De novo transcriptome assembly of the midgut glands of the herbivorous land crab *Chiromantes haematocheir*, and identification of laccase genes involved in lignin degradation

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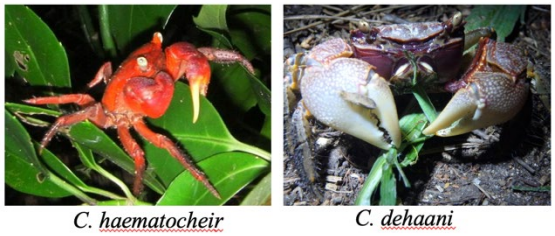
Research objective

Many land crabs are herbivores. *Chiromantes haematocheir* and *C. dehaani* are herbivorous land crabs that live in Japanese maritime forests, where they often eat decayed wood and fallen or green leaves (Fig. 1). Therefore, herbivorous land crabs are thought to degrade plant biomass, and in particular lignocellulose. The latter consists of three major components: cellulose, hemicellulose, lignin, and tannins. We recently reported guaiacol oxidase and cellulase activities in the midgut glands of the land crabs *C. haematocheir* and *C. dehaani* (Miyake et al. 2019). Since guaiacol oxidation is probably involved in lignin degradation, these land crabs seem to endogenously express lignin degrading enzymes. Detailed genetic information is required to study the molecular basis of the degradation of complex plant molecules such as lignin. However, the lack of genomic and transcriptomic resources for land crabs strongly limits molecular studies. RNA sequencing (RNA-seq) is a powerful tool that is widely used to profile the genomes of non-model species. In particular, *de novo* sequencing and assembly of the transcriptome represent the first step for the identification of genes involved in enzymatic activities in non-model animal species. In this study, we sequenced and assembled the transcriptome of *C. haematocheir* midgut glands. Genes encoding putative enzymes involved in the degradation of biopolymers, such as lignin and cellulose, were identified, and their expression was further characterized. Here we report the analysis of a laccase gene, which showed the highest expression level in the midgut glands of this land crab species. The properties of the corresponding laccase protein and its role in plant biomass degradation were studied. To assess whether this laccase could oxidize lignin-related substrates, we expressed this enzyme in *Escherichia coli,* purified it, and examined its activity.



C. haematocheir *C. dehaani*

Fig. 1 Land crabs eating plant diets

Methods

Total RNA was purified from 30 mg of midgut glands using an RNeasy Plus Mini kit (Qiagen) according to the manufacturer's instructions. RNA sequencing was performed by Hokkaido System Science Co., Ltd. (Sapporo, Japan). The libraries were subjected to 100-bp paired-end sequencing on an Illumina HiSeq 2500 instrument (Hokkaido System Science Co., Ltd).

To investigate laccase activity, we used 2,6-dimethoxyphenol (2,6-DMP) as a substrate. The reaction was initiated by mixing 100 μL of enzyme solution with 900 μL of a 4-mM substrate solution. To estimate the oxidation rate of 2,6-DMP to coerulignone at 25 °C, absorbance was measured at 470 nm every minute for 5 min using a UV-VIS Spectrophotometer UV-1280 (Shimadzu, Kyoto, Japan).

Laccase was produced as a C-terminal His_6-tagged enzyme. Transformant clones were cultured in 100 mL of LB medium supplemented with 100 μg/mL ampicillin at 37 °C. When the cultures reached an A_{600} of 0.6, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 0.5 mM $CuSO_4$ were added, and incubation was continued for 4 h. Cells were then collected by centrifugation at 8000 ×*g* for 10 min at 4 °C. Laccase was purified using a HisTALON™ gravity column purification kit (Clontech Laboratories, Inc., Mountain View, CA, USA).

Results

Land crabs were reared with plant or non-plant diets to study the effect of diet on laccase activity. After the preparation of crude extracts from digestive organs, enzyme activity was measured. As shown in Fig. 2, significant laccase activity against 2,6-DMP was detected only in land crabs fed with Zelkova leaves (plant diet). Laccase activity was mainly detected in midgut gland samples, although several cardiac stomach samples also showed significant activity. Nevertheless, laccase activity in the stomach was consistently lower than that in midgut glands. These results suggest that laccase is synthesized within the midgut glands and secreted into the digestive fluid of gut organs such as the cardiac stomach.

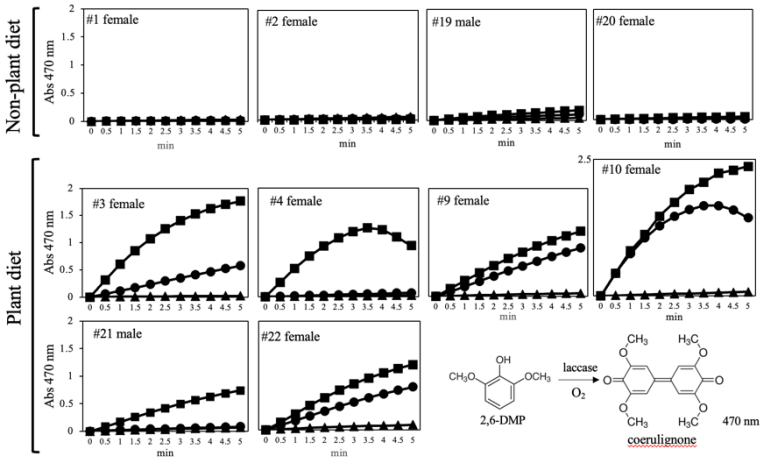


Fig. 2 The 2, 6-DMP oxidation activities of *C. haematocheir* land crabs. Six land crabs were reared with plant diets (Zelkova leaves) and four crabs with non-plant diets. Crabs were dissected, and crude extracts of cardiac stomachs (circles), midgut glands (squares), and hindguts (triangles) were analyzed.

Based on *de novo* assembly and annotation of RNA-seq reads, 22 contigs were identified

as laccase gene fragments. These were then consolidated into 11 different genes, among which the expression of TRINITY_DN24131 (24131) accounted for more than 99% of the total laccase expression. BlastP analysis against the latest version of the database showed that the amino acid sequence of the 24131-laccase was highly similar to that of the predicted laccase proteins of *Portunus trituberculatus* (swimming crab) (75.47%), *Callinectes sapidus* (blue crab) (74.6%), and *Penaeus vannamei* (Pacific white shrimp) (67.65%). The 24131-laccase consists of 613 amino acids, and exhibits a deduced molecular mass of 67.708 kDa and an estimated pI of 4.17. The search for a conserved domain based on BlastP analysis revealed a Cu-binding domain at the C terminus of this protein. This domain was similar to the cupredoxin domains of insect laccases, ascorbate oxidase, and multicopper oxidase. These laccase-like proteins harbor signal sequences in their N-terminal regions, indicating that they are secretory enzymes.

We attempted to verify the function of the 24131-laccase by introducing its cDNA into the pETBlue-2 expression vector. This enzyme was produced as a His_6-tagged protein in *E. coli* BL21 (DE3) and purified using a His_6-tag-binding resin. After purification of His_6-tagged 24131-laccase, we analyzed its enzymatic activity by performing oxidation assays using 4 mM 2,6-DMP. Notably, the purified 24131-laccase exhibited significant oxidation activity against 2,6-DMP. As expected, the addition of $CuSO_4$ to the culture medium was found to be essential for laccase activity.

Conclusion

The present study is the first to report the laccase activity of land crabs. Nevertheless, it is still unclear whether crab laccases are involved in the assimilation of lignin. We speculate that crabs may use laccases to remove barriers or deterrents, such as lignin or tannins, in order to utilize carbohydrates. In possible future biotechnological applications, these enzymes may be useful for the conversion of lignin structures, which may improve the extraction of carbohydrates such as cellulose and hemicellulose. For further applications of these putative lignin degrading enzymes, it is pivotal to improve the production of 24131-laccase and identify a guaiacol oxidase.

References

1） Miyake, K., Ura, K., Chida, S., Ueda, Y., Baba, Y., Kusube, T., and Yanai, S. (2019) Guaiacol oxidation activity of herbivorous land crabs, *Chiromantes haematocheir* and *Chiromantes dehaani. J. Biosci. Bioeng*. **128:** 316-322.

2） Miyake, K. and Baba Y. (2021) *De novo* transcriptome assembly of the midgut glands of herbivorous land crabs, *Chiromantes haematocheir*, and identification of laccase genes involved in lignin degradation. *J. Comp. Physiol. B* in press.