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Changes is genes coding for laccases 1 and 2 may contribute to deformation and reduction of wings in apollo butterfly (*Parnassius apollo*, Lepidoptera: Papilionidae) from the isolated population in Pieniny National Park (Poland)

Kinga Łukasiewicz[1,2] and Grzegorz Węgrzyn[1]✉

[1]Department of Molecular Biology, University of Gdańsk, Gdańsk, Poland; [2]Division of Molecular Biology and Clinical Genetics, Department of Medicine, Jagiellonian University Medical College, Kraków, Poland

An isolated population of apollo butterfly (*Parnassius apollo*, Lepidoptera: Papilionidae) occurs in Pieniny National Park (Poland). Deformations and reductions of wings in a relatively large number of individuals from this population is found, yet the reasons for these defects are unknown. During studies devoted to identify cause(s) of this phenomenon, we found that specific regions of genes coding of enzymes laccases 1 and 2 could not be amplified from DNA samples isolated from large fractions of malformed insects while expected PCR products were detected in almost all (with one exception) normal butterflies. Laccases (p-diphenol:dioxygen oxidoreductases) are oxidases containing several copper atoms. They catalyse single-electron oxidations of phenolic or other compounds with concomitant reduction of oxygen to water. In insects, their enzymatic activities were found previously in epidermis, midgut, Malpighian tubules, salivary glands, and reproductive tissues. Therefore, we suggest that defects in genes coding for laccases might contribute to deformation and reduction of wings in apollo butterflies, though it seems obvious that deficiency in these enzymes could not be the sole cause of these developmental improperties in *P. apollo* from Pieniny National Park.

Key words: apollo butterfly, deformed wings, reduced wings, genes of laccases

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INTRODUCTION

Parnassius apollo (Lepidoptera: Papilionidae), known also as apollo butterfly, is a species currently considered as near threatened (van Swaay *et al.,* 2010). Intriguingly, some 100 years ago it was quite common in Europe. The phases of its prosperity and extinction have been analyzed and published (Nakonieczny *et al.,* 2007; Łozowski *et al.,* 2014).

Because of the dramatically decreasing size of *P. apollo* global population, programs devoted to protect and save this butterfly have been initiated. In the Pieniny National Park (Poland) such a program was established in 1990s, and allowed to rebuild the population of this species from only 20-30 individuals which remained alive there (Witkowski & Adamski, 1996, Witkowski *et al.,* 1997). However, it appears that consequences of the restitution of a population from such a low number of individuals are developmental defects due to plausible accumulation of mutations in one or more genes (Jarvis *et al.,* 2011; Kawecki *et al.,* 2012). Currently, the *P. apollo* population from the Pieniny National Park is characterized by often appearance of butterflies with deformed or severely reduced wings (Adamski & Witkowski, 1999). The number of individuals in this population is estimated now for about 1 000 insects, but it changes every year, and therefore it is difficult to estimate the exact fraction of malformed butterflies. Nevertheless, based on the number of malformed insects in the collection of the Pieniny National Park, as well as field observations (our unpublished data), it is obvious that morphological abnormalities in wings of these insects are far more frequent than it could be expected based on spontaneous mutations. Interestingly, such abnormalities do not resemble phenotypes of apollo butterfly reported previously to bear mutations in genes directly involved in wing development (Descimon, 1988, Pierrat & Descimon, 2011). Therefore, we aimed to search for genetic defects which could be responsible for the specific deformation and reduction of *P. apollo* wings.

In this work, we have tested genes coding for laccases. These enzymes (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductases) are oxidases containing several copper atoms, and catalyse single-electron oxidations of phenolic or other compounds with concomitant reduction of oxygen to water (for reviews see: Claus 2004; Dittmer & Kanost, 2010, Jeon *et al.,* 2012, Moin & Omar, 2014). In insects, laccases were found in epidermis, midgut, Malpighian tubules, salivary glands, and reproductive tissues. It was demonstrated that in insects, these enzymes might have physiological roles in cuticle sclerotization, detoxification of food (particularly phenol-derived compounds), and maintenance of iron homeostasis (Coy *et al.,* 2010; Lang *et al.,* 2012; Prasain *et al.,* 2012; De Fine Licht *et al.,* 2013). We hypothesized that dysfunction(s) of laccase(s) might potentially contribute to developmental malformations due to either defective sclerotization of cuticle or toxic effects of inefficiently neutralized food metabolites, or both. If this is the case, one might assume that laccase activity deficiency could contribute to deformation or reduction of wings in *P. apollo.* Thus, we have asked if there are any differences in genes coding for laccases

✉e-mail: grzegorz.wegrzyn@biol.ug.edu.pl

Abbreviations: *P. apollo, Parnassius apollo*

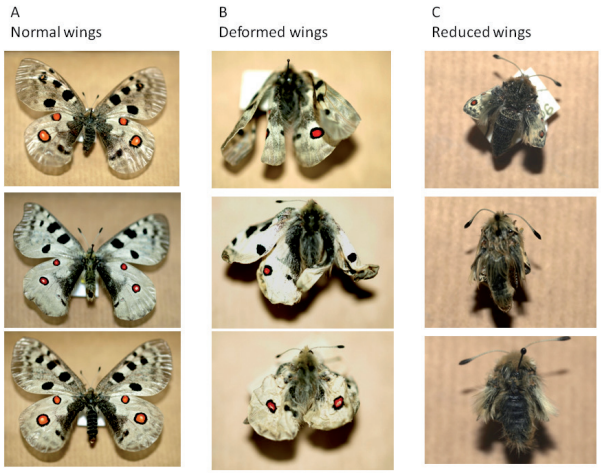

Figure 1. Examples of *P. apollo* individuals with normal (panel A), deformed (panel B), and reduced (panel C) wings. Photographs were made by the authors.

between normal and malformed apollo butterflies from the population occurring in Pieniny National Park.

MATERIALS AND METHODS

Insects. *P. apollo* individuals were from the collection of the Pieniny National Park (specimens were collected in years 1991-2007). The permission for the use of this material have been obtained from the Director of the Pieniny Natonal Park (permission no. PB-5232-24/07, topic ID: p0748). Photographs of examples of normal individuals and those with deformed or reduced wings are presented (Fig. 1).

DNA isolation, amplification and analysis. DNA was isolated from a material withdrawn from legs of investigated insects. The Sherlock AX Purification Kit (A&A Biotechnology) was used according to the manufacturer's instruction.

Fragments of genomic DNA, corresponding to coding regions of particular tested genes, were amplified by PCR with the use of primers listed in Table 1. For amplification of fragments of laccases' genes, following programs were employed: (i) for *laccase1*, initial denaturation at 94°C for 4 min, followed by 39 cycles of denaturation (94°C for 30 sec), annealing (45°C for 30 sec), and extension (72°C for 45 sec), and final extension at 72°C for 10 min; (ii) for *laccase2*, initial denaturation at 94°C for 4 min, followed by 39 cycles of denaturation (94°C for 30 sec), annealing (55°C for 30 sec), and extension (72°C for 45 sec), and final extension at 72°C for 10 min. Amplified DNA was separated by agarose gel electrophoresis and analyzed according to Sambrook and Russell (2001). The fragments of the coding sequences of *laccase1* and *laccase2* genes were verified by analysis of DNA sequences.

RESULTS AND DISCUSSION

Total DNA was isolated from samples of legs of either normal or malformed apollo butterflies. Because of the very limited amount of available biological material and low DNA concentrations obtained after the isolation, PCR assays with primers designed for *laccase1* or *laccase2* genes were performed on separate samples, derived from different individuals. Before these tests, the quality of each DNA template was proved by PCR reactions with primers for amplification of fragments of *decapentaplegic* (*dpp*), *hedgehog* (*hh*), and *patched* (*ptc*) genes (Table 1).

The primers for PCR-mediated amplification of fragments of *laccase1* and *laccase2* genes were designed on the basis of DNA sequences of other Lepidoptera, present in GenBank database, *Manduca sexta* and *Bombyx mori*, respectively (Table 1). The usefulness of these primers in amplification of the tested genes with DNA templates from *P. apollo* was confirmed by using samples from normal individuals. The sizes of amplified DNA products were either close to that expected for *M. sexta laccase1* gene (95 bp with *P. apollo* template vs. 112 bp for *M. sexta*) or exactly as expected for *B. mori laccase2* gene (131 bp).

The presence of the 95 bp PCR product after reactions with the *laccase1* primers and DNA templates from normal *P. apollo* individuals and those with deformed or reduced wings was tested. Among 21 normal apollo butterflies tested, the *laccase1*-specific product was detected in samples from 20 individuals (Table 2). Different results were obtained with samples from malformed *P. apollo* individuals, as the PCR product was present after reactions with templates derived from 9 out of 12 individuals with deformed wings, and only in 3 out of 17 individuals with reduced wings (Table 2).

Analogous experiments were performed with the use of primers specific for the *laccase2* gene. In these experiments, the 131 pb DNA reaction product was detected in samples from all tested (11 individuals) normal apollo butterflies, but only in 1 out of 7 insects with deformed wings and in none of those (11 individuals) with reduced wings (Table 3).

The results presented in Tables 2 and 3 indicate that in samples derived from a large fraction of apollo butter-

Table 1. Primers

Gene	Primers (forward and reverse)	Reference
dpp	5′ AGA GAA CGT GGC GAG ACA CTG 5′ GAG GAA AGT TGC GTA GGA ACG	Kapan *et al.,* (2006)
hh	5′ AAG GAA AAA CTG AAT ACG CTG GC 5′ CGA GAC GCC CCA ACT TTC C	Kapan *et al.,* (2006)
ptc	5′ CTC CGA AGA AGG TCT GCC GCA AG 5′ AAT TCG TGC TCG TCG TAT TTT C	Kapan *et al.,* (2006)
laccase1	5′ TTT ACA TTC CCG TTC ACA T 5′ GAT TCT AAG TAA ATA CGC AAT GA	This work, designed on the basis of the *laccase1* cDNA of *Manduca sexta* (Dittmer *et al.,* 2004), using the OLIGO 6.7 software (Molecular Biology Insights Inc., Colorado Springs, CO)
laccase2	5′ GTG CTG GGT TCC TTC TCA ATT CA 5′ GCT GAG TGG AGG CGT TAG GAA GA	This work, designed on the basis of the *laccase2* cDNA of *Bombyx mori* (Yatsu & Asano, 2009), using the OLIGO 6.7 software (Molecular Biology Insights Inc., Colorado Springs, CO)

Table 2. Results of PCR-mediated DNA amplification with the use of indicated templates and primers specific to the *laccase1* gene

Group of *P. apollo* individuals tested	Number of individuals used for DNA isolation[a]		
	All tested	With *laccase1* specific PCR product	Without *laccase1* specific PCR product
Normal	21	20	1
Deformed wings	12	9	3
Reduced wings	17	3	14

[a]Statistical significance of the differences between results of various experiments was assessed using the Fisher exact test. The *P* value for normal individuals *vs*. malformed insects (the sum of individuals with deformed and reduced wings) was 0.000062.

flies with deformed or reduced wings, it was not possible to detect specific PCR products characteristic for *laccase1* or *laccase2* genes. This suggest that in malformed *P. apollo* individuals from the Pieniny National Park, many have defects in genes coding for *laccase1* or *laccase2*, and possibly they are also deficient in activities of these enzymes. On the basis of the presented analysis, it is not possible to determine what kind of mutations occur in malformed butterflies. Large or small deletions are possible, as are point mutations, leading to allelic variations, that prevent efficient alignments of primers to DNA templates during PCR amplification. Nevertheless, some genetic defects in *laccase1* and *laccase2* loci appear correlated with deformation or reduction of wings in *P. apollo* individuals. On the other hand, we cannot exclude that a functional *laccase* gene is closely linked to another gene which dysfunction is responsible for the phenotype rather than laccase enzyme deficiency, and a large deletion resulted in the absence of this putative gene.

Genes coding for laccases are differentially located in genomes of insects. For example, in the *Tribolium castaneum* genome (GenBank accession no. NC_007417) different laccases' genes are completely separated, while in the *Papilio xuthus* genome (GenBank accession no. NW_013530391) such genes are linked, with the distance between genes up to 30 kb. The complete genome sequence of *P. apollo* is not known, however, since *Papilio* and *Parnasius* genera belong to the same family, it seems plausible that *laccase1* and *laccase2* genes are also linked in apollo butterfly. If so, a large deletion could result in defects of both genes.

Since some individuals with deformed and reduced wings appeared to had wild-type *laccase1* and *laccase2* genes, and we could not find a *laccase1*-specific PCR product in one normal apollo butterfly, it is obvious that dysfunctions of laccases cannot be the sole cause of malformations of *P. apollo* wings. However, *laccase2* is necessary for proper cuticle sclerotization, and *laccase1* was found in various insect tissues and organs (Claus 2004; Dittmer & Kanost, 2010, Jeon *et al.,* 2012, Moin & Omar, 2014). Moreover, these enzymes play roles in detoxification of phenol-derived compounds present in food and maintenance of iron homeostasis (Coy *et al.,* 2010; Lang *et al.,* 2012; Prasain *et al.,* 2012; De Fine Licht *et al.,* 2013). Therefore, one might speculate that deficiencies in activities of laccases could contribute to dysmorphology of insects due to either disturbed developmental processes as a result of improper cuticle sclerotization or toxic effects of food-derived compounds. The main food of *P. apollo* larvae from Pieniny National Park consist of *Sedum telephium* ssp. *maximum* and *S. acre* plants. These plants contain various bioactive compounds which, when not detoxicated, could be deleterious for insects; the examples are alkaloids, polyphenols, terpens, flavonols, tannins (Adamski *et al.,* 2000; Castilho & Kaplan, 2008; Altavilla *et al.,* 2008) or even alkaloids characteristic for *Sedum*, like sedinone, sederine, sedacryptine, sedacrine (Colau & Hootelé, 1983).

One might assume that potential disturbances in cuticle sclerotization could cause defects in structures of various insect organs, not only wings. We did not observe other severe morphological changes in the apollo butterfly specimens with deformed or reduced wings (Fig. 1). However, anatomical structures of these insects were not investigated due to the protection of *P. apollo* and the rules of the collection of the Pieniny National Park. Therefore, it is possible that other developmental defects are present in the malformed individuals.

In conclusion, the results presented in this report suggest that deficiency in laccases might contribute to deformation and reduction of wings of *P. apollo* individuals from Pieniny National Park. Nevertheless, this deficiency cannot be ascribed as a sole cause of these malformations. Thus, other factors or agents responsible for such phenotypes remain to be discovered.

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Table 3. Results of PCR-mediated DNA amplification with the use of indicated templates and primers specific to the *laccase2* gene

Group of *P. apollo* individuals tested	Number of individuals used for DNA isolation[a]		
	All tested	With *laccase2* specific PCR product	Without *laccase2* specific PCR product
Normal	11	11	0
Deformed wings	7	1	6
Reduced wings	11	0	11

[a]Statistical significance of the differences between results of various experiments was assessed using the Fisher exact test. The *P* value for normal individuals *vs*. malformed insects (the sum of individuals with deformed and reduced wings) was 0.000000.

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