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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.

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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 repertories 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 tBlastN 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 a 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 manufacture'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, Carlsbab, 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 dsRNAis 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 < e^{-20}) to TcWhite, TcScarlet and/or TcBrown (Table 1). Four of them (scaffold2319_cov114, scaffold435_cov164, scaf-

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

Tc_White (ABCG-9B)_AF422804		Tc_Scarlet (ABCG-9A)_KP120763		Tc_Brown_KP120764	
hit	e-value	hit	e-value	hit	e-value
scaffold699_cov144	1.00E-68	scaffold1097_cov196	2.00E-37	scaffold435_cov164	1.00E-26
scaffold684_cov202	3.00E-56	scaffold699_cov144	2.00E-33	scaffold1097_cov196	3.00E-26
scaffold1097_cov196	9.00E-48	scaffold435_cov164	3.00E-33	scaffold2319_cov114	2.00E-25
scaffold61_cov135	5.00E-38	scaffold684_cov202	9.00E-32	scaffold61_cov135	3.00E-24
scaffold435_cov164	3.00E-34	scaffold61_cov135	2.00E-29	scaffold684_cov202	4.00E-24
scaffold2319_cov114	8.00E-25	scaffold2319_cov114	4.00E-27	scaffold699_cov144	2.00E-22
scaffold14_cov190	5.00E-22	scaffold104_cov198	2.00E-22		

Tc_ABCG-4A_XP_971210		Tc_ABCG-4B_XP_971681		Tc_ABCG-4C_XP_001813184		Tc_Atet (ABCG-4D)_XP_973458	
hit	e-value	hit	e-value	hit	e-value	hit	e-value
scaffold435_cov164	6.00E-57	scaffold435_cov164	8.00E-59	scaffold2319_cov114	1.00E-84	scaffold435_cov164	3.00E-96
scaffold2319_cov114	3.00E-51	scaffold2319_cov114	4.00E-48	scaffold435_cov164	8.00E-68	scaffold2319_cov114	2.00E-59
scaffold480_cov117	4.00E-45	scaffold480_cov117	2.00E-40	scaffold10277_cov122	3.00E-49	scaffold104_cov198	9.00E-35
scaffold104_cov198	4.00E-34	scaffold104_cov198	2.00E-30	scaffold104_cov198	1.00E-35	scaffold10277_cov122	9.00E-27
scaffold3116_cov111	1.00E-24	scaffold1097_cov196	4.00E-23	scaffold480_cov117	1.00E-25	scaffold480_cov117	3.00E-26
scaffold1097_cov196	7.00E-23	scaffold684_cov202	7.00E-20	scaffold3116_cov111	2.00E-24	scaffold3116_cov111	2.00E-22
scaffold684_cov202	7.00E-21			scaffold684_cov202	9.00E-21	scaffold14_cov190	2.00E-22
				scaffold61_cov135	6.00E-20	scaffold684_cov202	1.00E-20
						scaffold1097_cov196	9.00E-20

Tc_ABCG-4E_XP_001811847.1		Tc_ABCG-4F_XP_971735		Tc_ABCG-4G_XP_973493.1		Tc_ABCG-4H_XP_973526.1	
hit	e-value	hit	e-value	hit	e-value	hit	e-value
scaffold435_cov164	8.00E-56	scaffold435_cov164	2.00E-58	scaffold435_cov164	4.00E-60	scaffold435_cov164	8.00E-62
scaffold2319_cov114	4.00E-39	scaffold2319_cov114	2.00E-49	scaffold2319_cov114	2.00E-48	scaffold2319_cov114	2.00E-48
scaffold2199_cov121	1.00E-28	scaffold104_cov198	8.00E-41	scaffold480_cov117	1.00E-36	scaffold480_cov117	6.00E-25
scaffold104_cov198	5.00E-26	scaffold3116_cov111	1.00E-23	scaffold104_cov198	2.00E-34	scaffold104_cov198	2.00E-21
scaffold480_cov117	9.00E-21	scaffold480_cov117	1.00E-23	scaffold3116_cov111	8.00E-30	scaffold1097_cov196	3.00E-21
scaffold684_cov202	4.00E-20	scaffold684_cov202	3.00E-21	scaffold10277_cov122	6.00E-24	scaffold684_cov202	7.00E-21
		scaffold1097_cov196	4.00E-21	scaffold14_cov190	4.00E-21	scaffold10277_cov122	1.00E-20
				scaffold61_cov135	7.00E-21		
				scaffold684_cov202	1.00E-20		

Tc_ABCG-8A_XP_975214.2		Tc_ABCG-9C_XP_968472.1		Tc_ABCG-9D_XP_968555.1	
hit	e-value	hit	e-value	hit	e-value
scaffold14_cov190	5.00E-88	scaffold104_cov198	4.00E-60	scaffold104_cov198	4.00E-46
scaffold435_cov164	2.00E-31				
scaffold2319_cov114	4.00E-23				
scaffold684_cov202	1.00E-22				
scaffold61_cov135	4.00E-21				
scaffold699_cov144	3.00E-20				

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 *H. 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/Scarlet/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-

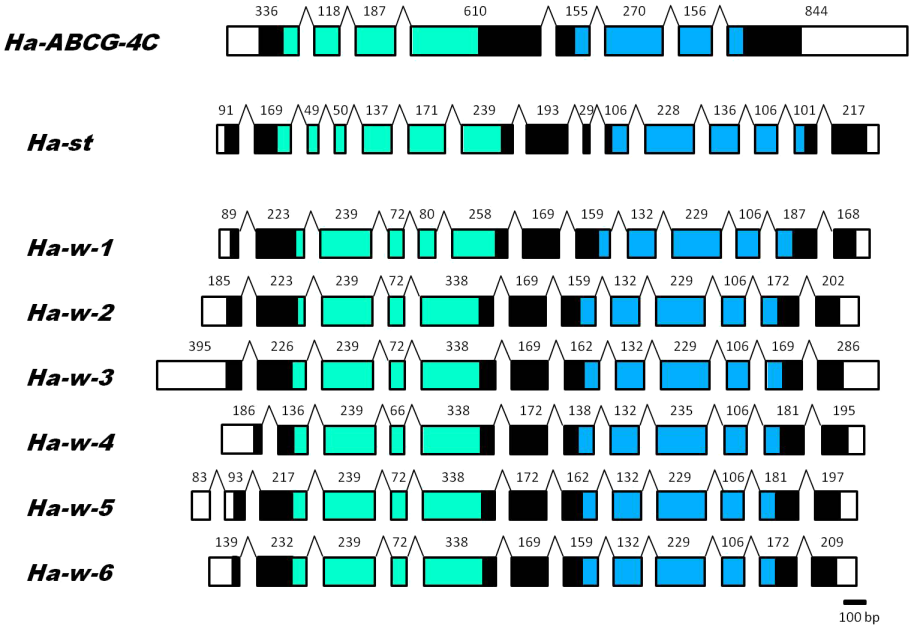

Fig. 1. Gene structures of identified eight ABCG genes from *Harmonia axyridis*. Boxes indicate exons. UTR regions were indicated as white open boxes and ORF regions were indicated as colored boxes. Conserved ATP binding domain and transmembrane domain were highlighted in light green and light blue, respectively.

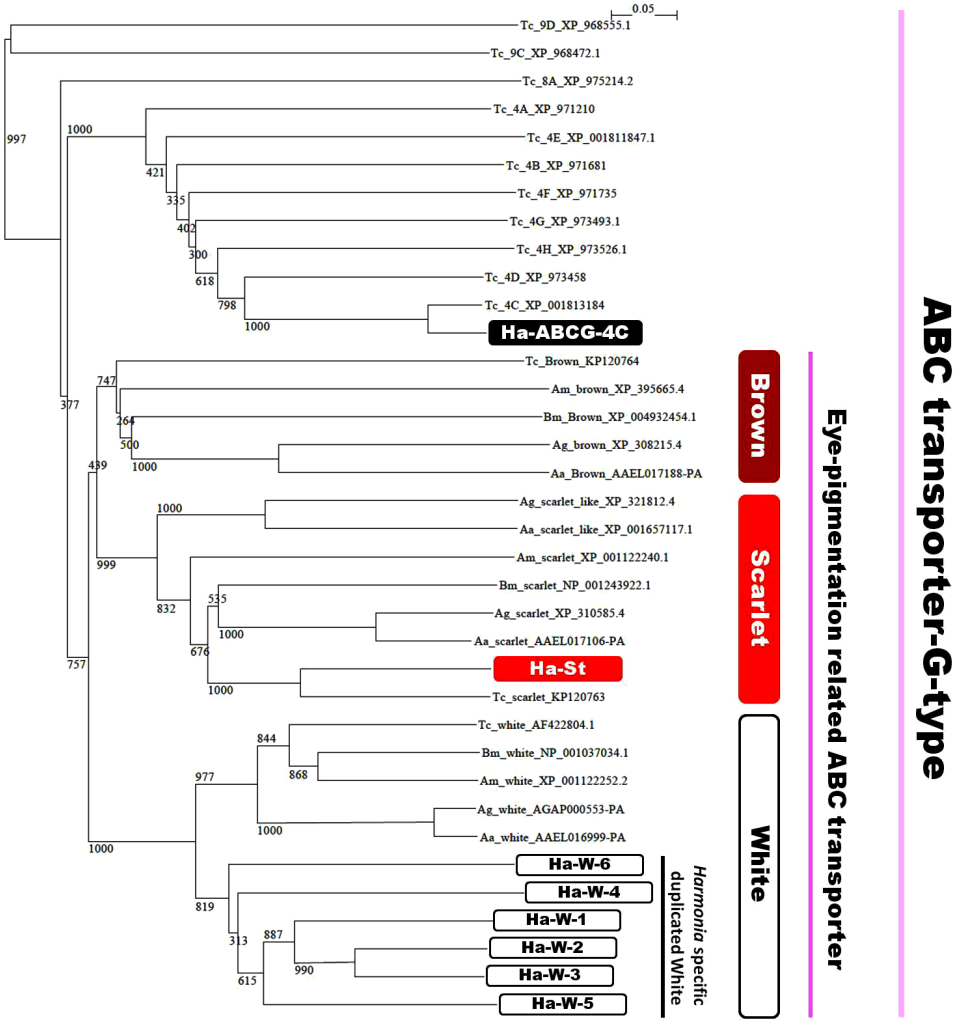

Fig. 2. Phylogenetic tree of ABCG genes from *H. axyridis* with other ABCG proteins from other insects. Six expressing *white* homologs and one *scarlet* homolog were found from *Harmonia* genome. We could not find homolog of *brown*. *Ha-ABCG-4C* was also experimentally identified as out group gene. Tc: *Tribolium castaneum*, Am; *Apis mellifera*, Bm; *Bombyx mori*, Ag; *Anopheles gambiae*, Aa; *Aedes aegypti*, Ha; *Harmonia axyridis*.

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

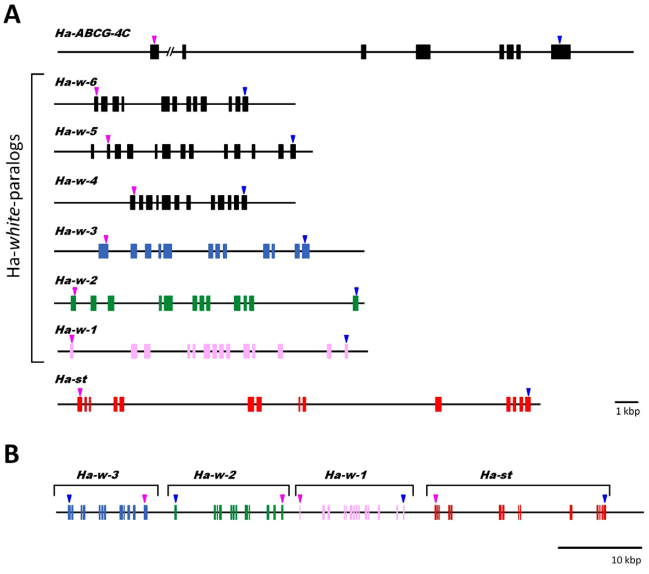

Fig. 3. Schematic gene structures of identified eight ABCG genes from *Harmonia axyridis*. **(A)** Schematic structure of each gene. Horizontal lines indicate genome and boxes indicate mapped exons. Pink and blue arrowheads indicate start and stop codons, respectively. Genes on the same scaffolds (scaffold684_cov202) were colored (blue, green, pink and red) and other genes are black. **(B)** Position and orientation relationship of tandemly repeated four genes (*Ha-w-1*, *Ha-w-2*, *Ha-w-3*, *Ha-st*).

Table 2. Summary of RNAi result.

Gene	Volume (μg)	Injected larvae	Successfully eclosed	White eye phenotype adult		Abnormal body color phenoype adults
				Strong (completely white eye)	Weak (Slightly pigmented eye)	
GFP (control)	1	9	8	0	0	0
Ha-w-1	1	9	8	0	0	0
Ha-w-2	1	9	8	7	1	0
Ha-w-3	1	9	8	0	0	0
Ha-w-4	1	9	9	0	0	0
Ha-w-5	1	9	7	0	0	0
Ha-w-6	1	9	7	0	0	0
Ha-st	1	9	5	4	1	0
Ha-ABCG-4C	1	9	2	0	0	0
quintuple (Ha-w-1, 3-6)	5	9	6	0	0	0

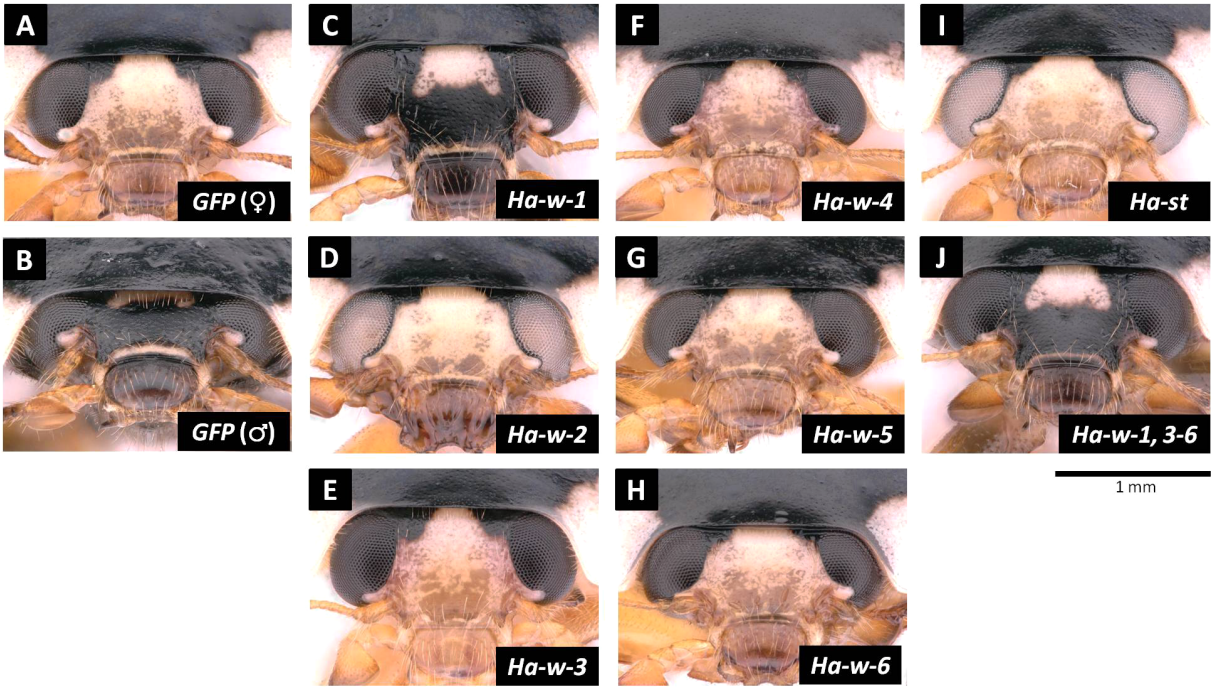

Fig. 4. Eye color phenotypes of gene knocked-down adults. Gene knockdown effect was observed in eye color. Knockdown of *Ha-w-2* **(D)** and *Ha-st* **(I)** caused white eye phenotype, while knockdown of other white homologs did not affect eye color **(C, E, F, G, H)** compared to *GFP* dsRNA injected animal **(A, B)**. Even, quintuple knockdown (*Ha-w-1, 3-6*) did not affect the eclosed adult eye color **(J)**. Head color exhibits sexual dimorphism; that is, males have black pigmented area in head **(B)**, while females do not **(A)**. Thus, head color difference between **(C, J)** (male) and **(D, E, F, G, H, I)** (female) is due to sexual difference, not a specific gene knockdown effect.

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 ommochrome 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.

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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.

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