTTTATTCATGATGCAATCCTTATTATTTCGATATTT
 TATTCCGAAGTCACATTTAGTGGCCAAAGATGCCATTCAAAGCATCCAGGGTTTGTATTCCTACTGTGGGACAGAAATTCATTTTACTAACATGTACGCAG
 TTATCTACATTTTCCCGAAGAGGTTGCCATTTTTACGTGAAAAAACTTTGTATTCAACTTTTTCGCTACTTTATCGCAAACTACTGTCTTTGATACC
 ACTTTCAATAGTGACCAATATGATTGCTTAGGAATCTTGTATTGTTTTCAACGTTGTACATGGATTTTGTGTTGTTGGCTCAAAATGACATATGTGGCG
 TTCTTAGTCTCTATAGTTTCTAGTTTAGGTCTAGCATTTTTCAGCAACGTTTTCAACCATTTGAACATGTGGACCTATTTTGGGTCCTAGGAATTTA
 TACTTCTCTTTTTTCCGCTTGTAGTTAAAGTTGATTCCGGTGAAGGGGCATTTAACTGGATCAATATATTTTACCGTTTTATTACGCTTTTGATAG
 TTTGTCAAATCTGTTTTGGAAGAGCTCGGCAAAATAGGTGAATGCACATTTAATCAGACCATCCCTGTTATCACACGTTAGCGAAGTTTTCGAAAGT
 TACGGCATTTCAAAACTTACGACACTGTGGCTTATAACATTTTGTTCCTACATATTTTAGGAGCGGTGTTTTGTTGCTTGGATTTCGCGGAATCGTAA
 GAAAAAGATGAGTCTTTCGCTGTAATGATCTGAATAAAATATAATTTAATGAGA

Figure S1 Nucleotide sequence of the longest *Tcw*, *Tcst* and *Tcbw* cDNAs. Bold letters indicate coding sequence, while boxes denote the start and stop codons. In *Tcw*, the underlined nucleotide marks the location of the single-base transversion (G→C) of the *ivory* mutant. In *Tcst*, the underlined bases are deleted from exon 11 in the *pearl* mutant.

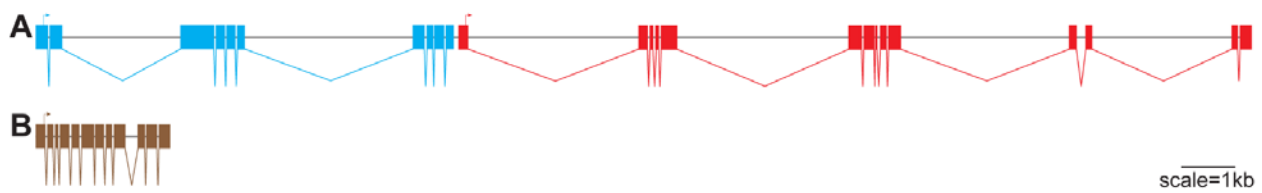


Figure S2 Gene structure of *Tcw*, *Tcst* and *Tcbw*. Exon arrangements are shown for (A) *Tcw* (cyan boxes) and *Tcst* (red boxes), as well as their relative positions on LG9 (black line), and for (B) *Tcbw* (brown boxes) on the X chromosome (black line). Arrows indicate relative position of start codon on first exon, as well as orientation of gene. Scale bar equals 1kb.

```

>Tcst pearl deletion region
gcattataatttgatttcagcacttgtagctcatcacaataactctgtttcagCTGCCTGGTTTAATAATCGAGCCGATTTGTTTCGTGATTATCGTCT
ATTGGCTTTCGGGCCTCCGTGCCACCACTT[ATGCCTTCCTAATGACAACTTTGGCGGGCATTCTTACCTTGAATTCGGCCGCTGCGTGCGgtaagtcgt
tcaagagccgtgattt]CGCACGATTTTTCACGT[TAA]aatttaaatacggcccaattcgggctattattagctctatatttgaattatggatttattccc

```

Figure S3 Genomic context of the *Tcst^{fl}* deletion. The wild-type sequence of the 85-bp deletion is shown between the two brackets. Exon 11 is shown in bold, capital letters while intronic sequence is non-bold, lower case. Intronic sequence that is incorporated into the *Tcst^{fl}* transcript is shown with capital letters, and the premature stop codon is boxed.

```

>Tcw red22 promoter region
aaaaatcggctctcaatttttatcaattatctgaacaattactttgcgggttaaactctgcttaaaactagcgaaggtgtgaccggttggttagatatggcct
gtattttgtagtggaacaaaaagattatcagttttctaacgccttacagctccaatatcgaataaaatcttagagggggttgaaacgcttcaaccgt
aaataagagcgttgcagtgagggaagacatatgatgatCATGACTACAACTATCAATTTACAAGGTATTTACAATTCAGACAGCGTCGACAGTT
AGCCTAAGTAAACAAATCATTTGTTCCAGATTATTTAAATACATAGTACAAGAGATAAATTTTCATGTGTACTACTTTTAAATTTGTGTTATTGTATTAG
TGTTCAGTGTAAAAATGGAAAAATGAACTGAACCACTTTTATCCGGAGTTGTATCACAATAAACGGGAATgtaagaatcatttaccttcgttaatgtttt

```

Figure S4 Sequence of the *Tcw* promoter and 1st exon in the *red²²* mutant. Transversions are marked with blue. A 5bp insertion is shown in red. No deletions were found in this sequence. All other markings are as in Figure 1.

Aa_White	1	MTINTDDHY-A-DNESKSNITS-----RRYS-----TSS-
Ag_White	1	MTINTDDQY-G-DAESKTTISS-----SRRY---SSSSY-
Am_White	1	MTATEETEP-LISSKSSTTCRN-----KSQT-----ILYD
Bm_White	1	MTAGNEEQEPLISTSVDNQRV-----YNN-----PQD-
Dm_White	1	MGQEDQELL-I-RGGSKHPSAE-----HLNN-----GDS-
Tc_White	1	MENETEPLL---SGVVSQINGN-----SGDS-----T---
Aa_Scarlet	1	MSVACISLA-A-SMVKGATSGKRRSGSLNSTAESFECQPVTVA--LP-
Ag_Scarlet	1	MVANGRKRH-S-SINEQQLVPL-----EPPPG--SC-
Am_Scarlet	1	MYVLKEGKW-----
Bm_Scarlet	1	MGKKADTTK-R-SADNSPEKSESP-----QPLQS--TSYELLP-----
Dm_Scarlet	1	MSDSDSKRI-D-VEAPERVEQ-----HELQVMPVG--ST-
Tc_Scarlet	1	MASDEEGTQ-L-SPLNTSWRN-----
Aa_Scarlet-like	1	MDGTTRL-----
Ag_Scarlet-like	1	MSAREAVVS-T-AVCCTV-----
Aa_Brown	1	MVS-----
Ag_Brown	1	MTANKPN-----
Am_Brown	1	ME-----
Bm_Brown	1	MELNKV-----
Dm_Brown	1	MQESGG-----
Tc_Brown	1	MS-----
Am_Ok	1	MKQQMK-----
Bm_Ok	1	MKEYNLKLP-T-RNVL-----
consensus	1	M

Aa_White	28	-----YQDV-DEGINSSFGS-NDKSTLIQVWPKPSYGAV
Ag_White	30	-----QDQS-MDDALNTTLT-NDKATLIQVWPKPSYGSV
Am_White	30	AISLEENERIAVSNKISSF-KLKPI-----HSRIG--ITSKDTLAQH
Bm_White	30	-----GQTP-NDSPRS-----SVGE--VTVAIPQNRN
Dm_White	28	-----GAAS-QSCINQGFQAKNYGTLR--PPSPPEDSG
Tc_White	25	-----SS-ATSIDL-----STFRV--PTYGTTSHPT
Aa_Scarlet	46	-----PPVSCL-SGAPAH-----PVGGS--RCYQSTLRSY
Ag_Scarlet	28	-----LSSPTA-PPPPA-----GSGTG--RCYQSSLRSY
Am_Scarlet	10	-----FRKKHNY
Bm_Scarlet	35	-----ADLVYI-DSGIKT-----SPVKY--DSLYPEVEEV
Dm_Scarlet	31	-----IEVPSL-DSTPKL-----SKRNS--SERSLPLRSY
Tc_Scarlet	20	-----FGTNESSG-----IFGES--DQFTRKIRTY
Aa_Scarlet-like	8	-----SESVKS
Ag_Scarlet-like	17	-----DNL-DGM-----PGVTVAQHE
Aa_Brown	4	-----
Ag_Brown	8	-----
Am_Brown	3	-----
Bm_Brown	7	-----
Dm_Brown	7	-----
Tc_Brown	3	-----
Am_Ok	7	-----YP
Bm_Ok	15	-----TG--AVVNWEDDEM
consensus	51	

Aa_White	60	KGQIPQHERLTYTWKEIDVFGETPGDTKKEP-LCSRLCCCFSRQKKDFNP
Ag_White	62	KGQIPQCERLTYTWKEIDVFGEAPTGDGKPREPLCTRLNCCTRQRKDFNP
Am_White	71	STGNTENESITYTWSDLNVYVAKVNEKPWE-----VF--FKKRKPIG
Bm_White	54	YGAIGGIEKVITYTWADVNAFATESRSRGRRF-W----SFWKNSSDRMFQQ
Dm_White	59	SGSGQLAENLTYAWHNMDIFGAVNQPGSGWRQLVNRTGLFCNERHIPAP
Tc_White	48	SKLVPPDERITYSWTEINAFANVSPPKTKF-----FNL-IKRKDSPVQ
Aa_Scarlet	73	NKWSPTEQGATLVWRDLCVYATGKQVGG-----SGDGGP
Ag_Scarlet	55	SKWSPTEQGATLVWRDLCVYATAGPAKGGGC-----GGGGGPPGCHRPT
Am_Scarlet	17	LVEDNDTEISVTLTWKDLVYAMDRGR-----KNV
Bm_Scarlet	62	FGVPRSPRPCTLVWRDVTVHVKLKN-----GR
Dm_Scarlet	58	SKWSPTEQGATLVWRDLCVYTNVGGs-----GQR
Tc_Scarlet	43	SQWSPMEEGVTLAWNDSVYIQTKKNG-----KTT
Aa_Scarlet-like	14	ELCTSNNDTVTLVWQNLTISSQTR-----NS
Ag_Scarlet-like	32	VAAAAADDDVTLIWQNLTIPIRSKPG-----AGE
Aa_Brown	4	-----
Ag_Brown	8	-DTSTPTGTVLLLEWKNLTVSVRSSSSGQPTSDTG---NHWYGRPGHQQKK
Am_Brown	3	-----DAGDMIWNNLTVTVRQKRDFFTN-----IYNKFQRREYEE
Bm_Brown	7	-LLDEFENKDAIIVRNLIKVWTPEEKSI-----W---RKVAKP
Dm_Brown	7	-SSGQGGPSLCLEWKQLNYVVPDQEQSNYS-----FW---NECRKKR
Tc_Brown	3	-----QQFFLSWHNINVKVSEKKHNF-----CKTTLT
Am_Ok	9	TLPLNVPNDLCLTWKNISYTVERTKNGGSLR-----AIFGFQYTE
Bm_Ok	27	GVMGDSPENLTLAWKDLVFRKKKIHTSMW-----RSAYYE
consensus	101	e ltl wkel vf

Aa_White	109	RKHLLKNVTGMAKSGELLAVM-----GSSGAGKTT
Ag_White	112	RKHLLKNVTGVAKSGELLAVM-----GSSGAGKTT
Am_White	111	RRHLLKDVCGVAYPGELLVIM-----GSSGAGKTT
Bm_White	99	RKQLLRNVNGAAYPGELLAIM-----GSSGAGKTT
Dm_White	109	RKHLLKNVCGVAYPGELLAVM-----GSSGAGKTT
Tc_White	90	KKHILKNVFGVAYPGELLAIL-----GSSGAGKTT
Aa_Scarlet	107	IKRIINNVS GAVTPGT LIALM-----GSSGAGKST
Ag_Scarlet	99	IKRIINNVS GAVTPGT LIALM-----GSSGA AKST
Am_Scarlet	46	RKRLIDNV RGAAEAGNLTAII-----GASGSGKSS
Bm_Scarlet	89	LKRLVNNVSGIAKPGTLIALM-----GPSGAGKTT
Dm_Scarlet	87	MKRIINNSTGAIQPGTLMALM-----GSSGSGKTT
Tc_Scarlet	73	CKRIINGVTGAVKAGSLVALM-----GASGAGKST
Aa_Scarlet-like	40	CSTIVHNVNGSLHSGSLVALM-----GPSGAGKST
Ag_Scarlet-like	62	QHPVLNDISGTLQPGTLVALM-----GPSGAGKTT
Aa_Brown	4	-----LSLSPSTLVRLFRSMRMTRCSPVRYKKNKSPSNLGAGKTT
Ag_Brown	54	ELTLRLNASGAVRSDNLVAIM-----GPSGAGKTT
Am_Brown	39	TLTILKGVSGYAMTGNLVAIM-----GSSGAGKTT
Bm_Brown	40	KTVIIDNVSACIREGEFAAII-----GPSGAGKTT
Dm_Brown	45	ELRILQDASGHMKTGDLIAIL-----GSSGAGKTT
Tc_Brown	30	EKIILEDVSGSVESGALNVIL-----GNSGCGKTT
Am_Ok	49	LIQLLHGVSGIVNSGMLMAIM-----GPSGAGKTT
Bm_Ok	63	EVKVLHGVSGSVSSGNLVALM-----GSSGAGKTT
consensus	151	khllknvsg v g llavm gssGagKtt

Aa_White	139	LLNALSFRRSPPGVKIAPT	SVRA	NGIPVNAEQ	LRARCAYVQ	QDDLFI	PAL
Ag_White	142	LLNALAFRRSPPGVKISP	NAVRA	NGVPVNAEQ	LRARCAYVQ	QDDLFI	PSL
Am_White	141	LLNALTFRRSGCGVI--	ASGVMA	ANGRRVSS	TILT	SRTAYVQ	QDDLFI
Bm_White	129	LLNTLTFRTTPGGVV--	ATGTRAL	NGQPATPD	ALTALSAYVQ	QDDLFI	GT
Dm_White	139	LLNALAFRRSPQGIQVSP	SGMRL	LLNGQPVD	AKEMQAR	CAYVQ	QDDLFI
Tc_White	120	LLNTLTFHTSSNLT--	VSGLR	CVNGIPV	SSKTLASQ	SAYVQ	QDDLFI
Aa_Scarlet	137	LMSALAYRMQPGTI--	VQGDV	LVNGQPIGP	-YMYRL	SGFVH	QDDLFI
Ag_Scarlet	129	LMSALAYRTPPGTV--	VQGDIL	LVNGQPVGP	-YMYRL	SGFVH	QDDLFI
Am_Scarlet	76	LIAALAFRTGSEHL--	IHGDIR	ANGRTID	SSYMMQ	NSGYMH	QEDIF
Bm_Scarlet	119	LMSALAHRRSPFGTV--	IDGEI	IMNGRPVCS	-YVDRE	SGYMH	QDDIFA
Dm_Scarlet	117	LMSTLAFRQPAGTV--	VQGDIL	INGRRIGP	-FMHRI	SGYVY	QDDLFI
Tc_Scarlet	103	LMSTLAHRITGGAF--	VEGDV	LINGRPIGN	-YMKYI	SGFMH	QEDIFI
Aa_Scarlet-like	70	LMGALAHRRSSAGIT--	TSGQI	RLNGKRI	GP-FMYN	VSGLI	YQDELL
Ag_Scarlet-like	92	LMSALAYRMSDKMT--	IAGDIR	VNGCP	IGP-YMYN	ISGYI	YQDELL
Aa_Brown	45	LLAAISMRLVAE---	VHGNV	LINGLYVS	QTQMKRL	SGFVP	QFEIAV
Ag_Brown	84	LLAAISMRTTGSTT--	VHGKV	LINGLYV	TRTQMKQ	LTFGV	PQYELI
Am_Brown	69	FLATLAGRIKS----	TIGSV	TINGQIIS	RTIMSV	MSGYL	PQFDAL
Bm_Brown	70	FLVSLAGKCTLP---	FEGTV	TINGRNV	RDL--TG	AEIVP	QFDVFT
Dm_Brown	75	LLAAISQRLRGN---	LTGDV	VINGMAM	ERHQMT	RIS	SFLPQF
Tc_Brown	60	LLTSISGRRK-----	KSGSL	KINNTT	ISDET	ARNVSG	LYQEDI
Am_Ok	79	LLATISRRVKGK----	ATGDV	LINGKPI	DEQMI	RISGF	VPTDLA
Bm_Ok	93	LLAAISRRDKSA----	LTGYL	MLNGRL	AGADLI	ARI	ISGFVP
consensus	201	ll alafr g vtg mllNg pv m lsgyv	Qddlfi	sl			

Aa_White	189	TTREHLVFFHAMLRMGKD	VPKSVK	-----	MNRVNE	VLQELS	LAKCA
Ag_White	192	TTREHLLFQAMLRMG	RDVPASVK	-----	QHRVQE	VLQELS	LVKCA
Am_White	189	TVKEHLLFQAMVRMDR	KIPMEQR	-----	FDRVHQ	VINEL	ALTCK
Bm_White	177	TVREHLVFFQAMVRMDR	HIPYAQR	-----	MKRVQE	VIQELS	LAKCQ
Dm_White	189	TAREHLIFQAMVRMPR	HLTYRQR	-----	VARVDQ	VIQELS	LAKCQ
Tc_White	168	TVKEHLIFQALLRMDR	DISYSQR	-----	MARVEE	VISDL	LAKCQ
Aa_Scarlet	184	TVTEHMYFMAKLKLD	RTVNKSTI	-----	NRLIEE	LLERT	GLSKCA
Ag_Scarlet	176	TVHEHMYFMAKLRLD	RRVGHRAI	-----	DQTI	RDLLER	VGLARCA
Am_Scarlet	124	TVIEHLWFMAARMKLD	GNLRVLDI	-----	ERKIDN	LLKDV	GLTSRR
Bm_Scarlet	166	TVIEHLTVMARLRMDR	RTSTVAR	-----	KRRVNQ	LMRQL	SLYESR
Dm_Scarlet	164	TVLEHLNFMAHLRLD	RRVSKEER	-----	RLITKE	LLERT	GLLSAA
Tc_Scarlet	150	TVSEHMNIMARLKLD	RKTQQR	-----	NSKIHE	ILKSL	GLTKCL
Aa_Scarlet-like	117	TVGEHMHLMACKL	GPSLSTHRK	-----	HLLIND	LLTQT	NLLQCY
Ag_Scarlet-like	139	TVQEHLLQMANLKL	GKSVTAERK	-----	RAMIAH	ILSRT	GLERCA
Aa_Brown	91	TVREHLVSFVSQ	LKGVQ	-----	NHRMNQ	VIKEL	QLDKCE
Ag_Brown	132	TVAEHLTFVLCLH	THNYLPSS	LR	SIHHEL	KNVGY	VAVLRIV
Am_Brown	114	TVEEHLLFSCALK	TDI--SRVQR	-----	KFLSMK	LLMEL	NLIDCK
Bm_Brown	113	TVMEQLVFMTEMK	LGNSTK-QPN	-----	KSIINT	TVIEE	FKLSAHV
Dm_Brown	121	TAYEHLFYFM	SHFKMHR	RTTKAEK	-----	RQRVAD	LLAVGL
Tc_Brown	105	TVFEHLQFITGL	QCSDK-NEKTR	-----	NFIITK	RQLSEL	SLERHA
Am_Ok	125	TIQEHMEFMACM	KMDRRLANFR	-----	RQRITV	LLREL	GLAKCI
Bm_Ok	139	TVAEHMEFMARLM	MDKRSTKIMR	-----	AKRVEQ	LLGEL	GVMSCT
consensus	251	Tv Ehl fma lrmdrk	v r		rv vl elal	kc	

Aa_White	229	DTIIGAP-GRMKGLSGGERKRLAFASETLTDPHLLLCDEPTSGLDSEMAH
Ag_White	232	DTIIGAP-GRIKGLSGGERKRLAFASETLTDPHLLLCDEPTSGLDSEMAH
Am_White	229	NTVIGQP-GRIKGLSGGEMKRLSFASEVLTDPPLMFCDEPTSGLDSEMAH
Bm_White	217	NTVIGIP-GRLKGLSGGEMKRLSFASEVLTDPPLMFCDEPTSGLDSEMAQ
Dm_White	229	HTIIGVP-GRVKGLSGGERKRLAFASEALTDPPLILCDEPTSGLDSEMAH
Tc_White	208	NTPIGIL-GRIKGLSGGEKKRLSFAAEVLTNPKLMFCDEPTSGLDSEMAH
Aa_Scarlet	224	NTRIGEV-GEGKMLSGGEKKRLAFATELLTKPTILFCDEPTTGLDSFSAQ
Ag_Scarlet	216	GTRIGEA-GDGKMLSGGEKKRLAFATELLTKPTILFCDEPTTGLDSYGAQ
Am_Scarlet	164	DVRIGNS-IDDKVLSGGEKKKLSFATELLTDPKILFLDEPTTGQDSHSAN
Bm_Scarlet	206	FTRIGGL-DGHKTLSGGERKRLAFATELLTDPGLLFCDEPTTGLDSSSAL
Dm_Scarlet	204	QTRIGSG-DDKKVLSGGERKRLAFAVELLNNPVILFCDEPTTGLDSYSAQ
Tc_Scarlet	190	NTRIGIN-GESKVLSGGEKKRLAFATELLTDPPIILFCDEPTTGLDSYSAQ
Aa_Scarlet-like	157	HTQIGQI-GVRKTLSGGERKRLAFAVELISRPKILFCDEPTTGLDSYSAG
Ag_Scarlet-like	179	NTKIADATGIGKTLSGGEKKRLAFAVELLSKPKFLFCDEPTTGLDSYSAR
Aa_Brown	124	DTRI-----SNLSGGERKKVNLAGELLTEPDILFCDEPTTGLDSFSAL
Ag_Brown	182	GTRI-----AQLSGGERKKVNLAGELLTEPEILFCDEPTTGLDSFNAA
Am_Brown	152	DVLI-----SNLSGGQKRKVSASEMISRPKILFLDEPTTGLDRFSAM
Bm_Brown	152	ETRI-----GSLSGGERRLLSLATSFLSNPQILILCDEPTTGLDSYNAS
Dm_Brown	161	HTRI-----QQLSGGERKRLSLAEELITDPIFLFCDEPTTGLDSFSAY
Tc_Brown	144	DTLI-----EKLSSGEKRRLSLAGELISNPISILFCDEPTTGLDSYNAF
Am_Ok	165	STKL-----SALSGGERKRVTLAVELLTEPSILFCDEPTTGLDSYGAM
Bm_Ok	179	KTKL-----KALSGGERKRVALAVQLLNDDPILFCDEPTTGLDSWAAS
consensus	301	trig g k lSgGerklafAselltdP ilfcDEPTtGLDsftA

Aa_White	278	SVLQVLKGMALKG-----
Ag_White	281	SVLQVLKGMAMKG-----
Am_White	278	QVVSVLKTLAARG-----
Bm_White	266	NVIQVLKGLAQKG-----
Dm_White	278	SVVQVLKKLSQKG-----
Tc_White	257	TVMQVLKEMAMTG-----
Aa_Scarlet	273	NLVSTLQLLAKRG-----
Ag_Scarlet	265	ALVSTLQQLARRG-----
Am_Scarlet	213	CVISQLKSFAAKG-----
Bm_Scarlet	255	KIVSLILRASAAQG-----
Dm_Scarlet	253	QLVATLYELAQKG-----
Tc_Scarlet	239	KIVTMMNTMASSG-----
Aa_Scarlet-like	206	QVVHMIRRLTRSG-----
Ag_Scarlet-like	229	QIVEMMKSLTRTG-----
Aa_Brown	167	AVLKTLLRKIALKGR-----
Ag_Brown	225	SVMKTLQCLCANGR-----
Am_Brown	195	QVVNALKIISSE-----
Bm_Brown	195	QVIGILKKLSASG-----
Dm_Brown	204	SVIKTLRHLCTRRRIAKHSLNQVYGEDSFETPSGESSASGSGSKSIEMEV
Tc_Brown	187	VVLEKLKTIATLG-----
Am_Ok	208	TVVRTLREVAASG-----
Bm_Ok	222	AVVSRLRKLAIGG-----
consensus	351	vv vlk la kg

Aa_White	311	ILLVAEGR-VAFLGSPYQASEFFSQLGIPC	PPNYPADFYVQMLAIAPNK
Ag_White	314	ILLVAEGR-VAFLGSPYQSAEFFSQLGIPC	PPNYPADFYVQMLAIAPAK
Am_White	311	ILLMAEGR-VAFMGITSQACTFFETLGAA	CPSNYPADFYVQMLAIVPGQ
Bm_White	299	LLIMADGR-VAFLGSSDEAFQFFKELGAAC	PANYNPADHFIQLLAGVPGR
Dm_White	311	ILLMAEGR-VAFLGTPSEAVDFFSYVGAQ	CPTNYPADFYVQVLAVVPGR
Tc_White	290	LLLMSEGR-TAFLGSPPEEAETFFRELEAP	CPRNYPADFYIQLLAIVPEK
Aa_Scarlet	306	VMLMADGR-VAFAGKPNDALIFFEQHGYS	CPSNYPAEFLIGVLATAPGY
Ag_Scarlet	298	VMLLAEGR-VAYAGRPEALAFFARHGCHAC	PPSYNPAEYLIGALATAPGY
Am_Scarlet	246	IILIAEGR-VAFSGRIDQAVEFFASQGYE	CPRKYNPADFLIAIVATGSKN
Bm_Scarlet	288	LVLLAEGR-IAFAGNASAALGFFESLGYH	CPLTYNPTDYFIKVLALTGPS
Dm_Scarlet	286	VMLLADGR-VAFTGSPQHALSFFANHCYY	CPEAYNPADFLIGVLATDPGY
Tc_Scarlet	272	LILMADGR-IAFIGSAASALDFFQKAGYRC	PTSYPNADFFIKTLATTPGF
Aa_Scarlet-like	239	VLLLSNGR-TAFMGKPHEAIQFFDRLGMIR	PGNCATAEHFIKCLSTCRD-
Ag_Scarlet-like	262	LILLTGGR-TGFIGAPSEAVQFLRLQGLE	CEAGYNTADFLCLKVLSSTTTT
Aa_Brown	201	IVLVRKGE-IYYQGPTTEBARTFFESIHF	PLPINCNPADHYFKLVCDYSQI
Ag_Brown	259	VILMQDGGTVIFYQGPTADRIDFFNSIG	KEVPANGNPADFYFQLVSPGATT
Am_Brown	227	VLLLSDGK-TGYFGSLKDATKFFLSLDYE	CPVGFDESEYYVKLLSRRNPI
Bm_Brown	228	ILLMAEGN-LLFHGTQDACKSFFESIDLH	CPLNYPAEFYIRAVSNHNGV
Dm_Brown	304	IILMDGGR-IVYQGRTEQAAKFFTDLGYE	PLNCPNADFYLKTLADKEGK
Tc_Brown	220	ITLMAEGK-IVFQGSKHESKLLFFDNLN	LHCPKAFNPAEFYINCLTKEDVS
Am_Ok	241	VLLLSGGR-VAFQGSMDATEFFDLSNL	SCPTFNSAEFYVSQLSIIRDK
Bm_Ok	255	IVFLANGR-TAFHGTIAQADQFSGSLNYK	CPLGFNAAEYYVSLGLIQIGK
consensus	451	illvaeGr vaf Gs ea Ff lg cp nypn	padfyi mla pq

Aa_White	360	-----EAECD-----TIKKICDSFAV-SPMAREVMEVANSCKNV
Ag_White	363	-----EAECD-----MIKKICDSFAV-SPIAREVLETASVAGKG
Am_White	360	-----ETSCRH-----AINTVCDFAQK-SEHGKIALEAEAINNE
Bm_White	348	-----EEVTRH-----TIDTVCTAFK-SEIGCRIAAEANALYN
Dm_White	360	-----EIESRD-----RIAKICDNFAI-SKVARDMEQLLATKN--
Tc_White	339	-----EESSRQ-----AVNLICDKFER-SNIGVKIALEAATTE
Aa_Scarlet	355	-----EKASQR-----SAQRICDLFAV-SEAAGQRDVLINLEMHM
Ag_Scarlet	347	-----EQASQR-----AAHRLCDLFAV-SEAAGQRDVLINLEVHM
Am_Scarlet	295	-----KDGE-Q-----VAHKICDIFSN-SKASNEIDRILERSQSI
Bm_Scarlet	337	-----EAASRH-----AKSICDRFAV-SDVAKELDMEIHLEYHL
Dm_Scarlet	335	-----EQASQR-----SAQHLCDQFAV-SSAAKQRDMLVNLEIHM
Tc_Scarlet	321	-----EENSKQ-----CIKRICDYFAV-SDYNKEVNVVVQYEFHM
Aa_Scarlet-like	287	-----ASDRI-----KPETICDEYER-SDIYHQQKLVISSELLL
Ag_Scarlet-like	311	-----TRKGNQFTTGAIGPKTICNNYSA-SEAARRQEALISVELYR
Aa_Brown	250	-----DHVEND-----HHLQ---QQQRKCHMENIG---KKCLM
Ag_Brown	309	-----FAASEA-----EEAIQRYEIVRKACRQNI---RKCLM
Am_Brown	276	MYATNPKPEDTGPSE-----LIDKICRAFSR-SPLSRIPET-----
Bm_Brown	277	-----CIKKML-----ENYQD-----QSLDCDETG---LEVNL
Dm_Brown	353	-----ENAGAV-----LR-AKYEHEHETDGLYSGSWLL
Tc_Brown	269	-----KMELVY-----KTQKNREP-----
Am_Ok	290	-----EAESYR-----KVNWICDQYK-SKYGLRVSKLIEYSCVT
Bm_Ok	304	-----ESESRE-----RIRRICDEYHR-SDIAAEIEARVGEVHDE
consensus	501	e t i k i c d f s a r v v l v

Aa_White	394	-EE-----QYYLQPMEGASRTGYRSTWWTQFYVVLWRSWLTVLKD
Ag_White	397	-MD-----EPYMLQQVEGVGSTGYRSSWWTQFYCILWRSWLSVLKD
Am_White	394	-FD-----DSIRDSKYSKNRSLYKASWCEQFRAVLWRSWLSVIKE
Bm_White	382	-ER-KIQAGLADAPWAMSSTTRARRSPYKASWCTQFRAVLWRSWLSVTKE
Dm_White	392	-----LEKPLEQPENGYTYKATWFMQFRAVLWRSWLSVLKE
Tc_White	373	-GG-----YHDIWMSGESFKSPYKASCWAQFKAVLWRSILAVFKE
Aa_Scarlet	389	-A-----ETGDFKI-TEESHLRSKSNWESTTFWLTYRAFLTIVRD
Ag_Scarlet	381	-A-----ESGDYRVTDEVQHLAGRPHWLHTTAWLTYRALLTVRD
Am_Scarlet	328	-S-----S--LTIK-TSSYRKKKRRHCCSRLFWLIYRHFLQVLRD
Bm_Scarlet	371	-M-----DNEVEDSRRLRGDSFRPPHFYTKIMWLVRYLIMIIRD
Dm_Scarlet	369	-A-----QSGNFPF-DTEVESFRGVAWYKRFHVWLRASLTLLRD
Tc_Scarlet	355	-G-----RAVESKI-YKLRTNFNEMFFWQKLYWLTYRWFLDLWRD
Aa_Scarlet-like	320	-S-----EYGYRRP-LEMEDSQQRHSWFYTLNCLIRRNFLCAHRN
Ag_Scarlet-like	351	-TT-VD-----SGGDEAF-RRRLTESRDRCWFYTLYWLMYRHVLQSHRN
Aa_Brown	277	-G-----PYHQNDVIDKLCRDNHACWPSQLQLLLRRGVIDSVRN
Ag_Brown	339	-T-----RYHQAKIIQKLANDKHRVCRAQLMILLHRTTLDMSRK
Am_Brown	311	-----KNTRYFEIEPQRKSGCMTQFFWLIWRIWVQNRRT
Bm_Brown	302	IY-----KNYGSVRTVPSSFRPQRNWLKQVQLLLWRSSLSLKSD
Dm_Brown	378	-A-----RSY-SGDYLNKQVNFKKIRWYIYQVYLLMVRFMTEDLRN
Tc_Brown	283	-----QNHVHFDNLFLKKQTKNCIFYDLKWLWRCYLNTRKN
Am_Ok	324	-ES-ME-----L-PSIFSDVSLSLKNFKKARELTQLHVLVWRIYLDYKRN
Bm_Ok	338	-VDYFN-----GTLDEKNEYFEKYLTLLVKVNYFVQFYWLMWRNIQQMKNH
consensus	551	r ww ql yllwR wl vlrd

Aa_White	433	PML--VKVRLQQT-AMVATLIGSIYFGQ---KLDQDGVNMINGALFLFLT
Ag_White	437	PML--VKVRLQQT-AMVATLIGSIYFGQ---VLDQDGVNMINGSLFLFLT
Am_White	433	PIL--IKVRLQQT-VMVSLLVGIVYFNQ---RLDQDGVNMINGALFIFLT
Bm_White	430	PML--IKVRFLQT-IMVSILIGVIYFGQ---NLDQDGVNMINGAIFMFLT
Dm_White	428	PLL--VKVRLIQT-TMVAILIGLIFLQ---QLTQVGVNMINGAIFLFLT
Tc_White	412	PLL--IKVRLQQT-LIISLVIGAIYFGQ---DLNQDGVNMINGVLFVFLT
Aa_Scarlet	427	PTV--QYLRLQK-IAIALMAGLCFSGA--ISLDQLGVQAIQGIILFIVS
Ag_Scarlet	420	PTV--QYLRLQK-IAIALMAGLCFTGA--IEPTQLGVQATQGLLFIILS
Am_Scarlet	364	PSV--QIIRIFQK-VSVATIGGLCFMGA--VNFDQLGIQAAQGVIFILVS
Bm_Scarlet	410	PRV--QLVRILQK-LAIALTAGVCFLGT--PRLTQAGVQDVQALFIIIA
Dm_Scarlet	407	PTI--QWLRFIQK-IAMAFIIGACFACT--TEPSQLGVQAVQALFIMIS
Tc_Scarlet	393	PTL--QATKISEK-IVIGIMIGLCYLCT--DFTTQVGIQNVGIIIFLLVS
Aa_Scarlet-like	358	PQL--QYMKLAQR-LVIAVLVGLCFSS--IDLSQSGAQAQVGIIIFLIVS
Ag_Scarlet-like	392	PNL--QYFKIVQR-IAIAVLVGLCFSDA--IELSQRGVQAMQGVIFLIVS
Aa_Brown	316	IRQ--HVIIVTLL-FLITSITISALYFHV--TPTSQTATQDIRGALFLMVC
Ag_Brown	378	LRE--YLTVTAI-FLFTSVVIASLYYDV--RPVSQTSIQDIRGALFLMIS
Am_Brown	345	IFDSGDWISWFS-YFLSMAVVTTFYMG--NPRTQEGVQVQARGALYMMSS
Bm_Brown	342	LKS--YVFQLLLSVVVTASVLGTVYSGV--TGTTQRGIQDVRGFLWLVT
Dm_Brown	416	IRS--GLIAFGF-FMITAVTSLMYSGI--GGLTQRTVQDVGGISIFMLSN
Tc_Brown	320	KIT--NLGTYFYS-MMQILIIISIFYSEV--TFSGQDAIQSIQGLLSYCGT
Am_Ok	366	YTT--LFLRFITY-MCIGVLIGLPFMNISGEAMNQDTIQNMQGLLYLVVV
Bm_Ok	382	STI--WIAEFLLL-MFVGFIIISFPYIGHF-KELDQORDIQNVGGLLYLTIT
consensus	601	p 1 vrlq vmvalligi y g ltQ gvq inG lflllt

Aa_White	477	NMTFQNVFAVINVFSAELPVFLREKRSRLFRVDTYFLGKTIAEVPLFLAV
Ag_White	481	NMTFQNVFAVINVFSAELPVFLREKRSRLYRVDTYFLGKTIAELPLFIIV
Am_White	477	NMTFQNVFAVINVFCALPIFLREHRNGMYRTDVYFLCKTLAEAPIFIAV
Bm_White	474	NMTFQNIFAVINVFCSELPIFIREHHSQMYRADVYFLSKTLAEAPVFATI
Dm_White	472	NMTFQNVFATINVFTSELPVFMREARSRLYRCDTYFLGKTIAELPLFLTV
Tc_White	456	NMTFQNVFAVINVFSGELPVFLQEHNRNGMYRPSIYFISKTLAESPIFIII
Aa_Scarlet	472	ENTFSPMYSVLSVFPDTPFLFMRETSGLYRTSQYYVANALAMLPGLIFE
Ag_Scarlet	465	ENTFTPMYAVLAVFPETFPFLFMRETKNGLYHPSQYYVANVAAMLPGLVLE
Am_Scarlet	409	ENAFFPMYATLALIPQELPLLRREYRAGMPIYLYYIARIFSLIPGLIIE
Bm_Scarlet	455	ENTFSPMYSVLHMFPEEFPLFNRELKAGLYSTPVYYTARMIALFPGLLIE
Dm_Scarlet	452	ENTYHPMYSVLNLFPOGFPLFMRETRSGLYSTGQYYAANILALLPGMIIE
Tc_Scarlet	438	ENTFTPMYSILDEFQPKYPLFLREYNSGLYSSFLYFLSRIMAMLPGLIIE
Aa_Scarlet-like	403	ENTFLPMYAVLSVFPESFPLFLRERKANLYGTGQFYIAQIVAMLPFVLLLE
Ag_Scarlet-like	437	ENTFLPMYAALSIFPERFPLFQREKKANLYSTAQFYISTIMSMTPFVLLLE
Aa_Brown	361	ELIYTISYAVFYVFSYEMPLLRREVGEQMYRLSAYYVHKALLTVPKAIIFH
Ag_Brown	423	ELVYTISYGVFYTFPAEMPLIRREVGEKSYTSLSMYYLHKVLYSVPRAFLE
Am_Brown	392	EISFTVAYSVIYEFPGQLLIYLR--DGIYSCGPYYVATFCGLVPKAILK
Bm_Brown	388	EVCYGLAYSTLYVFKYEVTLEFRRE--VGMYKCSAYFVSKFLSFIPRCVIW
Dm_Brown	461	EMIETFSYGVTYIFPAALPIIRREVGECTYSLSAYYVALVLSFVPVAFK
Tc_Brown	365	EFTFTNMAYAVIYIFPEEVAIFLREK--NLYSTFAYFIAKLLSLIPLSIVT
Am_Ok	413	ETVETFNAYAVFYTFPRELPLLRDIASGLYGPAPYYFSKVIVLIPGAIQ
Bm_Ok	428	ETIFLFIYAVFITFPSEVPILLRETASGLYSPLPYYSKMIFFWIPRAVIE
consensus	651	emtf vyavi vfp elplflre r gly yylgkviamvP ii

Aa_White	527	PFVFTSITYPMIGLKSGATYYLTALLIVVLVANVATSF	GYLISCASSSIS
Ag_White	531	PFVFTSITYPMIGLRTGATHYLTTLFIVTLVANVSTF	GYLISCASSSIS
Am_White	527	PLLFTIITAYPMIGLYPGIDHFFITAGIVALVANVSTF	GYLISCISNNLS
Bm_White	524	PLVFTTITAYYMIGLNPDPKRFFIASGLAALVTNVATSF	GYLISCASSSVS
Dm_White	522	PLVETAITAYPMIGLRAGVLHFFNCLALVTLVANVSTF	GYLISCASSSTS
Tc_White	506	PVTLTSVCYFMIGLNSHGFRFYIACGIMILVANVAISF	GYLISCVSRVS
Aa_Scarlet	522	PLVFVIIAYWLAALRPTFGAFMVTVIASTLVMNVSTAC	GCFFSAAFNSLP
Ag_Scarlet	515	PLVFVLIAYWLAALRPTLHAFIVTAAAATLVMNVSTAC	GCFFSAAFNSLP
Am_Scarlet	459	PLLFTAILYWLAGLRDNIETFGFTLLVLLLTINVSTAC	GCFFSTAFESVP
Bm_Scarlet	505	PVLFTGVVYWLAGLRYSAYAIGLTIFIISILVLNVAIAC	GSFFSCAFGSMP
Dm_Scarlet	502	PLIFVIIICYWLTGLRSTFYAFGVTAMCVLVLMNVATA	CGFFSTAFNSVP
Tc_Scarlet	488	PILFVIIIVYWLSSGLRATTYAFIMTTLAGILTLNSAAAC	GIFFSNAFDSVP
Aa_Scarlet-like	453	STTFILIVYYLAHLRPTILGLLCTVAACTLVMNVSMAC	GCFFSTMFSSVP
Ag_Scarlet-like	487	TCAFILIVYFLANLRPTLLGLLVTVVVSVLVMNVSIAC	GCFFSVLFPTVA
Aa_Brown	411	SYLFIGLIYGFVQFSTGFATYVGMAAVCTVASLLGVSY	GYLFTCITGSLE
Ag_Brown	473	SFLFIGVAYAFVGFSTDFITYCCMSLVSSGASVLAMAY	GYLLSCTTGTMN
Am_Brown	440	AVLETTVIYFILISQIDLLNLFYCLITSTAAICGTAYGL	MISIMIENID
Bm_Brown	436	PIALVCTTTVAIELPNHVITTFEFIVVLIFAAIASMAY	GLGMSALFTSTG
Dm_Brown	511	GYVFLSVIYASIIYYTRGFLLYLSMGFLMSLSAVA	AVGYGVFLSSLFESDK
Tc_Brown	413	NMICLGLIFMFSNVLHGFCLWLKMTYVAFVLSIVSS	SSLGLAFSATESTIE
Am_Ok	463	PLLYSAFIFAITGLKGGLLGFFVYFALPVVCAISASA	FGLFLSASFKSME
Bm_Ok	478	PVLFGSLIFIVAE LRGGFVGWLGFCFVCVLCANYAN	AYGSFLSSVFDKME
consensus	701	plvfv ilyymiglr fl i lv nvatafG fis f si	

Aa_White	577	MALSVGPPIIIPFLIFGGFFLNSASVPSYFEYLSYF	SWFRYANEALLINQ
Ag_White	581	MALSVGPPIIIPFLIFGGFFLNSASVPAYFKYLSYLS	SWFRYANEALLINQ
Am_White	577	MALSIGPPVPIIPFLLFGGFFLNTASVPFYFEWFSYLS	SWFRYANEALLINQ
Bm_White	574	MAASVGPPPIIIPFMLFGGFFLNSGSPVPPYLSWISYLS	SWFHYGNEALLINQ
Dm_White	572	MALSVGPPIIIPFLLFGGFFLNSGSPVPLYLKWLSYLS	SWFRYANEGLLINQ
Tc_White	556	MALSIGPPPIIIPFLLFGGFFLNVSSIPIYFKWLSFL	SWFRYANGALMINQ
Aa_Scarlet	572	LAMAYLVPDFYILMITSGVFIQLSSMPKAISWTPYIS	WMMYANEAMSIAQ
Ag_Scarlet	565	LAMAYLVPDFYILMITSGVFIHLNTMPAATRWLPYIS	WMMYANEAMSIAQ
Am_Scarlet	509	LAMAYLIPFDYILMITMGPFKLGLSLPVYIQWKYIS	WLLHSTEALTILO
Bm_Scarlet	555	LAIAYLVPDFYILMITSGIFIKLSSIPRYVAWIRYLS	WLMYSNEAMSIVQ
Dm_Scarlet	552	LAMAYLVPLDYIFMITSGIFIQVNSLPVAFWWTQFL	SWMLYANEAMTAAQ
Tc_Scarlet	538	AAMAYLVPDFYVLMITSGVFVKLSTLPRVFSWTKYLS	WLMYSTESISTVQ
Aa_Scarlet-like	503	MAMSYLVPDFYILMITSGIFIRIWTIPTVLRWMPFI	SWMMFASEAISVAQ
Ag_Scarlet-like	537	SAMSYLVPDFYILMITSGIFIKIWSMPTYLQWMPFI	SWMMFASEAISIAQ
Aa_Brown	461	MSLEAANLIFLLYNLLGGLYLNVAFPV----	SKYLSFFFFASEGVSIIYY
Ag_Brown	523	MAIETSNIIFLAFMLLGGLYLNLRAPPL----	LKYLSFFFFASEGVSIIYY
Am_Brown	490	IATSIMVPIDMLFLLTAGMFYNLRSIPTYLTCFKYFS	IFFYLNALSIIYY
Bm_Brown	486	NMGDVMPCFDLPLFLMSGAFLLTSTLPILWLYPVKF	ISHFYAMDTISNLY
Dm_Brown	561	MASECAAPFDLIFLIFGGTYMNVDTVPG----	LKYLSLFFYSNEALMYKF
Tc_Brown	463	HVDLFLGPLEFILLFSGLLVKVDSVKGAFNWIKYIS	PFYYAFDSLNLFL
Am_Ok	513	TASLFSVPLDFLGLMFCCGIYHLGYLTSYIAWLKYL	SQFYGLEAVSLTQ
Bm_Ok	528	TAALVSVPFDLIGTMFSGLYLNLGSVSPYFSWLR	FVSAFYGIESISILQ
consensus	751	mal lvpvdiifli Gvflnv svp y wlkyLSw f yanealsi q	

Aa_White	627	WSTVQEGDIA-CTR-----ANVTCPSSGQIILETFNFKVE--DFGFDIA
Ag_White	631	WSTVVDGEIA-CTR-----ANVTCP-RSEIILETFNFRVE--DFALDIA
Am_White	627	WSEVES--IA-CTR-----SNATCPKSGRMVLQTFNFKQE--HFWMDIV
Bm_White	624	WAGVET--IA-CTR-----ENFTCPASGQVVLQTLNFSQD--DFAMDVV
Dm_White	622	WADVEPGEIS-CTS-----SNTTTPSSGKVILETLNFSAA--DLPDLYV
Tc_White	606	WENVTN--IQ-CPN-----ADLPCPKDGHVILETFHFSEA--DFVMDVV
Aa_Scarlet	622	WEGVSN--IT-CFVED---PNLPCMRTGEEVLAHYSFDES--HLWRNIW
Ag_Scarlet	615	WEGVSN--IT-CPAVD---DKLPCLRTGGEVLEHYSFSET--HLAPNLW
Am_Scarlet	559	WNNVHN--IS-CEETD---PELPCITDGIQVLQRYDFDET--NFWIDII
Bm_Scarlet	605	WDGVEN--IT-CTNSN---STGVPCVSTGDEVLMQYDFTSS--NLWLDIS
Dm_Scarlet	602	WSGVQN--IT-CFQES---ADLPCFHTGQDVLDKYSFNES--NVYRNLL
Tc_Scarlet	588	WNGIKN--IT-CDISD---QEIPCLTADTQVLEKYSFSED--NLSRDLW
Aa_Scarlet-like	553	WDGIDY--LD-CEGI-----PDRACLHDGDDVLQQYSFGRT--HLMDFI
Ag_Scarlet-like	587	WDGVKS--IE-CSNI-----IPSVCLHNGEQVLDQYSFSRQ--HLRTDLV
Aa_Brown	507	WQGVQN--IT-CDEG---RNVTCLRNGEAVLQDYGYGTSLDTVYFNYL
Ag_Brown	569	WLP IQS--IP-CNGTSSRLNETITCLANGQAVLEDAGYATSYEALHNYL
Am_Brown	540	WSRIDD--ID-CQVS-----SDLPCLKNGEQVLSEYGFKEN--NLIWDMS
Bm_Brown	536	WRQILY--ID-CPVN-----TTTTCTSSGEAVLYEIGYSNN--FVLQNSL
Dm_Brown	607	WIDIDN--ID-CPVN-----EDHPCIKTGVEVLQQGSYRNADYTYWLD CF
Tc_Brown	513	WKDV GK--IGECTFN-----QTIPCYHNVSEVLQSYGIYKTYDTVAYN IL
Am_Ok	563	WLLIDH--IN-CSSD-----PEEPCISSGLEVLKYG YLPT--HYTMDII
Bm_Ok	578	WDSIES--ID-CVKL-----PGIPCIKTGPDVLNRYGYSES--HFWRNCC
consensus	801	W v i C vpCmksg vLe y f s l ldiv

Aa_White	668	CLCMLIVIFRLGALFCLWLRSR---SKE
Ag_White	671	CLFALIVIFRLGALLCLWLRSR---SKE
Am_White	666	CLFSLIIAFRFLAFLALLLKTRGNYKQR
Bm_White	663	NMILLFVGFERFLAYLALLWRTR---RAK
Dm_White	663	GLAILIVSFRVLAYLALRLRAR---RKE
Tc_White	645	MLAVLIVGFRLVAFLLLVK TW---RFK
Aa_Scarlet	663	AMVVIYFGFHVLCVFLWRKTK----HG
Ag_Scarlet	656	AMVLLYFGFHLLGYLFLWRKTK----R
Am_Scarlet	600	LMVTIYFVFHIFAYICLWNRCR---WK
Bm_Scarlet	647	ALLILYITFHLLALLALRYRTR---RK
Dm_Scarlet	643	AMVGLYFGFHLLGYCLWRRAR---KL
Tc_Scarlet	629	SMLFLCIIIFHCLSFICLWLKIR---KR
Aa_Scarlet-like	593	ALITQYFLYHALALLFLHRRAS---KS
Ag_Scarlet-like	627	VLVGQYFIYHLMAMLC LARRVS---RN
Aa_Brown	549	VMAAEILVIHFAAYLCLRRFVRR-VGFY
Ag_Brown	616	VMAVEIVLVHLVAYMLLRKFVRK-AGFY
Am_Brown	580	GLLILTIIAMNIIGYFGLRRRRKI-QTIL
Bm_Brown	576	GLLLVTTMWGLLGYG MKREEKK-GYAY
Dm_Brown	649	SLVVAVIFHIVSEGLVRRYIHR-SGY Y
Tc_Brown	556	FLHILGAVFCLLGFAGIVRKKM--SLSL
Am_Ok	603	GLLVIFSFSHLAGFLVIRHRSRK-EPVY
Bm_Ok	618	CLATMYCVAHFVAFIMVIKRSRG-TPVY
consensus	851	liml vifhllafl l rr r

Figure S5 Full alignment of ABC eye-color transporter homologs. Amino acids in black highlight are seen in that position in more than half of aligned sequences; grey highlight marks similar amino acids. The consensus line shows the most likely amino acid for a given position, with invariable positions marked with a capital letter.

Table S1 Primers used

Name:	Sequence:	Purpose:
5'WR1	CGGTGTGTTTTGACACTTTG	5' RACE
5'WR2	TGACTGGCCAAGGTTTTC	5' RACE
brownRi-F	TAATACGACTCACTATAGGGAGCTTAGTCTGGA	dsRNA template
brownRi-R	TAATACGACTCACTATAGGGCGGGATTGA	dsRNA template
RT-Uni	CGTCAGCTTGATTAAGTCAACGATCTTTTTTTTTTTTTTTTTTTTV	Full-length gene
Scar RiF	TAATACGACTCACTATAGGGCCGGGAAAAGTA	dsRNA template
Scar RiR	TAATACGACTCACTATAGGGCTGAGTATGAGTCG	dsRNA template
St-utr F2	ACTAAATGTCTAGGAGCG	Full-length gene
Tcbw FL F1	TAAGCATTTCGGATAATGTGCG	Full-length gene
Tcbw FL F2	ACAATTCTTTTTGTCGTGGCA	Full-length gene
Tcst 5'RACE inner	GGTCGTGGGTTTCGTCGCAGA	5' RACE
Tcst 5'RACE inner2	GTTTCGCTCCTGTTGTGTCGTT	5' RACE
Tcst 5'RACE outer	GATGAGGGTTGATGGATTGTGC	5' RACE
Tcst FL F1	GTGTTTCGGTGTTTGCTTCTA	Full-length gene
Tcw3'UTR	TTTATTAATTACAAAAGTACAAC <u>TCAACTATTTGAAACGCC</u>	Full-length gene
Tcw5'UTR	CAGACAGCGTCGACAG	Full-length gene
Uni-linker	CGTCAGCTTGATTAAGTCAACGATC	Full-length gene
WH-11	TTCTGTGGCGGTCCATTTTGGCAG	mapping
WH-12RC	CCATCCTGGTTCAATCTTG	mapping
wRiF	TAATACGACTCACTATAGGGCTCCCCGTCTTCCT	dsRNA template
wRiR	TAATACGACTCACTATAGGGGTTTGGGCACTGA	dsRNA template