Molecular Characterization of Eye Pigmentation-Related ABC Transporter Genes in the Ladybird Beetle Harmonia axyridis Reveals Striking Gene Duplication of the white Gene

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Molecular Characterization of Eye Pigmentation-Related ABC Transporter Genes in the Ladybird Beetle *Harmonia axyridis* Reveals Striking Gene Duplication of the white Gene

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Many species of ladybird beetles (Coccinellidae) possess vivid body colors. These colors and patterns show diversity between coccinellid species, or even within species. However, the molecular underpinnings of these striking body colors are scarcely understood. One of the candidate pigmentation molecules responsible for ladybird body color is ommochrome pigment, which is well known as the red pigment molecule responsible for the red eyes of *Drosophila*. Various insects also use ommochrome in body coloration. It is known that ommochrome pigment precursors are imported into appropriate cells by the ATP binding cassette (ABC) transporter proteins White and Scarlet. Thus, these ABC transporter genes are potentially involved in various color and pattern expressions seen in ladybird beetle species. In this study, in order to identify the repertory of ABC transporter genes responsible for such body colors, we performed molecular characterization of pigment-related ABC transporter genes, especially white and scarlet, in the coccinellid *Harmonia axyridis*. By using whole genome data for *H. axyridis* and subsequent RACE-PCR, six white orthologs and one scarlet ortholog were successfully identified. According to the results of functional analyses via RNA interference (RNAi), only one of these genes had a major function in eye pigmentation. Specific effects on body color and pattern were not detected by our RNAi experiments of any of these genes. This is the first report of this striking duplication of white genes and their functional analyses in insects.

Key words: ABC transporter, color pattern, ladybird beetle, white, scarlet, gene duplication, RNAi, *Harmonia axyridis*

INTRODUCTION

Color patterns of beetles

Color patterns of insects show striking diversity. Color patterns have many ecological functions, such as camouflage, aposematism and sex signals. Recent studies have explored the molecular developmental underpinnings of such color pattern formation in insects, especially in *Drosophila* and some butterflies (Wittkopp and Beldade, 2009). In addition to these groups, Coleoptera is also known to contain species with remarkable color patterns on their bodies and elytra, such as in the various patterns of vivid coloration of ladybird beetles.

Ommochrome as potential source of beetle body color

One of the potential candidate substances responsible for coloration in various beetles is ommochrome pigment. Ommochrome pigments are known as the pigmentation molecules responsible for eye coloration in various insects (Linzen, 1974; Grubbs et al., 2015), but are also used for body and wing coloration (Reed and Nagy, 2005; Futahashi et al., 2012; Shamim et al., 2014). It is widely known that ommochrome pigment precursors are imported into appropriate cells by ATP binding cassette (ABC) transporters.
ABC-transporters as candidate genes underpinning various color expressions in insects

ABC transporters are a superfamily of transmembrane transporter proteins that utilize ATP energy (Broehan et al., 2013; Dermauw and Van Leeuwen, 2014). The ABC transporter superfamily can be divided to eight subfamilies, type A to H (Broehan et al., 2013). The proteins known to be involved in pigment molecule transport in insects are members of the ABC-transporter G-type (ABCG) subfamily. ABCG proteins are characterized by possessing a single ATP-binding domain and a single transmembrane domain, located at the N-terminal and C-terminal sides of the protein, respectively. Other ABC-transporters typically have two ATP-binding domains and two transmembrane domains (Broehan et al., 2013; Dermauw and Van Leeuwen, 2014).

One of the most well-known pigment-transporting ABC transporter genes is the *white* gene. In *Drosophila*, *white* protein forms a heterodimer with *Scarlet*, which is coded by the *white* paralog *scarlet*, and this heterodimer transports ommochrome precursors (Ewart and Howells, 1998). This mechanism is thought to be conserved across many insects, including *Tribolium* (Broehan et al., 2013; Grubbs et al., 2015). Knockdown of the *white* or *scarlet* genes leads to defective eye color phenotypes in *Tribolium* (Broehan et al., 2013; Grubbs et al., 2015). In addition to White and Scarlet, another ABCG member protein, Brown, also functions in eye pigmentation in *Drosophila*, by forming a heterodimer with White to transport pteridine (Ewart and Howells, 1998). In *Tribolium* (and many other insects including *Bombyx*, *Aedes*, *Anopheles* and *Apis*), pteridines do not play an important role in eye pigmentation, which is instead mainly determined by ommochrome pigments (Grubbs et al., 2015).

Characterization of eye pigmentation-related ABC-transporters in the ladybird beetle *Harmonia axyridis*

Considering that insects use ommochrome pigments for eye pigmentation and for coloration of other body parts, ABC-transporter-dependent pigment substrate transport is one of the candidate molecular bases for color expression in ladybirds. However, the gene repertoires and functions of eye pigmentation-related ABC transporters in ladybird beetles are not well understood. In order to describe the ABC transporter gene sets and characterize their potential function in mediating eye pigmentation and body coloration in ladybird beetles, we used *Harmonia axyridis* as a model species. This species shows genetic polymorphism of color pattern and is amenable to various molecular tools, such as RNAi (Niimi et al., 2005), transgenesis (Kuwayama et al., 2006, 2014; Osanai-Futahashi et al., 2012), and molecular sexing (Gotoh et al., 2015). Moreover, our group has recently completed whole-genome next generation sequencing (Ando et al., in revision), which enabled us to search for ABC transporter genes (*white, scarlet, brown*) over the entire *Harmonia* genome. We confirmed the identity of candidate transporter genes by cloning full length transcripts through reverse transcription PCR (RT-PCR) and rapid amplification of cDNA ends (RACE)-PCR. We then performed functional analyses on these ABC transporters via RNAi to identify any that functioned in mediating eye and body color. 

Materials and Methods

**Insects**

*Harmonia axyridis* individuals were reared and housed in the lab, as described in Kuwayama et al. (2014).

**Blast search of eye-color related ABC transporter genes against draft *Harmonia* genome database**

We searched the *Harmonia axyridis* draft genome database (Ando et al., in revision) using full length *Tribolium* homologues of ABC transporter G-type (ABCG) genes as query sequences using the blastN program. Query sequences were from Broehan et al. (2013) (TcABCG-4A to 4H, 8A, 9A to 9D) and Grubbs et al. (2015) (TcWhite, TcScarlet and TcBrown).

**Identification of full-length ABC transporter genes from *Harmonia axyridis***

We identified the full-length sequence of seven candidate *white/scarlet/brown*-homolog genes and one out group ABCG gene via RT-PCR and RACE-PCR. The template cDNA was prepared from single *Harmonia axyridis* pupa. Total RNA was extracted with RNeasy Mini kit (Qiagen, Hilden, Germany) using QIAcube instrument (Qiagen). We synthesized cDNA for RACE-PCR with SMART RACE kit (Clontech, Mountain View, CA, USA) according to the manufacturer’s protocol, using 4.5 μg of extracted total RNA. Using these templates, we performed RT-PCR and RACE-PCR. Primer pairs for RT-PCR were designed based on predicted conserved eye pigment and drug resistance transporter subfamily G of the ATP-binding cassette superfamily (ABCG-EPDR) regions from the *Harmonia* draft genome described above. Primers for RACE-PCR were also designed based on predicted conserved ABCG-EPDR regions from the draft genome and/or on partial gene sequences identified by RT-PCR and subsequent sequencing. Primers used for RT-PCR and RACE-PCR are listed in Supplementary Table S1. PCR was performed with KOD FX NEO (Toyobo, Osaka, Japan) and AmpliTaq 360 DNA polymerase (Applied biosystems, Waltham, MT, USA) according to the manufacturer’s protocol. Amplified PCR products were separated by electrophoresis in 2% agarose gels and purified using MagExtractor (Toyobo). Purified PCR products were directly sequenced or subcloned into TOPO vector (pCR4-TOPO) with TOPO TA cloning Kit (Invitrogen, Carlsbad, CA, USA). Sequencing was performed by an automatic DNA sequencer (DNA sequencer 3130 genetic analyzer; Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using BioEdit software (IBIS Biosciences Carlsbad, USA). Identified sequences were searched by BlastX in the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic neighbor-joining (NJ) tree was constructed by using Clustalx (Thompson et al., 2002) using putative amino acid sequences of identified *Harmonia* ABCG proteins and previously identified insect homologs of White, Scarlet, Brown and *Tribolium* ABCG proteins described in Broehan et al. (2013) and Grubbs et al. (2015). Identified sequences are registered to DDBJ (Accession Nos. LC322265 to LC322272).

**Functional analyses of ABC transporters via RNAi**

In order to characterize the function of ABC transporters in postembryonic development of *Harmonia axyridis*, we performed functional analyses via larval RNAi (Niimi et al., 2005) which is very effective in focal species. Using T7 promoter sequences attached to primer pairs designed for each target gene (Supplementary Table S2), partial sequences of targets were amplified by PCR. Amplified PCR products were purified by electrophoresis, as described above. dsRNAs were synthesized by MEGAScript T7 kit (Ambion, Austin, TX, USA) according to the manufacturer’s protocol.

Injections of dsRNA were performed according to Niimi et al. (2005). Approximately 1 μg of dsRNA was injected into each late
last instar larva. An amount of dsRNAs sufficient for effective gene knockdown as described in our previous (Niimi et al., 2005) and current studies (Ando et al., in revision). EGFP dsRNA was injected as a negative control. Nine larvae were used per one focal gene or control EGFP knockdown.

RESULTS

Identification of full sequences of white homologs from Harmonia axyridis

First, we searched pigmentation-related ABC transporter homologs against our draft genome database of Harmonia axyridis (Ando et al., in revision), using amino acids sequences of Tribolium White, Scarlet and Brown homologs. According to the search, there were eight scaffolds (scaffold2319_cov114, scaffold61_cov135, scaffold699_cov144, scaffold435_cov164, scaffold14_cov190, scaffold1097_cov196, scaffold104_cov198 and scaffold684_cov202) containing a region which showed high similarity (e-value < 1.00E-19) to TcWhite, TcScarlet and/or TcBrown (Table 1). Four of them (scaffold2319_cov114, scaffold435_cov164, scaffold61_cov135, scaffold684_cov202) were selected for further analysis as they showed the highest similarity to the target sequences.

Table 1. Results of local tBlastN search using Tribolium ABC-transporter G-type proteins.

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<th>Tc_Brown_KP120764</th>
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</tr>
<tr>
<td>scaffold14_cov190</td>
<td>5.00E-22</td>
<td>scaffold104_cov198</td>
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Tc_ABCG-4A_XP_971210 | Tc_ABCG-4B_XP_971681 | Tc_ABCG-4C_XP_001813184 | Tc_Atet (ABCG-4D)_XP_973458 |
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<td>scaffold10277_cov122</td>
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Tc_ABCG-4E_XP_001811847.1 | Tc_ABCG-4F_XP_971735 | Tc_ABCG-4G_XP_973493.1 | Tc_ABCG-4H_XP_973526.1 |
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<td>scaffold684_cov202</td>
<td>1.00E-22</td>
<td>scaffold699_cov144</td>
</tr>
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Only scaffolds with high similarity (e-value < 1.00E-19) were shown.
fold14_cov190 and scaffold104_cov198) also showed higher similarity (e-value < e^{−50}) to other Tribolium ABCG genes (TcABCG-4C, TcABCG-4D, TcABCG-8A and TcABCG-9C, respectively) than to White, Scarlet, and Brown. Thus, we excluded genes on those four scaffolds from candidate white/scarlet/brown-homolog genes.

Next, we searched the remaining four scaffolds (scaffold61_cov135, scaffold699_cov144, scaffold1097_cov196, and scaffold684_cov202) for the conserved ABCG-EPDR domain of the Tribolium White, Scarlet and Brown proteins using the tBlastN program. Three of the scaffolds (scaffold61_cov135, scaffold699_cov144 and scaffold1097_cov196) contained only one predicted protein sequence that aligned with the query, while one scaffold (scaffold684_cov202) contained four predicted protein sequences that aligned with the query.

We confirmed the expression of these seven putative ABCG genes via RT-PCR and identified the full-length expressed sequences by subsequent RACE-PCR (Fig. 1). We also identified a full-length sequence of one other ABCG-4C-like gene which was predicted to be an outgroup from the white/scarlet/brown homologs.

The sequences of the eight genes identified indicate that all eight genes share a similar structure. The nucleic acid sequences are of similar size, ranging from 2022 bp to 2676 bp. Putative amino acid lengths are from 619 aa to 673 aa. All of the putative proteins possess two conserved domains, the ATP binding domain on the N-terminal side and the transmembrane domain on the C-terminal side (Fig. 1), which are characteristic of ABC transporter G-type subfamily proteins (Broehan et al., 2013).

We constructed a molecular phylogenetic tree using identified the full putative amino acid sequences for the eight genes, and putative proteins of other insect ABCG genes including White, Scarlet and Brown. The result indicates that six of the putative _Harmonia axyridis_ proteins grouped with White proteins from other insect species (Fig. 2). One of the putative _H. axyridis_ proteins grouped with Scarlet proteins from other insects (Fig. 2). Orthologs of Brown protein were not found from _H. axyridis_. As predicted, the putative product of the ABCG-4C-like gene from _H. axyridis_ was located outside of the White/Sharcet/Brown group (Fig. 2) and grouped with TcABCG-4C protein with high bootstrap value. Thus, we concluded that we had identified six white homologs, one scarlet homolog and one ABCG-4C homolog from _Harmonia axyridis_. We refer to these as Ha-white-1 (Ha-w-1) to Ha-white-6 (Ha-w-6), Ha-scarlet (Ha-st) and Ha-ABCG-4C (Figs. 1, 2).

Gene structure, such as number of predicted exons, varied among these groups. The white/scarlet group genes had 12 or 15 exons, while Ha-ABCG-4C had just eight exons (Fig. 1). Also, the gene region of each white/scarlet group gene was 10 to 20 kbp, while ABCG-4C was approximately 40 kbp (Fig. 3).

From their mapped positions, interestingly, four genes (Ha-w-1, Ha-w-2, Ha-w-3 and Ha-st) showed tandem repeat structures. The interval of each gene is approximately 500 bp to 3000 bp (Fig. 3) and all four genes lie inside of a 60 kbp region of the scaffold (Fig. 3).

Functional analyses of identified ABCG genes via RNAi

In order to reveal function for each identified ABCG transporter gene, we performed gene knockdown via RNAi for the eight identified genes (Table 2, Fig. 4).

Knockdown of the out-group Ha-ABCG-4C gene was lethal. Most of the RNAi animals died during the pupal period (Table 2), characterized by shrinking of the body probably due to desiccation. Knockdown of the other seven genes did not cause significant mortality or block development to the adult stage.

The two phenotypes we examined in the adults were eye pigmentation and body color. Among the six duplicated white homologs (Ha-w-1 to 6) only knockdown of Ha-w-2 caused a distinct adult phenotype in eye color. _Ha-w-2 RNAi_ adults had a white eye phenotype lacking the black pigmentation in each ommatidium found in controls (Fig. 4). On the other hand, knockdown of any of the other white homolog genes (Ha-w-1, 3 to 6) did not affect eye color compared to controls (Table 2). Moreover, we knocked down those five genes (Ha-w-1, 3 to 6) simultaneously (i.e., quintuple knockdown), but we could not detect any effect on eye color in eclosed adults (Fig. 4). Knockdown of Ha-st also resulted in the white eye phenotype similar to knockdown of the Ha-w-2 gene (Fig. 4).

In terms of other effects on col-

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**Fig. 1.** Gene structures of identified eight ABCG genes from *Harmonia axyridis*. Boxes indicate exons. UTR regions were indicated as white open boxes and ORF regions were indicated as colored boxes. Conserved ATP binding domain and transmembrane domain were highlighted in light green and light blue, respectively.
oration, we could not detect any difference in adult body color after knockdown of any of the genes individually or in combination compared to controls (Table 2).

**DISCUSSION**

Identification of a functional white gene and scarlet gene mediating eye pigmentation in *Harmonia axyridis*

In this study, we identified six duplicated white homologs and one scarlet homolog from the *Harmonia* genome. Based on our RNAi results, only one of the six white homologs, *Ha-w-2*, had a function in eye pigmentation. We could not detect any function for the other five white homologs during pupal and adult development, including eye pigmentation. Considering that both *Ha-w-2(RNAi)* and *Ha-st(RNAi)* resulted in indistinguishable phenotype of white eyes, it is likely that the products of these two genes act together by forming a heterodimer for transport of ommochrome pigment precursors into cells, as is the case in other insects. It is known that ommochrome pigments are the major source of eye pigmentation in various insects, including *Tribolium* (Broehan et al., 2013; Grubbs et al., 2015). Our results are consistent with these previous studies. Also, the lack of any detectible body color phenotype in knockdowns of any of these ABCG-transporter genes, including both *Ha-w-2(RNAi)* and *Ha-st(RNAi)*, suggests that ommochrome are not a major source of body color in *Harmonia axyridis*.

Potential loss of the brown gene in *Harmonia axyridis*

In this study, we could not identify any gene sequences grouped with the brown gene clade (Fig. 2). This could be due to a technical issue (such as the assembly quality of our
Harmonia genome) or natural characteristics of the gene (i.e., the sequence of the brown gene was not well conserved or is not present in the Harmonia genome). It is known that Brown protein is required for pteridine pigment transport, for which it forms a heterodimer with White protein (Ewart and Howells, 1998; Tatematsu et al., 2011). Brown is also necessary for normal eye coloration where it functions in transport of red pigments in Drosophila (Ewart and Howells, 1998). However, in other insects including Bombyx and Tribolium, pteridines are not involved in eye pigmentation (Tatematsu et al., 2011; Grubbs et al., 2015). Consistent with this, RNAi knockdown of brown did not affect eye color in Tribolium (Grubbs et al., 2015). Except in Drosophila, a clear function of the brown gene is not known, and brown is the less conserved compared to white and scarlet (Grubbs et al., 2015). In fact, brown was initially not recognized in the Tribolium and Bombyx genomes (Broehan et al., 2013; Tatematsu et al., 2011). Thus, it is possible that the homology-based search strategy used in this study was unable to detect brown homolog in the Harmonia genome.

**Striking duplication of the white gene in Harmonia axyridis**

We found six white homologs in the Harmonia genome. Such striking duplication of the white gene is also seen in the water flea Daphnia pulex and the spider mite Tetranychus.
urticae (Dermauw et al., 2013; Dermauw and Van Leeuwen, 2014), but the function of those duplicated white homologs has not yet been analyzed. Our study is the first characterization of duplicated white homologs in an insect as long as our knowledge. Interestingly, three of them (Ha-w-1 to 3) are on the same scaffold with Ha-st. The orientation of these genes differs between Ha-w-2 and 3 from Ha-w-1 and Ha-st (Fig. 3). Given that white and scarlet are on closely located in the genome with same orientation in Tribolium and Bombyx (Tatematsu et al., 2011), we hypothesize that the original copy of the white gene of H. axyridis would be located next to scarlet and share the same orientation (Fig. 3). Among the six Harmonia white homologs, only Ha-w-1 meets these criteria. Thus, Ha-w-1 might be the original ortholog of the white gene in Harmonia axyridis. Also, the molecular phylogenetic tree of the duplicated white genes indicated that Ha-w-2 and Ha-w-3 showed the highest similarity and grouped with the highest bootstrap value. Thus, we suggest that Ha-w-2 and Ha-w-3 are the most recently duplicated pair of white genes. Also, considering that Ha-w-1 is grouped together with Ha-w-2 and Ha-w-3 with high bootstrap values, they might have resulted from duplication of Ha-w-1. Ancestral Ha-w-1 likely exhibited a function in eye pigmentation, but its function was lost in Ha-w-1 and kept in Ha-w-2 after gene duplication due to gene redundancy.

Why did the white gene undergo such striking duplication in ladybird beetles? It is known that duplicated genes are able to gain new functions (Innan and Kondrashov, 2010; Albalat and Cañestro, 2016). On the other hand, duplicated genes often became pseudogenes due to the overlapping function between two paralogs (pseudogenization) (Albalat and Canestro, 2016). In H. axyridis, we could not detect any developmental function of the duplicated white homologs except the Ha-w-2 gene, at least during pupal-adult development, although all of those white homologs are certainly transcribed. It is possible that we could not detect their function in Harmonia due to deficiencies in experimental observation/design depth. For example, White is suggested to be involved in the regulation of biogenic amines in the brain in Drosophila beyond simply a function in eye pigmentation (Borycz et al., 2008). Such non-morphologic phenotypes and internal morphologic phenotypes can be overlooked in our observation. Also, timing of dsRNA injection (at last larval instar) in our experimental design is not able to detect the gene function in embryonic and early larval development. Although our RNAi experimental conditions are well established (Niimi et al., 2005; Ando et al., in revision), the knockdown efficiency needed to obtain a phenotype is potentially different among target genes. Thus, further studies, including expression analyses along with the developmental stages and various tissues, combined with appropriate timing of gene knockdown analyses, are necessary for investigating unknown functions of white paralogs in Harmonia axyridis.

It is also possible that the duplicated white genes are redundantly conserved in the Harmonia genome. Although white homolog genes are not likely to have major role in establishing the adult body color pattern in Harmonia axyridis, some other ladybird species are known to use omochrome for their adult elyra color pattern (Niimi, unpublished data) and lineage specific duplicated white homologs are potentially involved in such color pattern formation in these species. In order to test this hypothesis, identification, expression analyses and functional analyses of the duplicated white homologs should be performed in other ladybird species in future studies.

ACKNOWLEDGMENTS

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COMPETING INTERESTS

The authors have declared that they have no competing interests.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: HG, TY, TN. Designed and performed the experiments: TT, HG, SM, JH. Technical contribution to genome data: YM, AT. Wrote the manuscript: HG. All authors reviewed and revised the manuscript and approved the final version of the paper.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online (URL: http://www.bioone.org/doi/suppl/10.2108/zs170166).

Supplementary Table S1. List of primer sequences for gene cloning and sequence.

Supplementary Table S2. List of primer sequences for dsRNA synthesis.

REFERENCES


ABC transporter genes in ladybird beetle


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