RESEARCH ARTICLE

Evidence of *Bombyx mori* (Lepidoptera: Bombycidae) odorant receptors related to oviposition behavior

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Abstract: The silkworm, *Bombyx mori*, is an insect that is economically important for silk production, cosmetics, medical applications, food, and scientific research. The oviposition behavior of the female moth affects the number of eggs and the volume of silk production. This research aimed to investigate the relationship between the various treatment conditions of mulberry odor, the expression levels of *Bombyx mori* odorant receptor genes (*BmOrs*) in the antenna of female *Bombyx mori* moths, and the moths' oviposition behavior. Four treatment conditions (fresh mulberry leaves, 2% mix, mulberry leaf juice, and 2% powder) showed a higher oviposition rate than that of the control. Our results revealed that the expression levels of *BmOr44*, *BmOr54*, *BmOr56*, and *BmOr63* might play a major role in oviposition. The predicted three-dimensional structures of BmOr44, BmOr54, BmOr56, and BmOr63 proteins were found similar and some active compounds of mulberry leaf could virtually bind to these proteins. The expression patterns of *BmOr19* and *BmOr30*, the specific female adult moth odorant receptor genes, were similar in almost every treatment.

Keywords: Silk moth, olfactory receptors, egg laying

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1 Introduction

The silkworm, *Bombyx mori* (L.) (Lepidoptera: Bombycidae) is essential for silk production, has been used as a model organism in scientific research, and is used in the medical and cosmetic industry for the manufacture of, for example, surgical thread. Silk has also been used to manufacture parachutes, tire lining, electrical insulation, and artificial blood vessels (Saad et al., 2019). The silkworm has also been used as a bioreactor to produce recombinant proteins (Li et al., 2022). The production of silk is a major industry in many developing countries, and China produces about 80% of the world's raw silk (Saad et al., 2019).

Moreover, the larvae and pupae of silkworm are one of the insect-based nutritious food sources with high vitamins and proteins (Zhou et al., 2022). Silkworm eggs showed a high level of vitamin B, sugars, and fat (Buhroo et al., 2018). Previous studies demonstrated that silkworm pupae protect the liver, inhibit apoptosis, enhance immunity, inhibit cancer, and regulate blood glucose and blood lipids (Mahanta et al., 2023).

Insects, including *B. mori*, use their olfactory responses to environmental odorants to locate mates, find suitable places for oviposition, and avoid predators (Tanaka et al., 2009). The antennae of insects serve as an essential peripheral olfactory system, housing different types of sensilla, the sensory structures that play an important role in detecting and processing odorants (Qiu et al., 2018). The various types of odors bind to the corresponding odorant receptors (Ors). In previous studies, 62 Ors were found in *Drosophila melanogaster* (Clyne et al., 1999, Gao and Chess, 1999, Vosshall et al., 1999), 79 Ors were found in the *Anopheles gambiae* mosquito (Hill et al., 2002), and 170 Ors were found in Apis mellifera (Robertson and Wanner, 2006).

Sixty-eight Ors were identified in the *Bombyx mori* genome. The expression levels of some *BmOr* genes were found in both male and female adult silkworms. Other *BmOrs* genes were found to have their expression exclusively in specific stages. A previous study showed that silkworm behavior was affected by natural chemoattractants and their corresponding Ors. For example, Tanaka et al. (2009) found that *Bombyx mori* silkworms prefer the cis-jasmone compound in mulberry leaves, which might be mediated by *BmOr56*.

The protein structures of Ors possess seven transmembrane receptors (Sakurai et al., 2014, Del Mármol et al., 2021, Wicher and Miazzi, 2021, Ha and Smith, 2022) that differ from the G-protein-coupled receptor (Del Mármol et al.,

2021). It was found that MhOr5 of Machilis hrabei showed the function of odorant-gated ion channels (Del Mármol et al., 2021) suggesting that Ors are activated by the odors. One type of odor can activate various kinds of Ors, while many types of odors can activate the same Ors (Wicher and Miazzi, 2021).

Bombyx mori preferentially lives on the leaves of the white mulberry. After the female moth has been fertilized, it flies to the host plants to spawn (Tanaka et al., 2009). In this study, the oviposition behavior and the expression levels of ten candidate *BmOr* genes, *BmOr2*, *BmOr14*, *BmOr19*, *BmOr29*, *BmOr30*, *BmOr36*, *BmOr44*, *BmOr54*, *BmOr56*, and *BmOr63*, were investigated upon the various conditions of mulberry odors. Some *BmOrs* were found to correspond to the oviposition behavior; their functions will be further investigated. The expression levels of the other *BmOrs* and their roles in oviposition behavior will also be elucidated.

2 Materials and methods

2.1 Moth treatment conditions for oviposition

Moth of silkworm strain J108 was obtained from The Queen Sirikit Department of Sericulture (QSDS), Bangkok, Thailand. This research was done under the certificate of approval for animal care and use for scientific research of Kasetsart University (ACKU65-SCI-031). Silkworms were raised in the rearing rooms (6x8 meters) with controlled temperature and relative humidity. The 1^{st} to 3^{rd} instar larvae were raised at 25-28ºC with relative humidity 80-90%. The 4^{th}-5^{th} instar larvae were raised at 23-26ºC with humidity 70%. The photoperiod used natural light. During the oviposition, the moths were covered with black cloth (Lim et al., 1990).

Eight treatment conditions were used in the experiments. Two of the eight conditions were mulberry leaf powder in different concentrations which were 2% powder 3% powder by dry weight (w/v). The mulberry leaves were dried, powdered, and soaked in hot water for six minutes. The filter cloth was sprayed with each concentration of mulberry powder and then dried out for use in the experiments. The other three treatment conditions were mulberry leaf powder in three different concentrations by dry weight of powder (w/v) mixed with 4% wet cassava glue (1% mix, 2% mix, 3% mix). The filter cloth was sprayed with a mixture of each concentration and was then used in the experiments as it was drying. The other two conditions were fresh mulberry leaves and mulberry leaf juice. For the former, the filter cloth was placed on fresh mulberry leaves and, in the latter, the filter cloth was soaked in leaf juice and used after it had dried. For the control condition, moths were placed on a drying filter cloth that was soaked in 4% wet cassava glue. After fertilization, the female moths were left on the prepared filter cloths (Lim et al., 1990). After six hours, the female moths were preserved in 95% ethanol and the oviposition eggs and the eggs remaining in the abdomen were counted. Ten moths were used for one replication of each condition, and three replications were performed. The oviposition rates were then calculated by [oviposition eggs/(oviposition eggs + eggs remaining in the abdomen)] x 100. The moths' antennae were collected for RNA extraction.

2.2 Expression levels of *Bombyx mori* odorant receptor (*BmOrs*) genes by qRT-PCR

The total RNA was extracted from the antennae using TRIzol® reagent (Thermo Fisher Scientific, MA, USA). The extracted RNA was converted to cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA). The cDNA from each treatment condition was used as the template for qRT-PCR. The qRT-PCR reactions were performed in 10 µL of a mixture containing 1 µL of cDNA template, 5 pmol of each primer, and 1X of iTaq universal SYBR® green supermix (BIO-RAD, CA, USA). The amplification condition was two minutes at 95°C, followed by 35 cycles of 20 sec at 95°C, 15 sec of an annealing temperature at 55°C, and 20 sec at 72°C. The melting curve analysis was performed at 60–95°C (0.5°C increments at 5 sec/step). Ten *Bombyx mori* odorant receptor (*BmOrs*) gene-specific primers (Table 1) were used, and the *actin* gene (Gene ID: 100145915) was used as the reference for transcript quantification. Six antennae were used for one replicate in each condition. Three replicates were conducted. The $\triangle\triangle C_T$ was determined and the gene expression levels were calculated by the $2^{-\triangle\triangle C}{}_T$ method using actin as the control.

Table 1. Specific primers for *olfactory receptor (OR)* gene and *actin* gene for qRT-PCR

Gene ID	Name	Sequence (5'-3')
100145915	*actin*	F: CTCGCCTCCCTCTCTACCTT R: CAACAACAACATTCCGTTCG
692613	*BmOr2*	F: CAGCGGGCCACTTCTTATTC R: TGTTGATGCCCATGCAGATG
100101192	*BmOr14*	F: GAGCGACCTCAACAACACAA R: TAAGCTAGATGGTCGCGGTT
778490	*BmOr19*	F: GTAACCGTTGTCCTGATGGC R: TGGTTGCTGTGCTTTGTTCA
100127042	*BmOr29*	F: CTGGCACGGTAATGACGTTT R: CATGTCTGACGCGAAGTTGT
778491	*BmOr30*	F: GTTAACCAGAGCCACAGAGC R: CCCAGGCAGCGTTCATTAAG
100127030	*BmOr36*	F: AGCGTTTGCATCGTTCAGAT R: ACTAGTTCGCTTTGAACCGT
100127045	*BmOr44*	F: ATGCAGATGGTTTGGATGGC R: CAAATTACGCCAAGGACCGT
100144598	*BmOr54*	F: AGTTTGGCTGGGTTCTCGAT R: AACCACCAGCTGTTATTGCC
100379311	*BmOr56*	F: TGGCGTGACCTCATTGGTTA R: TGCCACGGTCGTATACATCA
100379315	*BmOr63*	F: ACCCTTACGACACCTCCAAG R: TTCAAGTCTTGGCCTAGCGA

*F = forward

R = reverse

2.3 Statistical analysis

The oviposition rates of moths across eight conditions were assessed by setting the oviposition rates as variables and the factors were conditions. The relative gene expression levels for the eight treatments were compared. The variables were relative expression levels of *BmOr* genes and the factors were treatments. The statistical analysis included calculation of standard error of the mean (SEM), F values, and P values were examined using ANOVA (IBM SPSS ver. 23, 2015). Furthermore, the least significance difference (LSD) test was employed as the post hoc test.

2.4 Amino acid sequence alignment and three-dimensional structures of *BmOr* proteins

The amino acid sequences of BmOr44, BmOr54, BmOr56, and BmOr63 with the UniProtID C4B7X3, B1B1Q4, C4B7Y4, and B1B1Q6, respectively, were aligned using Clustal Omega (Madeira et al., 2024). The predicted 3D structures of these proteins were obtained from AlphaFold (Jumper et al., 2021, Varadi et al., 2024).

2.5 Molecular docking of *BmOr* proteins with major active compounds in mulberry leaf

Five major active compounds, phytene-2, phytol, hexanal, cis-jasmone, linalool, in mulberry leaf were selected for molecular docking with BmOr44, BmOr54, BmOr56, and BmOr63 proteins. The 3D structures of these compound were obtained from ChemSpider database (https://www.chemspider.com). The molecular docking procedure was performed by iGEMDOCK (Hsu et al., 2011).

3 Results

3.1 Oviposition rates of moth

The observed oviposition rate of moths in each condition was shown in Table 2. Four treatment conditions: fresh mulberry leaves, 2% mix, mulberry leaf juice, and 2% powder, showed a higher oviposition rate than that of the control. The highest oviposition rate was 94.2% from moths that were treated with fresh mulberry leaves followed by the 2% mix, leaf juice, and 2% powder conditions. Moths treated with 1% and 3% mix, and 1% and 3% powder showed oviposition rates of less than 90%, which was lower than that of the control. The variance of oviposition rate among eight conditions were not significantly different (df between groups = 7, df within groups = 16, F = 4.31, P =0.07). The LSD post hoc test revealed that the oviposition rate on 3% powder, the lowest value, was significantly different from all treatments except 1% mix. The oviposition rate of fresh mulberry leaves was significantly higher than that of the 3% powder, 1% and 3% mix. The average total egg numbers per moth was 493.8±18.1.

Table 2. *Bombyx mori* oviposition rates of eight conditions

Condition	Oviposition rate (%) Mean±SEM
3% powder	85.5±1.5
1% mix	88.9±1.7
3% mix	89.7±1.1
Wet glue (control)	91.9±0.8
2% powder	92.5±1.4
Mulberry leaf juice	92.5±1.8
2% mix	92.7±1.4
Fresh mulberry leaves	94.2±0.5

One-way ANOVA analysis
df between groups = 7
df within groups = 16
F = 4.31, P = 0.07 % powder: dried mulberry leaf powder (w/v) % mix: dried mulberry leaf powder (w/v) mixed with 4% wet cassava glue

3.2 Expression levels of *Bombyx mori* odorant receptor (*BmOrs*) genes

The expression levels of *BmOr* genes in all conditions were examined by qRT-PCR. The results of ten *BmOr* relative gene expression levels in each treatment condition were shown in Table 3. The F and *P* values of each gene were shown in Table 4 with the degrees of freedom (df) = 23 (df between groups = 7, df within groups = 16). *All P* values obtained from each gene were less than 0.05 indicating that the expression levels of each gene in at least two treatments were statistically significant difference.

Table 3. The mean of relative expression levels of ten candidate *BmOrs* genes of *Bombyx mori* in eight conditions. The actin gene was used as an internal control. Wet glue was the control condition

	BmOrs									
Treatments	02	14	19	29	30	36	44	54	56	63
3% powder	0.84	0.84	0.47	0.81	0.81	0.39	2.93	1.91	1.62	2.02
1% mix	4.50	18.90	1.02	16.06	5.86	2.25	22.84	19.90	9.04	4.10
3% mix	0.71	4.98	0.30	3.47	0.29	0.21	3.41	2.07	1.22	1.10
wet glue (C)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
2% powder	6.23	2.50	1.68	0.94	2.45	1.69	10.56	4.35	1.95	5.20
mulberry leaf juice	1.87	14.02	1.33	5.89	1.29	0.76	3.57	10.93	5.47	11.73
2% mix	2.20	7.26	0.83	22.33	1.98	1.21	8.64	9.89	36.70	3.75
fresh mulberry	0.32	0.49	0.40	0.30	0.32	0.36	0.36	0.29	0.42	0.35

% powder: dried mulberry leaf powder (w/v)
% mix: dried mulberry leaf powder (w/v) mixed with 4% wet cassava glue

The highest gene expression level was found in *BmOr56* at 2% mix whereas the lowest one was *BmOr36* at 3% mix. The expression levels of seven *BmOrs* (*BmOr2*, *BmOr14*, *BmOr29*, *BmOr44*, *BmOr54*, *BmOr56*, *BmOr63*) in fresh mulberry leaves, which gave the highest oviposition rate, were found to be lower than other conditions. In the 3% powder condition (lowest oviposition rate), six *BmOr* genes, *BmOr2*, *BmOr14*, *BmOr19*, *BmOr29*, *BmOr30*, *BmOr36*, were found to be lower than those of the control, whereas four other *BmOr* genes, *BmOr44*, *BmOr54*, *BmOr56*, *BmOr63*, were higher.

Except for a 1% mix and 2% powder, *BmOr19* and

Table 4. The analyzed statistical values based on relative gene expression levels of ten candidate *BmOrs* genes of *Bombyx mori* in eight conditions.

Gene	F value	*P*
BmOr2	4.9	0.004
BmOr14	4.6	0.006
BmOr19	4.1	0.009
BmOr29	3.9	0.012
BmOr30	6.5	0.001
BmOr36	7	0.001
BmOr44	24.1	0.001
BmOr54	19.7	0.001
BmOr56	6.3	0.001
BmOr63	4.7	0.005

One-way ANOVA analysis
df between groups = 7
df within groups = 16

BmOr30 expression levels shared similar patterns in other treatments as shown in Figure 1. In contrast, the expression patterns of *BmOr14* and *BmOr36*, which were expressed in both male and female adult moth antennae (Tanaka et al., 2009) were not similar. The expression levels of *BmOr14* in all treatment conditions were higher than those of *BmOr36* as shown in Figure 2. In almost all conditions, except fresh mulberry leaves, the expression levels of four *BmOr* genes, *BmOr44*, *BmOr54*, *BmOr56*, and *BmOr63* were higher than those of the control. The expression of these four genes was also found in larval, adult male, and adult female antennae (Tanaka et al., 2009).

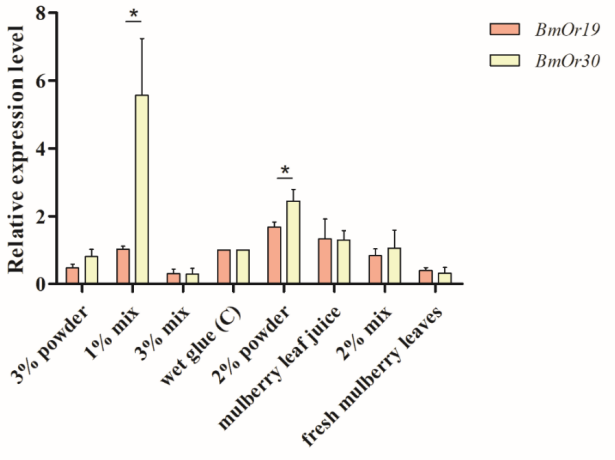

Figure 1. Relative gene expression levels of *Bombyx mori BmOr19* and *BmOr30*. The expression levels of *BmOr19* and *BmOr30* genes were significantly different in 1% mix and 2% powder. * P 0.05. (% powder: dried mulberry leaf powder (w/v); % mix: dried mulberry leaf powder (w/v) mixed with 4% wet cassava glue)

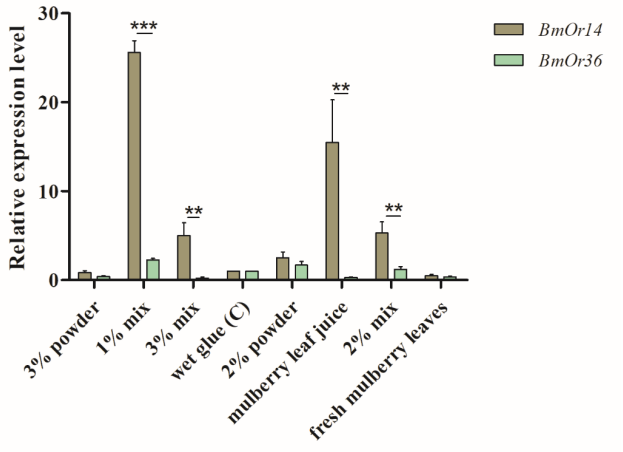

Figure 2. Relative gene expression levels of Bombyx mori *BmOr14* and *BmOr36*. The expression levels of *BmOr14* were found to be significantly higher than those of *BmOr36* genes in 1% mix, 2% mix, 3% mix and mulberry leaf juice. ** P 0.01, *** P 0.001. (% powder: dried mulberry leaf powder (w/v); % mix: dried mulberry leaf powder (w/v) mixed with 4% wet cassava glue)

3.3 The comparison of *BmOr* gene expression levels among 3% powder, wet glue, and fresh mulberry leaves conditions

The *BmOr* gene expression patterns of 3% powder (the lowest oviposition rate), wet glue, and fresh mulberry leaves (the highest oviposition rate) were compared. The expression levels of six *BmOr* genes, *BmOr2*, *BmOr14*, *BmOr19*, *BmOr29*, *BmOr30*, and *BmOr36*, from the 3% powder condition were higher than those of the fresh mulberry leaves. Nonetheless, all of them were still lower than those of wet glue (control) as shown in Figure 3. Interestingly, the relative expression levels of *BmOr44*, *BmOr54*, *BmOr56*, and *BmOr63* in the fresh mulberry leaves condition were lower than those for 3% powder and wet glue.

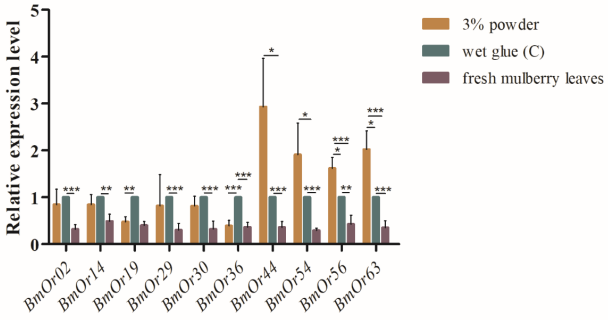

Figure 3. Relative gene expression levels of ten *Bombyx mori BmOrs* in 3% powder, wet glue, and fresh mulberry leaves conditions. * P 0.05, ** P 0.01, *** P 0.001. (% powder: dried mulberry leaf powder (w/v))

3.4 Amino acid sequence identity and three-dimensional structures of *BmOr* proteins

The percentages of amino acid sequence identity of *BmOr44*, *BmOr54*, *BmOr56*, and *BmOr63* were shown in Table 5. All the percentages of amino acid sequence identity were found lower than 20%. However, the 3D structures of these *BmOr* proteins were similar as shown in Figure 4. These proteins were composed of 4-7 helical transmembrane domains.

Table 5. Percentage of amino acid sequence identity of four BmOr proteins

	BmOr44	BmOr54	BmOr56	BmOr63
BmOr44		12.6	14.8	14.7
BmOr54			15.0	11.8
BmOr56				19.1
BmOr63				

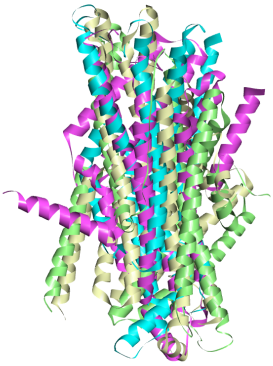

Figure 4. The superimposed of predicted 3D structures of four BmOr proteins, BmOr44 (cyan), BmOr54 (magenta), BmOr56 (yellow), and BmOr63 (green). The illustration was made by BIOVIA, Discovery Studio Visualizer v.21.

3.5 Molecular docking of *BmOr* proteins with major active compounds in mulberry leaf

Five major active compounds, phytene-2, phytol, hexanal, cis-jasmone, linalool, in mulberry leaf could interact with the *BmOr* proteins. The binding free energy values of these compounds with *BmOr* proteins at non-transmembrane regions were shown in Table 6. The lowest free energy value at -84.95 kcal/mol was found between phytol and *BmOr63*. The binding poses of compounds and *BmOr* proteins were shown in Figure 5 A-D.

Table 6. The binding free energy values of active compounds with BmOr proteins at non-transmembrane regions

BmOr proteins	Active compounds	Binding free energy
BmOr44	Hexanal	-48.81
	Phytol	-81.89
BmOr54	Phytene	-77.3
	Linalool	-55.64
	Phytol	-76.43
BmOr56	Linalool	-57.32
	Hexanal	-43.58
	Phytol	-84.95
BmOr63	Cis-jasmone	-58.35
	Linalool	-53.41
	Hexanal	-43.79

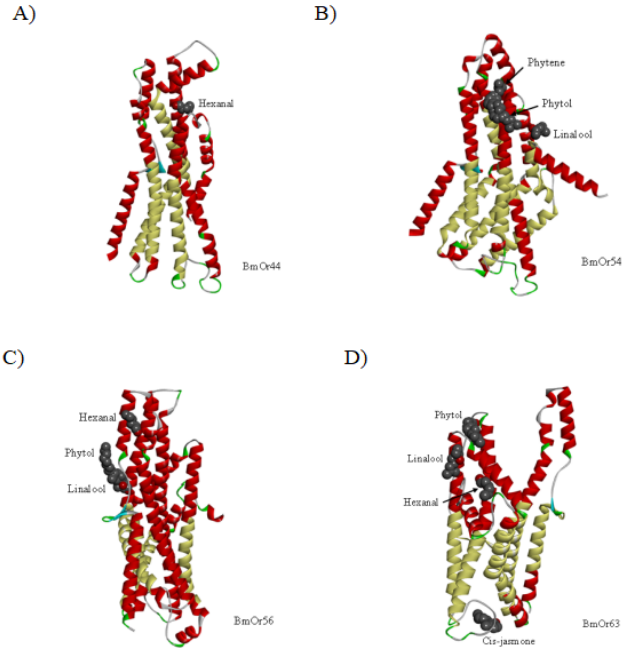

Figure 5. The binding poses of compounds and BmOr proteins A) BmOr44 with hexanal B) BmOr54 with phytol, phytene, linalool C) BmOr56 with phytol, linalool, hexanal D) BmOr63 with phytol, cis-jasmone, linalool, hexanal. The yellow color showed the transmembrane regions. The illustration was made by BIOVIA, Discovery Studio Visualizer v.21.

4 Discussion

In a previous study, moths preferred mulberry leaves as oviposition sites due to the scent compounds that significantly induce egg oviposition. Two mulberry leaf volatiles, valencene,

and alpha-humulene, were revealed as the active compounds affecting egg laying (Damodaram et al., 2014). Another scent compound that was identified as a potent attractant in mulberry leaves for silkworms was cis-jasmone (Tanaka et al., 2009). For the conditions of mulberry mix and mulberry powder, 2% mix and 2% powder were shown to increase the oviposition rate of moths, while 1% and 3% were shown to reduce the oviposition rate. These results implied that the level of active compounds at 2% was more suitable for egg laying. However, the fresh mulberry leaf treatment was the condition the moth preferred for egg laying.

The fresh mulberry leaves contained various types of active compounds (Tanaka et al., 2009, Win et al., 2022), i.e. phytene-2, phytol, hexanal, cis-jasmone, linalool. Different *BmOr* genes could respond to several odorants resulting in the diverse behaviors of moths. It is possible that one kind of odor could affect many types of *BmOrs*, and many types of odors could stimulate the same *BmOrs*. The types and the expression levels of these receptors might affect each other depending on the odorants. This outcome could determine the functions of the receptors and lead to the moths' behavior. Deletion of *BmOr2* or *Bombyx mori* odorant receptor co-receptor (*BmOrco*) decreased the response to bombykol and revealed the defective selection in mulberry leaves in silkworms (Liu et al., 2017).

Our result demonstrated that *BmOr19* and *BmOr30* expression levels shared similar patterns in almost all conditions. *BmOr19* and *BmOr30* appear to be specifically expressed in female antennae (Tanaka et al., 2009) and might detect specific odors that are critical to female behaviors, such as oviposition cues or male-produced courtship pheromones (Wanner et al., 2007). It was found that *BmOr19*, *BmOr29*, and *BmOr42* responded to linalool, one of the mulberry fresh leaf odors (Anderson et al., 2009, Tanaka et al., 2009). However, *BmOr30* was found to be down-regulated in the domestic silkworm (Qiu et al., 2018).

The expression levels of four *BmOr* genes, *BmOr44*, *BmOr54*, *BmOr56*, and *BmOr63*, were higher than those of the control in almost all conditions, except fresh mulberry leaves. This result implied that these four genes might play an important role in egg laying. Disruption of their expression levels might affect the oviposition rate of moths. It was found that cis-jasmone which is the most potent attractant emitted from mulberry leaves, was recognized strongly by *BmOr56* and weakly by *BmOr54* in silkworms. Deletion of *BmOr56* could completely abolish the response of silkworms to cis-jasmone (Morinaga et al., 2023). The *BmOr63* did not respond to cis-jasmone but strongly responded to Henkel100 (Tanaka et al., 2009). Moreover, our results corresponded to the study of Qiu et al., 2018, which demonstrated that *BmOr44* and *BmOr56* were found down-regulated in domestic silkworms treated with mulberry leaves in an indoor environment. *BmOr44*, *BmOr54*, and *BmOr63* might interact with the other odorant compounds of fresh mulberry leaves. The fresh mulberry leaves contained various types of active compounds that could induce the highest oviposition rate of female moths. The other treatments in this study were extracts from mulberry leaves so some of the active compounds might have become degraded or lost during the processing. This could disturb the functions of the odorant receptors that affect oviposition behavior.

Although the amino acid sequence identity among these four BmOr proteins, BmOr44, BmOr54, BmOr56, and BmOr63, were less than 20%, but the predicted 3D structures of them were similar implying the related functions of these odorant receptors. In silico analysis indicated that the active compounds in mulberry leaf interact differently with each odorant receptor, thereby revealing the distinct functions of these receptors.

Our results show that four *BmOr* genes, *BmOr44*, *BmOr54*, *BmOr56*, and *BmOr63*, might play an important role in the oviposition mechanism. This research is the first step in screening the relationship between *BmOr* genes and moth oviposition so it could be used to study the role of odorant receptor genes in the future.

Conflict of Interest

Authors declare no conflict of interests while preparing this article.

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