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Transcriptome Analysis and Characterization of Chemosensory Genes in the Forest Pest, *Dioryctria abietella* (Lepidoptera: Pyralidae)

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In Lepidoptera, RNA sequencing has become a useful tool in identifying chemosensory genes from antennal transcriptomes, but little attention is paid to non-antennal tissues. Though the antennae are primarily responsible for olfaction, studies have found that a certain number of chemosensory genes are exclusively or highly expressed in the non-antennal tissues, such as proboscises, legs and abdomens. In this study, we report a global transcriptome of 16 tissues from *Dioryctria abietella*, including chemosensory and non-chemosensory tissues. Through Illumina sequencing, totally 952,658,466 clean reads were generated, summing to 142.90 gigabases of data. Based on the transcriptome, 235 chemosensory-related genes were identified, comprising 42 odorant binding proteins (OBPs), 23 chemosensory proteins (CSPs), 75 odorant receptors (ORs), 62 gustatory receptors (GRs), 30 ionotropic receptors (IRs), and 3 sensory neuron membrane proteins (SNMPs). Compared to a previous study in this species, 140 novel genes were found. A transcriptome-wide analysis combined with PCR results revealed that except for GRs, the majority of other five chemosensory gene families in Lepidoptera were expressed in the antennae, including 160 chemosensory genes in *D. abietella*. Using phylogenetic and expression profiling analyses, members of the six chemosensory gene repertoires were characterized, in which 11 DabiORs were candidates for detecting female sex pheromones in *D. abietella*, and DabiOR23 may be involved in the sensing of plant-derived phenylacetaldehyde. Intriguingly, more than half of the genes were detected in the proboscises, and one fourth of the genes were found to have the expression in the legs. Our study not only greatly extends and improves the description of chemosensory genes in *D. abietella*, but also identifies potential molecular targets involved in olfaction, gustation and non-chemosensory functions for control of this pest.

Keywords: *Dioryctria abietella*, transcriptome, chemosensory gene, phylogenetic analysis, expression profile

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INTRODUCTION

Over the last decade, Illumina sequencing has been applied to a diverse range of insects regarding tissue transcriptomes (Kawahara et al., 2019; McKenna et al., 2019). Taking the Lepidoptera as an example, we refer to the studies on the identification of chemosensory-related genes derived from antennal transcriptomes, and find that at least 55 moth species, including *Dioryctria abietella* in this

study, have described chemosensory gene families involved in olfaction, gustation, development, insecticide resistance and even the sensation of humidity and temperature, i.e., odorant binding proteins (OBPs), chemosensory proteins (CSPs), odorant receptors (ORs), gustatory receptors (GRs), ionotropic receptors (IRs) and sensory neuron membrane proteins (SNMPs) (Agnihotri et al., 2016; Pelosi et al., 2018; Robertson, 2019; Xu, 2020; Montagné et al., 2021). Such great attention to chemosensory genes in moth antennae is attributable to their importance during the processes of smell and taste reception, as they mediate chemosensory-related behaviors of moths, such as the seeking of feeding or ovipositing host plants, the escape of dangerous signals and the recognition of conspecific partners (Cury et al., 2019; Zhang et al., 2019; Liu X. L. et al., 2020; Fleischer and Krieger, 2021). Through transcriptomic sequencing and analyses, we are able to explore the adaptation and specialization of moths to host, non-host plants or the constantly changing habitats, and identify candidate molecular targets associated with chemosensation, such as OBPs, CSPs, ORs, GRs, IRs, and SNMPs facilitating the screening of behaviorally active compounds by a reverse chemical ecology strategy.

During the process of olfactory reception in insects, two classes of binding proteins of OBPs and CSPs are responsible for filtering a variety of odorant molecules in the surrounding environment, as the first barriers for receiving and sensing chemical signals (Leal, 2013; Pelosi et al., 2018). Outside the olfactory roles, some members of OBPs or CSPs that are exclusively or highly expressed in gustatory or non-sensory organs have been found to be involved in additional physiological roles, such as taste, development, flight and insecticide resistance (Pelosi et al., 2018; Wang et al., 2020).

With the discovery of three chemosensory receptor gene families in *D. melanogaster* (Clyne et al., 1999, 2000; Benton et al., 2009), to date a large number of ORs, GRs and IRs have been identified and characterized in Diptera (Nozawa and Nei, 2007; Croset et al., 2010), Lepidoptera (Engsontia et al., 2014; Xu, 2020; Yin et al., 2021), Coleoptera (Andersson et al., 2019; Mitchell et al., 2020; Mitchell and Andersson, 2021), Hemiptera (Smadja et al., 2009; Liu Y. et al., 2021), and Hymenoptera (Ferguson et al., 2021; Obiero et al., 2021). In insects, ORs are expressed in sensilla basiconica and sensilla trichoidea separately responsible for general odorants (i.e., general ORs) and sex pheromones (i.e., pheromone receptors, PRs) (Krieger et al., 2009; de Fouchier et al., 2017; Guo et al., 2020), while olfactory sensory neurons expressing IRs are distributed in sensilla coeloconica sensitive to amines, acids, alcohols and aldehydes (Yao et al., 2005; Silbering et al., 2011; Rytz et al., 2013). Beyond olfaction, IRs are dedicated to the detection of humidity, temperature or tastants (Stewart et al., 2015; Enjin et al., 2016; Knecht et al., 2016; Hou et al., 2021). Compared with ORs and IRs, this GR gene repertoire possesses more variable gene numbers, ranging from 45 in *Manduca sexta* to 237 in *Spodoptera litura* (Koenig et al., 2015; Cheng T. et al., 2017). This variation is possibly correlated with host diversity, as members of an expanded bitter GRs clade primarily respond to plant secondary metabolites (Xu et al., 2016; Kasubuchi et al., 2018; Zhang et al., 2019). Besides the bitter receptors, other GR members in moths could detect sugars, fructose and carbon dioxide (CO_2), and share relatively high conservation in sequences and numbers (Zhang et al., 2011; Xu et al., 2012; Xu and Anderson, 2015; Ning et al., 2016; Xu, 2020).

Aside from the ORs, GRs, and IRs described above, a relatively small number of transmembrane proteins, SNMPs, are described in insects. In most species, two SNMPs are ubiquitous, representing SNMP1 and SNMP2 (Vogt et al., 2009). In Lepidoptera, the third SNMP gene subfamily was identified, bringing the numbers of SNMPs to three in some species (Liu et al., 2015; Zhang et al., 2020). More recently, a study reported 4 SNMP-encoding genes in a zygaenid species, *Achelura yunnanensis*, with two gene variants in the SNMP2 subfamily (Li G. C. et al., 2021). Although SNMP genes have been reported for over two decades, their functional studies are limited to members of the SNMP1 group which are involved in the sensing of sex pheromones (Pregitzer et al., 2014; Xu et al., 2021).

The coneworm, *D. abietella* (Lepidoptera: Pyralidae), is an oligophagous pest where its larvae feed on cones of the Pinaceae family, including the genus *Pinus*, *Abies fabri* (Mast.) Craib., *Picea abies* (L.) Karst., and *P. likiangensis* var. *linzhiensis* (Rosenberg and Weslien, 2005; Song et al., 2020; Tang et al., 2020). Early studies have reported sex pheromone components [(3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)–pentacosapentaene and (9*Z*,11*E*)–tetradecadienyl acetate] of this species and their application in fields (Löfstedt et al., 1983, 2012). Apart from that, terpene compounds produced by host plants have been implicated to be attractive to this pest (Tang et al., 2020). However, little is known about molecular mechanisms underlying intraspecific communication between female and male moths, as well as interspecific interaction between this pest and host plants. More recently, Xing et al. (2021) described six chemosensory gene repertoires from *D. abietella* by antennal transcriptome analysis (15 *OBPs*, 18 *CSPs*, 65 *ORs*, 5 *GRs*, 24 *IRs* and 5 *SNMPs*), but the majority of which were partial sequences. Moreover, after our transcriptome-wide sequencing and comparative analyses of 16 tissues including the antennae in this species, the numbers of *ORs*, *GRs*, *IRs,* and *SNMPs* reported in the previous study were summed down to 42, 4, 14, and 3, respectively. Thus, our study has expanded upon those results here with the identification of 42 *OBPs*, 23 *CSPs*, 75 *ORs*, 62 *GRs*, 30 *IRs* and 3 *SNMPs* in *D. abietella*, and provides an extensive resource for studying putative functional roles of chemosensory genes in this pest, coupled with expression profiling analyses.

MATERIALS AND METHODS

Insects and Tissue Collection

The last instar larvae and pupae of *D. abietella* were collected from pine cones of *Pinus armandii* in Zixi Mountain, Chuxiong city, Yunnan Province, China. Next, the larvae were reared in the laboratory until pupae emergence, and pupae were sexed and individually kept in cages. Emerged adults were supplied with 10% honey solution. Rearing conditions were as follows: 25 ± 1°C, 60 ± 5% relative humidity and a 12-h light/dark cycle.

For RNA sequencing (RNA–Seq) and expression profiling analyses, 16 tissues including 50 antennae, 100 proboscises, 20 heads without antennae and proboscises, 5 thoraxes, 3 abdomens, 30 legs, 50 wings of female or male moths, as well as 30 female pheromone glands with ovipositors and 30 male hairpencils were collected from 3–day–old moths. These collected tissues were immediately frozen in liquid nitrogen and ground by glass homogenizers. After completely homogenized, 1 mL of TRIzol reagent (TaKaRa, Dalian, Liaoning, China) was added to each tissue. The prepared samples were stored at −70°C until RNA extraction.

RNA Extraction and cDNA Preparation

Total RNAs of various tissues were extracted by using TRIzol reagent (TaKaRa, Dalian, Liaoning, China), according to the manufacturer's protocols. Next, genomic DNA was digested with gDNA Eraser at 42°C for 2 min. The purified RNA samples were used for construction of cDNA libraries and expression profiling analyses. In brief, cDNA templates of different tissues were synthesized with the PrimeScript™ RT reagent Kit (TaKaRa, Dalian, Liaoning, China).

Library Construction and Sequencing

First, we assessed the quality and quantity of RNA samples by using agarose gels, the NanoPhotometer® Spectrophotometer (IMPLEN, CA, United States), the Qubit® 2.0 Fluorometer (Life Technologies, CA, United States), and the Bioanalyzer 2100 System (Agilent Technologies, CA, United States), respectively. Next, 1 μg of total RNA for each tissue evenly mixed by three biological pools was used to constructed a cDNA library using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB Inc., United States). Lastly, the prepared libraries were sequenced with 150-bp paired-end reads on an Illumina HiSeq™ 2000 instrument.

After cDNA libraries were sequenced, redundant sequences were discarded from the resulting raw reads, including adapter, low quality and containing poly–N sequences. The quality-filtered reads (clean reads) were assembled into transcripts by Trinity v2.5.1 (Grabherr et al., 2011) as the transcript transcriptome. Further, the assembled transcripts were clustered by Corset v1.05 (Davidson and Oshlack, 2014), and the longest transcript for each cluster was selected as one unigene. All the unigenes were pooled into the unigene transcriptome.

Functional Annotation and Gene Expression Estimation

To predict putative functional roles of unigenes, we annotated the unigenes into different databases, including NR [National Center for Biotechnology Information (NCBI) non-redundant protein sequences], NT (NCBI non-redundant nucleotide sequences), PFAM (Protein family), KOG/COG (EuKaryotic Orthologous Groups/Clusters of Orthologous Groups), SwissProt, KO (KEGG Ortholog) and GO (Gene Ontology).

Expression levels of unigenes in different tissues were calculated by mapping clean reads to the reference transcriptome of unigenes using RSEM v1.2.15 with default parameters (Li and Dewey, 2011). The numbers of read counts for each gene were used to determine the expression levels by FPKM (fragments per kilobase of transcript sequence per millions base pairs sequenced) method (Trapnell et al., 2010). If one gene was identified only from the transcript transcriptome, its expression values in tissues were unavailable because the FPKM values were computed based on the reads mapped to the unigene transcriptome.

Gene Identification and Comparison

For the identification of chemosensory genes in *D. abietella*, two *de novo* transcriptomes were used, i.e., the transcript transcriptome and the unigene transcriptome. First, a homology-based search with TBLASTN, implemented in Geneious v10.1.3[1], was employed to identify genes from the transcriptomes, with queries of chemosensory genes from *D. abietella*, *Galleria mellonella*, *Ostrinia furnacalis,* and *Bombyx mori* (Zhang T. et al., 2015; Guo et al., 2017; Liu et al., 2018; Zhao et al., 2019; Jiang et al., 2021; Xing et al., 2021; Yin et al., 2021). In search of each query, we set *e*-value and maximum blast hits as $1e^{-5}$ and 20, respectively. In addition, the identified genes were iteratively blasted against the two stand-alone transcriptomes of *D. abietella* until no new genes were found.

To compare the numbers of chemosensory genes expressed in antennae of adult Lepidoptera, we summarized the studies on chemosensory genes identified from antennal transcriptomes. Specially, if one gene was identified only from antennal transcriptomes, it was thought to have the expression in the antennae. Alternatively, when one gene was found from the non-antennal tissues, but its transcription in the antennae was evidenced by reverse transcription (RT)–PCR or quantitative real-time PCR (qPCR), this gene was also counted as antennae-expressed genes. Detailed information on sequencing tissues, gene numbers and references were seen in **Supplementary Table 1**.

Phylogenetic Analysis

In the trees of OBPs and CSPs, the proteins from *B. mori*, *D. abietella* and *S. litura* were selected (Gong et al., 2009; Xiao et al., 2021). The OR tree was inferred with the ORs from *D. abietella*, *Helicoverpa armigera,* and *O. furnacalis* (Zhang T. et al., 2015; Guo et al., 2020). Of these, 35 HarmORs previously characterized were included (Liu et al., 2013; Jiang et al., 2014; Guo et al., 2020; Yang and Wang, 2021). The IRs from *B. mori*, *D. abietella*, *H. armigera* and *O. furnacalis* were used to infer the tree (Zhang T. et al., 2015; Liu et al., 2018; Xing et al., 2021). In the GR tree, we selected the GRs from *B. mori*, *D. abietella* and *H. armigera*, in which 15 representatives in *H. armigera* (CO_2, sugar, GR43a–like and some bitter GRs known ligands) were included (Xu et al., 2016; Guo et al., 2017). In the SNMP tree, considering a relatively small gene repertoire in each species, we used the SNMPs from 14 lepidopteran species and *D. melanogaster* (Zhang et al., 2020). For the selection of SNMPs used in Lepidoptera, if possible, all SNMPs from the pyralid and crambid species were included. Multiple

[1]https://www.geneious.com/

alignments of the protein sequences were conducted under the L–INS–i algorithm using MAFFT v7.450 with default parameters (Katoh and Standley, 2013). The aligned sequences were used to construct the maximum-likelihood trees under the Whelan and Goldman (WAG) model, implemented in FastTree v2.1.11 (Price et al., 2010). In the phylogenetic analysis, chemosensory receptors and SNMPs with less than 200 amino acids were discarded, and OBPs and CSPs showing below 100 amino acids were removed. Tree edition and visualization were conducted using FigTree v1.4.4[2].

Expression Profiling Analysis With PCR Approaches

In the expression profiles of genes, full-length transcripts encoding DabiORs and GRs were selected, including *DabiOR1–OR54*, *Orco,* and *GR1–GR16*. In the CSP, IR, and SNMP gene families, except for a pseudogenized *DabiIR2*, all the genes were selected. In the OBP gene family, we selected 32 *DabiOBPs* with specific or high expression in antennae. RT–PCR was employed to determine tissue- and sex-specific expression of genes in sequenced tissues. The reactions were performed on a TAdanced 96 G instrument (Analytik Jena AG, Jena, Germany) with an annealing temperature of 58°C and 35 cycles. For quality control of cDNA templates, a reference gene, ribosomal protein L10 (*DabiRPL10*), was used. In the RT–PCR analyses, one of three biological templates used for transcriptome sequencing was first used. If the expression of one gene in tissues was inconsistent with its corresponding FPKM results, at least two biological replicates were conducted. Gene-specific primers were designed using Primer Premier 5 (**Supplementary Table 2**). RT–PCR raw data of genes including uncropped gel pictures were seen in **Supplementary Figure 1**.

Based on the phylogeny of ORs, we selected 11 candidate *DabiPRs* in *D. abietella*, and further detected their relative expression levels in antennae and other tissues using qPCR. Each reaction contained a total volume of 20 μL mixture, consisting of 2 μL of cDNA, each 0.5 μL of forward and reverse primers (10 μM), and 10 μL of Bestar® SybrGreen qPCR mastermix (DBI® Bioscience, Germany). The reactions were run with an annealing temperature of 58°C and 40 cycles on a LightCycler® 96 System (Roche Diagnostics). For each gene, three biological pools were conducted with three technical replicates for each pool. Two reference genes, ribosomal protein S4 and L8 (*DabiRPS4* and *RPL8*), were used to compute the relative expression of target genes using the Q-GENE method (Muller et al., 2002; Simon, 2003). The amplification efficiencies of primers for target and reference genes were determined using fivefold dilutions of antennal cDNA. The specificity of the products was evaluated by a melting curve analysis. The primers were designed using Beacon Designer 8.14 (**Supplementary Table 2**). qPCR raw data of target and reference genes were seen in **Supplementary Figure 2** and **Supplementary Table 3**, including cycle threshold (CT) values, melting curves and primer amplification efficiencies. Data represented mean ± SEM. Statistical significance of gene expression levels among tissues was compared using a one–way analysis of variance (ANOVA), followed by the Fisher's least significant difference (LSD) test, implemented in IBM SPSS Statistics 21.0 (SPSS Inc., Chicago, IL, United States). $P < 0.05$ was considered as significant difference.

[2]http://tree.bio.ed.ac.uk/software/figtree/

RESULTS AND DISCUSSION

The Transcriptome of Various Tissues in *Dioryctria abietella*

Through the Illumina HiSeq® 2000 sequencing technique, we constructed and sequenced 16 RNA–Seq libraries of female and male moths. This sequencing resulted in the generation of variable raw data, ranging from 52,058,094 reads in male heads to 75,443,066 reads in male proboscises. After cleaning, approximately 99.85% of raw reads (952,658,466) were obtained and set as clean reads, representing 463,121,204 in female tissues and 489,537,262 in males. The size of all the clean reads was summed to 142.90 gigabases (G). Such sequencing and analysis yielded a low error rate (0.03%), high Phred values (Q20 = 98.26% and Q30 = 94.69%) and a moderate GC content (45.24%) (**Table 1**). With an emphasis on the antennae responsible for olfaction, here our transcriptome sequencing obtained more raw data from *D. abietella* antennae (females: 58,464,276 sequences and males: 71,979,886 sequences) compared to previous studies in *D. abietella* (47,005,976 and 50,818,590 reads for females and males, respectively) (Xing et al., 2021), and other two pyralid moths, *G. mellonella* (41,665,706 and 37,238,352 reads for females and males, respectively) (Zhao et al., 2019) and *Plodia interpunctella* (21,937,207 and 22,682,896 reads for females and males, respectively) (Jia et al., 2018). This may explain, to some extent, why *D. abietella* here possessed a larger number of ORs as members of this gene family were primarily enriched in the antennae (70 ORs in this study, 42 ORs in a previous study from *D. abietella*, 46 ORs in *G. mellonella* and 47 ORs in *P. interpunctella*) (Jia et al., 2018; Zhao et al., 2019; Xing et al., 2021).

All clean reads were subject to sequence assembly using Trinity v2.1.5. As a result, 435,990 transcripts were generated with the majority of below 301 bp. This Corset clustering further led to the yields of 172,189 unigenes, 57.71% of which were categorized into the sizes of 301–1000 bp (**Supplementary Figure 3A**). Functional annotation of all the unigenes showed that the NCBI NR database had the largest number of genes (41,456) among seven databases, followed by GO (33,051) and PFAM (31,976). As many as 60,504 unigenes were presented in at least one database, whereas a relatively small number of genes (4546) were common to all seven databases (**Supplementary Figure 3B**). In the analyses of GO functions, 33,501 unigenes were annotated into different categories, representing 25, 20, and 10 functional terms in biological process, cell component and molecular function, respectively. In biological process, cellular process and metabolic process were the most abundant terms. Both cell and cell part accounted for the largest proportion in cell component. GO categories of molecular function were mainly composed of binding and

TABLE 1 | Statistics of the transcriptome of various tissues in *D. abietella*.

Tissue	Raw read	Clean read	Size (G)	Error(%)	Q20(%)	Q30(%)	GC content(%)
FA	58,464,276	58,395,372	8.76	0.02	98.60	95.31	44.84
FPro	54,190,778	54,111,656	8.12	0.03	97.97	94.11	45.04
FH	52,180,720	52,056,692	7.80	0.03	97.91	94.10	42.89
FT	72,497,030	72,382,434	10.86	0.03	97.94	94.06	45.08
FAb	57,724,882	57,653,700	8.64	0.02	98.65	95.51	46.54
FL	59,214,614	59,126,418	8.86	0.03	97.92	93.85	45.86
FW	54,597,756	54,509,940	8.18	0.02	98.53	95.21	45.36
FPG	54,964,100	54,884,992	8.24	0.02	98.64	95.49	46.21
MA	71,979,886	71,892,116	10.78	0.03	97.94	94.03	44.49
MPro	75,443,066	75,325,052	11.30	0.03	97.86	93.92	44.42
MH	52,058,094	51,949,624	7.80	0.03	97.91	94.09	43.73
MT	63,764,016	63,682,986	9.56	0.02	98.18	94.54	43.76
MAb	58,079,532	57,989,368	8.70	0.02	98.47	95.04	46.56
ML	55,969,010	55,875,530	8.38	0.02	98.64	95.53	45.74
MW	56,054,934	55,973,766	8.40	0.03	98.45	94.94	46.10
MHp	56,913,050	56,848,820	8.52	0.02	98.56	95.28	47.19
Total/Mean	954,095,744	952,658,466	142.90	0.03	98.26	94.69	45.24

FA and MA, female and male antennae; FPro and MPro, male and female proboscises; FH and MH, female and male heads without antennae and proboscises; FT and MT, female and male thoraxes; FAb and MAb, female and male abdomens; FL and ML, female and male legs; FW and MW, female and male wings, MHp, male hairpencils and FPG, female pheromone glands.

catalytic activities (**Supplementary Figure 3C**). In search of unigenes against the NCBI NR protein sequence database, *D. abietella* genes shared the highest homology to those from *Amyelois transitella* (52.65%) (**Supplementary Figure 3D**).

Identification of Chemosensory Genes in *Dioryctria abietella*

We identified 235 chemosensory-related transcripts from the *de novo* assembled transcriptomes, comprising 42 *OBPs*, 23 *CSPs*, 75 *ORs*, 62 *GRs*, 30 *IRs,* and 3 *SNMPs*. Notably, *DabiOR70* and 10 *DabiGRs* were found exclusively in the transcript transcriptome, but were partial sequences. Of the identified genes, 148 were predicted to have complete open reading frames (ORFs), with variable sizes of 139–340 amino acids for DabiOBPs, 102–290 for DabiCSPs, 357–476 for DabiORs, 372–482 for DabiGRs, 494–844 for DabiIRs, and 523–526 for DabiSNMPs. The remaining 87 genes were fragments, more than half of which (46/87) belonged to members of the GR gene family, including a conserved CO_2 receptor (*DabiGR34*) (**Supplementary Table 4**).

In comparison with a previous study on chemosensory genes in this species identified from the antennal transcriptome (15 *OBPs*, 18 *CSPs*, 42 *ORs*, 4 *GRs,* and 14 *IRs*) (Xing et al., 2021), our current transcriptome did not retrieve one CSP gene, namely *DabiCSP2*, but newly identified 140 genes encoding 27 OBPs, 6 CSPs, 33 ORs, 58 GRs, and 16 IRs. Of the identified *OBPs* and *CSPs*, we followed the previous nomenclature systems, except for *DabiPBP3*, *GOBP1,* and *GOBP2* that were designated according to their orthology to OBPs in other lepidopterans. For the chemosensory membrane protein gene families, due to their fragmented sequences and some incorrect genes in the previous study, we merged a number of gene fragments, corrected some genes and re-named them. This re-annotation led to the yields of 42 *ORs*, 4 *GRs*, 14 *IRs,* and 3 *SNMPs* in the previous study (Xing et al., 2021). Among these, *DabiOR5* and *OR25* did not belong to the OR gene family and were identified as two parts of the ortholog of GR1 in the CO_2 subclade, *DabiIR19* was a member (*DabiiGluR10*) of ionotropic glutamate receptors, and *DabiSNMP5* was discarded because it did not belong to SNMPs (**Supplementary Table 4**). Together, except for *DabiSNMPs*, our current study identified more chemosensory genes in other five gene families, largely attributed to transcriptome-wide sequencing of multi-tissues. Meanwhile, this possibly reflected that some genes were expressed in non-antennal tissues, especially the GR gene repertoire.

Antennae-Expressed Chemosensory Genes in Adult Moths

With the emergence of RNA–Seq techniques, a large number of tissues in moths have been sequenced. Currently, we focused on a principle olfactory organ of adults, antenna, and compared the numbers of chemosensory genes expressed in the antennae. By using "antennae" and/or "transcriptome" and/or "chemosensory genes" as keywords to search web of science, we found that antennal tissues of 55 moth species including *D. abietella* have been sequenced, emphasizing the identification and characterization of chemosensory gene families. Subsequently, we combined antennal transcriptome, RT–PCR and qPCR data to determine the numbers of chemosensory genes in the antennae (**Figure 1** and **Supplementary Table 1**).

This comprehensive and comparative analysis led to the identification of 1212 *OBPs*, 728 *CSPs*, 2698 *ORs*, 317 *GRs*, 844 *IRs,* and 80 *SNMPs* in adult antennae from 55 moths belonging to 17 families, including 37 *DabiOBPs*, 20 *DabiCSPs*, 70 *DabiORs*, 6 *DabiGRs*, 24 *DabiIRs,* and 3 *DabiSNMPs*. Expectedly, numerous OR members were detected in the antennae. However, the numbers of OR genes were variable among different species. For example, the OR numbers in 14 noctuid moths varied from 13

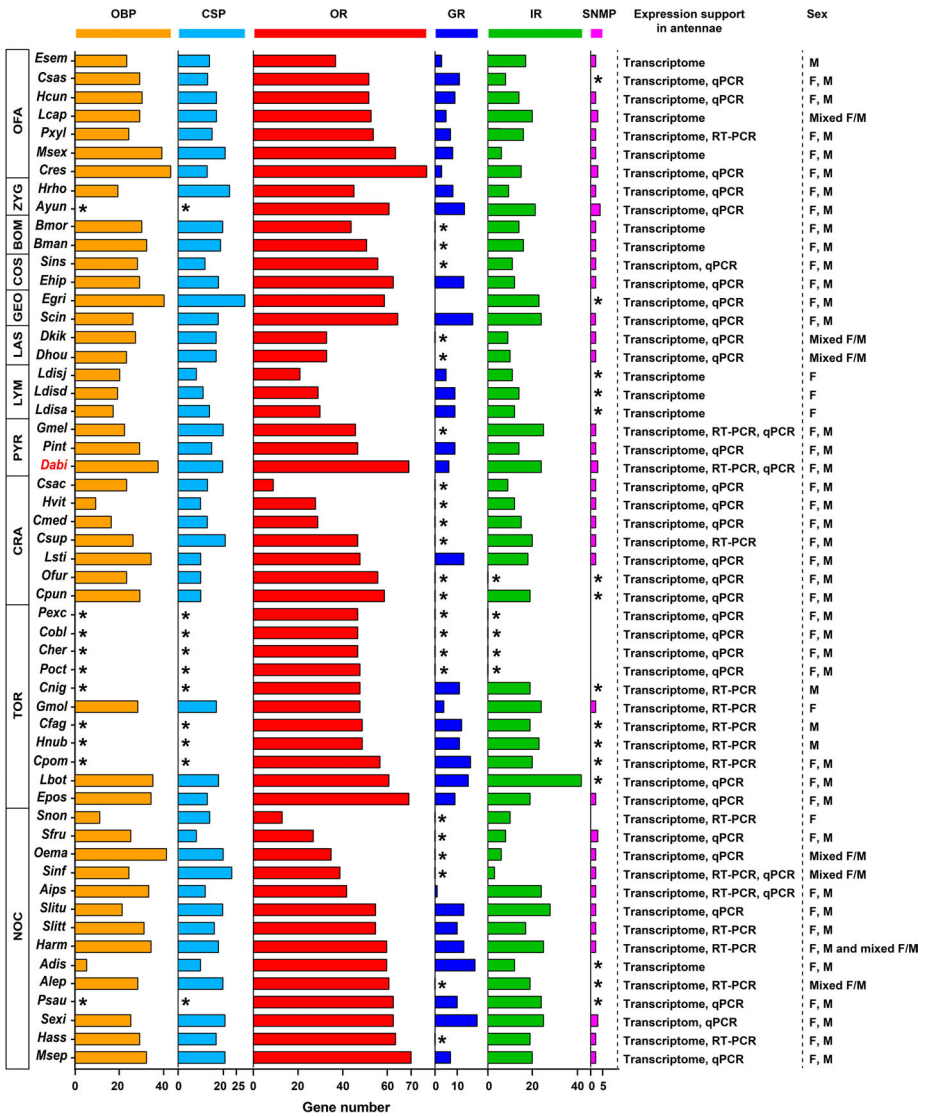

FIGURE 1 | Chemosensory gene repertoires of 55 lepidopteran species derived from antennal transcriptomes and PCR data. Based on RNA–Seq data and PCR results of genes in antennae, six chemosensory protein families of *OBPs*, *CSPs*, *ORs*, *GRs*, *IRs,* and *SNMPs* were counted. Asterisks represent that these genes were not reported in the corresponding studies. "F, M" represent that female and male antennae were sequenced by RNA–Seq, respectively; while "Mixed F/M" mean that a mixed sample of female and male antennae were sequenced. In addition to that, F or M represent that the antennal transcriptomes of only female or male moths were sequenced. BOM, Bombycidae including *Bombyx mandarina* and *Bombyx mori* (Qiu et al., 2018); COS, Cossidae including *Eogystia hippophaecolus* (Hu et al., 2016) and *Streltzoviella insularis* (Yang et al., 2019); CRA, Crambidae including *Chilo sacchariphagus* (Liu J. et al., 2021), *Chilo suppressalis* (Cao et al., 2014), *Cnaphalocrocis medinalis* (Zeng et al., 2013, 2015); *Conogethes punctiferalis* (Jia et al., 2016; Xiao et al., 2016), *Loxostege sticticalis* (Wei et al., 2017), *Heortia vitessoides* (Cheng et al., 2019) and *Ostrinia furnacalis* (Yang et al., 2015; Zhang T. et al., 2015); GEO, Geometridae including Ectropis grisescens (Li et al., 2017) and Semiothisa cinerearia (Liu P. et al., 2020; Zhu et al., 2020); LAS, Lasiocampidae including *Dendrolimus houi and Dendrolimus kikuchii* (Zhang et al., 2014); LYM, Lymantriidae including *Lymantria dispar asiatica*, *Lymantria dispar japonica and Lymantria dispar* (Clavijo McCormick et al., 2017); NOC, Noctuidae including *Agrotis ipsilon* (Gu et al., 2014), *Athetis dissimilis* (Dong et al., 2016; Sun et al., 2016), *Athetis lepigone* (Zhang et al., 2016, 2017), *Mythimna separata* (Chang et al., 2017; Du et al., 2018), *Oraesia emarginata* (Feng et al., 2017), *Helicoverpa armigera* (Liu et al., 2012; Zhang J. et al., 2015), *Helicoverpa assulta* (Zhang J. et al., 2015), *Sesamia nonagrioides* (Glaser et al., 2013), *Spodoptera exigua* (Llopis-Gimenez et al., 2020; Zhang et al., 2018), *Spodoptera frugiperda* (Qiu et al., 2020), *Spodoptera littoralis* (Walker et al., 2019), *Spodoptera litura* (Feng et al., 2015; Li L. L. et al., 2021), *Sesamia inferens* (Zhang et al., 2013) and *Peridroma saucia* (Sun et al., 2020); PYR, Pyralidae including *Dioryctria abietella* (Xing et al., 2021; This study), *Galleria mellonella* (Jiang et al., 2021; Zhao et al., 2019) and *Plodia interpunctella* (Jia et al., 2018); TOR, Tortricidae including *Ctenopseustis herana*, *Ctenopseustis obliquana* (Steinwender et al., 2015), *Cydia fagiglandana*, *Cydia nigricana* (Gonzalez et al., 2017), *Cydia pomonella* (Bengtsson et al., 2012; Walker et al., 2016), *Epiphyas postvittana* (Corcoran et al., 2015), *Grapholita molesta* (Li et al., 2015), *Hedya nubiferana* (Gonzalez et al., 2017), *Lobesia botrana* (Rojas et al., 2018), *Planotortrix excessana* and *Planotortrix octo* (Steinwender et al., 2016); ZYG, Zygaenidae including *Achelura yunnanensis* (Li G. C. et al., 2021) and *Histia rhodope* (Yang et al., 2020a,b); and OFA, Other families including *Carposina sasakii* (Tian et al., 2018), *Clostera restitura* (Gu et al., 2019), *Eriocrania semipurpurella* (Yuvaraj et al., 2018), *Hyphantria cunea* (Zhang et al., 2016), *Lampronia capitella* (Yuvaraj et al., 2018), *Manduca sexta* (Grosse-Wilde et al., 2011; Howlett et al., 2012; Vogt et al., 2015) and *Plutella xyllostella* (Yang et al., 2017). Detailed information presented in this picture are listed in **Supplementary Table 1**.

in *Sesamia nonagrioides* to 71 in *Mythimna separate*, while 9 in *Chilo sacchariphagus* and 78 in *Clostera restitura* were observed among species of the different families. Similarly, the numbers of the OBP and IR gene repertoires in the antennae were different across moths, with the range of 5 OBPs in *Athetis dissimilis* to 43 OBPs in *C. restitura*, and 3 IRs in *Sesamia inferens* to 42 IRs in *Lobesia botrana*. In contrast to the families of OBPs, ORs and IRs, the CSP, GR, and SNMP gene families harbored smaller members in the antennae. In particular, GR genes in 23 species were not identified or missed in the antennae, suggesting extremely low or no expression of the genes (**Figure 1** and **Supplementary Table 1**).

Further, we concentrated on gene numbers of three gene families primarily involved in olfaction, i.e., OBPs, ORs, and IRs. Difference in the repertoire sizes of antennae-expressed OBPs, ORs, or IRs were possibly attributed to the following reasons: (1) sequencing depth as discussed above; (2) sequencing sexes such as *S. nonagrioides,* and (3) sequencing platforms or techniques (**Figure 1** and **Supplementary Table 1**). In addition, it was also possible that a wide diversity of host plants drove the evolution of olfactory genes (Pelosi et al., 2018; Robertson, 2019; Auer et al., 2020; Yin et al., 2021). Although lepidopteran GRs, except for CO_2-sensing receptors, were mainly expressed in gustatory-related tissues like proboscises and legs, they were indeed present in the antennae from *D. abietella* and other lepidopterans, including sugar, GR43a–like and some bitter receptors (Xu et al., 2016; Guo et al., 2017; Li G. C. et al., 2021). This may be correlated with contact chemosensation of antennae, as lepidopteran insects were able to touch or tap host plants using their antennae before feeding nectar or laying eggs (Ozaki et al., 2011; Briscoe et al., 2013; Agnihotri et al., 2016; Xu et al., 2016; Guo et al., 2017).

Phylogenetic and Expression Profiling Analysis of *Dioryctria abietella* Odorant Binding Proteins

Previously, lepidopteran OBPs were classified into six subfamilies based on sequence homology and conserved cysteines, i.e., pheromone binding proteins/general odorant binding proteins (PBP/GOBPs), antennal binding protein I (ABP I), ABP II, chemical sense-related lipophilic ligand binding proteins (CRLBPs), Minus–C OBPs and Plus–C OBPs (Gong et al., 2009). In the present study, 42 DabiOBPs were distributed in each subfamily, representing each five in PBP/GOBPs and Minus–C OBPs, each seven in ABP IIs and Plus–C OBPs, and each nine in ABP Is and CRLBPs. In most orthologous clades, there were 1:1:1 orthologs among three species. However, two subfamily expansions were observed in some species, including 9 BmorOBPs in Minus–C OBPs (each five in *D. abietella* and *S. litura*) and 17 SlitOBPs in ABP Is (8 in *B. mori* and 9 in *D. abietella*). In addition, the ortholog of PBP4, which was previously defined as a novel subclass of the Lepidoptera-specific PBP/GOBPs (Xiao et al., 2021), was not found in *D. abietella* (**Figure 2A**).

Expression profiles with RNA–Seq data revealed that the majority of *DabiOBPs* exhibited tissue-specific expression. Nearly half of the genes were enriched in antennae, with *DabiPBP1* showing the highest values in males (FPKM = 24511.21) (**Supplementary Table 4**), suggesting their olfactory roles involved in the reception of sex pheromones and plant odorants.

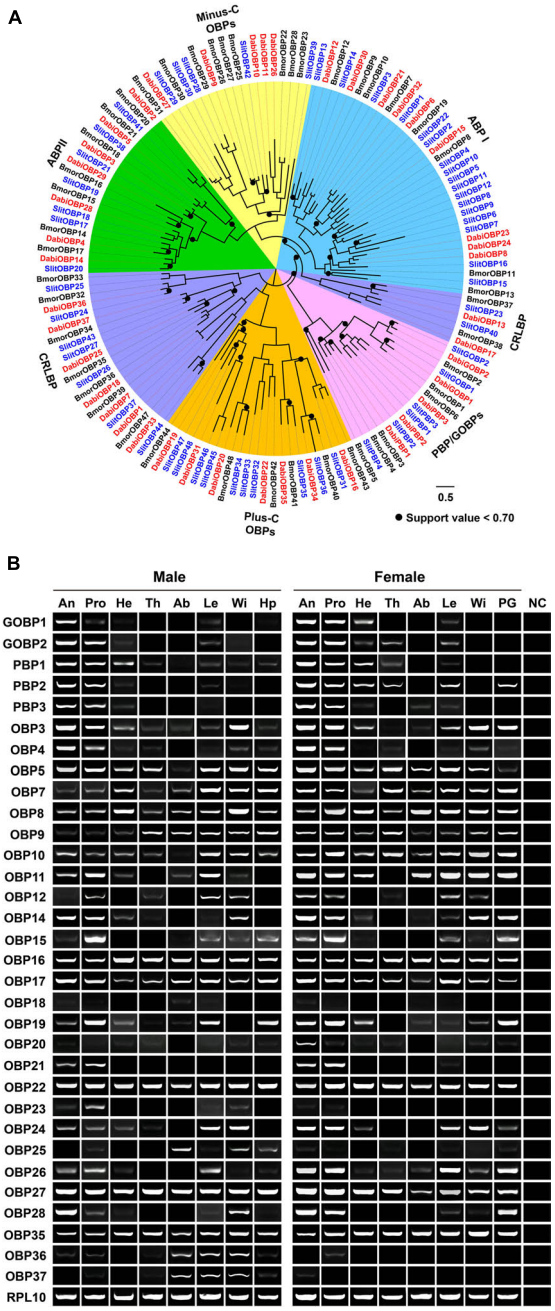

FIGURE 2 | The candidate OBP gene family in *D. abietella*. **(A)** The phylogeny of OBPs in *S. litura* (Slit, blue), *D. abietella* (Dabi, red) and *B. mori* (Bmor, black). According to the classification system of OBPs in Lepidoptera, DabiOBPs clustered into six subfamilies. **(B)** Expression profiles of *DabiOBPs* in different tissues of both sexes from *D. abietella*. A reference gene, *DabiRPL10*, was used to check the quality and quantity of cDNA templates. NC, negative control using sterile water as the template. The full names of tissues are listed in **Table 1**.

Some genes (*DabiOBP11*, *OBP15*, *OBP19,* and *OBP31*) were primarily expressed in gustatory organs such as proboscises and legs (**Supplementary Table 4**), possibly associated with taste reception as implied in previous studies (Guo et al., 2018; Koutroumpa et al., 2021). Intriguingly, 5 *DabiOBPs* (*DabiOBP9*, *OBP18*, *OBP25*, *OBP36,* and *OBP37*) had obviously high expression in male abdomens (FPKM > 13) but very low levels in females (FPKM < 1) (**Supplementary Table 4**). As indicated in *S. litura*, a number of *SlitOBPs* were abundantly presented in male reproductive tissues (Xiao et al., 2021). Thus, the 5 *DabiOBPs* may be candidate reproductive-related genes for modulating female or male reproductive-associated behaviors. On the other hand, some *DabiOBPs* appeared to have sex-biased expression in the antennae, for example, *DabiPBP1* had 10.25-fold higher transcription in males compared to females, *DabiOBP19* and *OBP31* transcription was female-biased with 17.69-fold and 21.53-fold higher than males, respectively (**Supplementary Table 4**).

To validate the results of RNA–Seq, we employed RT–PCR to construct the expression profiles of 32 *DabiOBPs* in 16 tissues of both sexes. Among them, the expression of all the genes appeared to be detectable in the antennae, but 5 genes (*DabiOBP18*, *OBP23*, *OBP25*, *OBP36,* and *OBP37*) exhibited extremely weak bands. Interestingly, most of the genes were also transcribed in the proboscises. In addition, a number of *DabiOBPs* were expressed in non-chemosensory tissues (**Figure 2B**). Combining RNA–Seq and RT–PCR results, it was found that *DabiOBPs* were mainly transcribed in the antennae (female: 36 and male: 34) and proboscises (female: 34 and male: 35). In 14 other tissues, a comparable number of *DabiOBP* genes were obtained, ranging from 15 in female thoraxes and abdomens to 28 in male legs (**Table 2**). These results well evidenced the presence of *DabiOBPs* in tissues, suggestive of their functional diversification beyond olfaction.

TABLE 2 | Summary of chemosensory genes expressed in various tissues of *D. abietella* (FPKM > 1 or RT–PCR and qPCR support).

Gene	OBP	CSP	OR	GR	IR	SNMP	Total
tissue							
FA	36	19	65	6	24	2	152
MA	34	15	68	5	18	2	142
FPro	34	18	31	19	12	2	116
MPro	35	18	30	15	9	2	109
FH	24	14	3	2	4	1	48
MH	26	14	3	2	1	1	47
FT	15	19	6	4	6	2	52
MT	16	17	5	3	2	2	45
FAb	15	16	5	3	2	2	43
MAb	17	15	5	4	7	2	50
FL	27	20	3	2	0	1	53
ML	28	20	3	1	6	1	59
FW	21	17	0	0	0	1	39
MW	26	19	2	0	1	1	49
FPG	22	18	3	6	2	2	53
MHp	20	18	4	2	7	1	52

*The full names of tissues are seen in **Table 1**.*

Phylogenetic and Expression Profiling Analysis of *Dioryctria abietella* Chemosensory Proteins

With 67 CSP protein sequences from *B. mori*, *D. abietella,* and *S. litura*, we inferred the maximum-likelihood phylogeny of CSPs. A high degree of conservation of CSPs among three species was obtained, with 14 orthologous clades (1:1:1) sharing over 50% average amino acid identities among orthologs (**Figure 3A**). Compared with the numbers of CSPs in other lepidopterans annotated in the genomes (Gong et al., 2007; Gouin et al., 2017; Pearce et al., 2017), our current transcriptome may identify most, if not all, of the CSP gene family in *D. abietella*, coupled with phylogenetic analyses of CSPs in the three moths (**Figure 3A**).

Combining FPKM and RT–PCR approaches, the expression profiles of 23 *DabiCSPs* were determined. In line with those from other moths (Gong et al., 2007; Xiao et al., 2021), *DabiCSPs* were broadly expressed in various tissues, and none of the tissue-specific genes were found (**Figure 3B**). In particular, some of them displayed a very high transcription in each tissue (FPKM > 180), such as *DabiCSP5*, *CSP9,* and *CSP13*. However, *DabiCSP11* and *CSP21* appeared to be expressed in some tissues at a low level (FPKM < 2.2) (**Supplementary Table 4**). Together, female and male legs had the largest number of *DabiCSPs* among tissues with 20 relatives, whereas each 14 genes were presented in heads of both sexes (**Table 2**).

Phylogenetic and Expression Profiling Analysis of *Dioryctria abietella* Odorant Receptors

Using 160 protein sequences from *D. abietella*, *H. armigera,* and *O. furnacalis*, we built the maximum-likelihood tree of ORs, rooted with a highly conserved Orco clade across insects (Vosshall and Hansson, 2011). Seventy-two DabiORs excluding 3 short DabiORs (<200 amino acids, DabiOR68, OR69, and OR74) were phylogenetically split into various small clades. In the tree, 10 DabiORs clustered into a conserved pheromone-sensing PR clade as previously defined (Krieger et al., 2004; Zhang and Löfstedt, 2015), and thus were identified as candidate DabiPRs in *D. abietella* (**Figure 4A**). In addition, Bastin-Héline et al. (2019) recently described a novel PR lineage, which was not close to this classical PR clade in phylogeny. To check whether DabiORs clustered into this novel clade, we reconstructed the tree of DabiORs, together with *H. armigera* and *S. littoralis* PRs, and novel PRs identified in a previous study (Bastin-Héline et al., 2019). As a result, DabiOR4 were presented in this novel clade and shared 39.29% amino acid identity to BmorOR30 in *B. mori* (**Supplementary Figure 4**). Therefore, this *DabiOR4* gene might be also a PR candidate for the detection of sex pheromones in *D. abietella*. In our phylogenetic analysis, it was noticed that DabiOR23 was homologous to HarmOR42 in *H. armigera*, with 71.72% amino acid identities (**Figure 4A**). In this conserved monophylogenetic group including HarmOR42, a recent study indicated that most members of this group, at least 12 orthologous ORs functionally studied, could detect phenylacetaldehyde, a floral volatile common to flowering plants

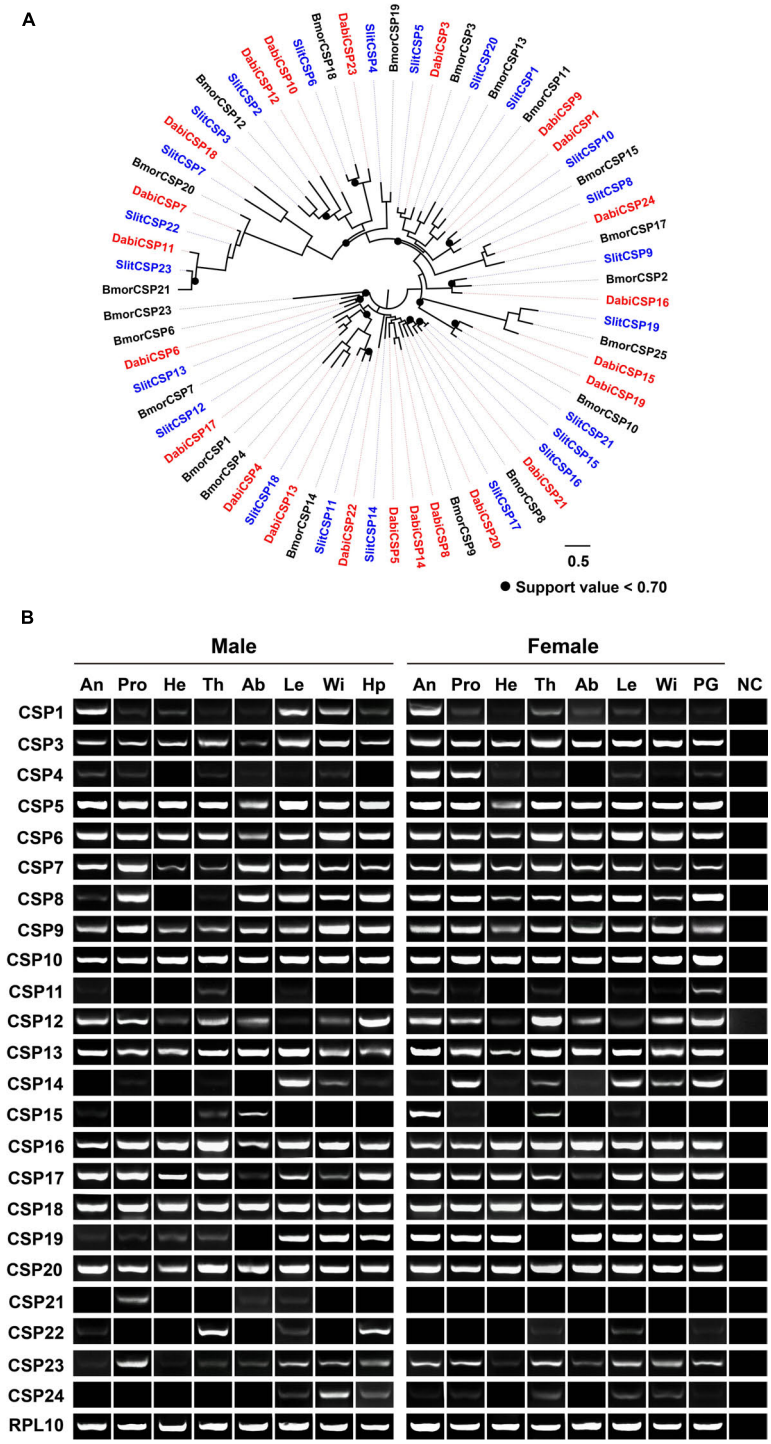

FIGURE 3 | The candidate CSP gene family in *D. abietella*. **(A)** The phylogeny of CSPs in *S. litura* (Slit, blue), *D. abietella* (Dabi, red), and *B. mori* (Bmor, black). **(B)** Expression profiles of *DabiCSPs* in different tissues of both sexes from *D. abietella*. A reference gene, *DabiRPL10*, was used to check the quality and quantity of cDNA templates. NC, negative control using sterile water as the template. The full names of tissues are listed in **Table 1**.

(Guo et al., 2020). Therefore, we postulated that DabiOR23 may also recognize this compound facilitating the searching of nectar sources.

In the analysis of expression profiles with FPKM values, we found that except for *DabiOR70* with unavailable expression data, the majority of *DabiORs* (67/74) were detected in female or male

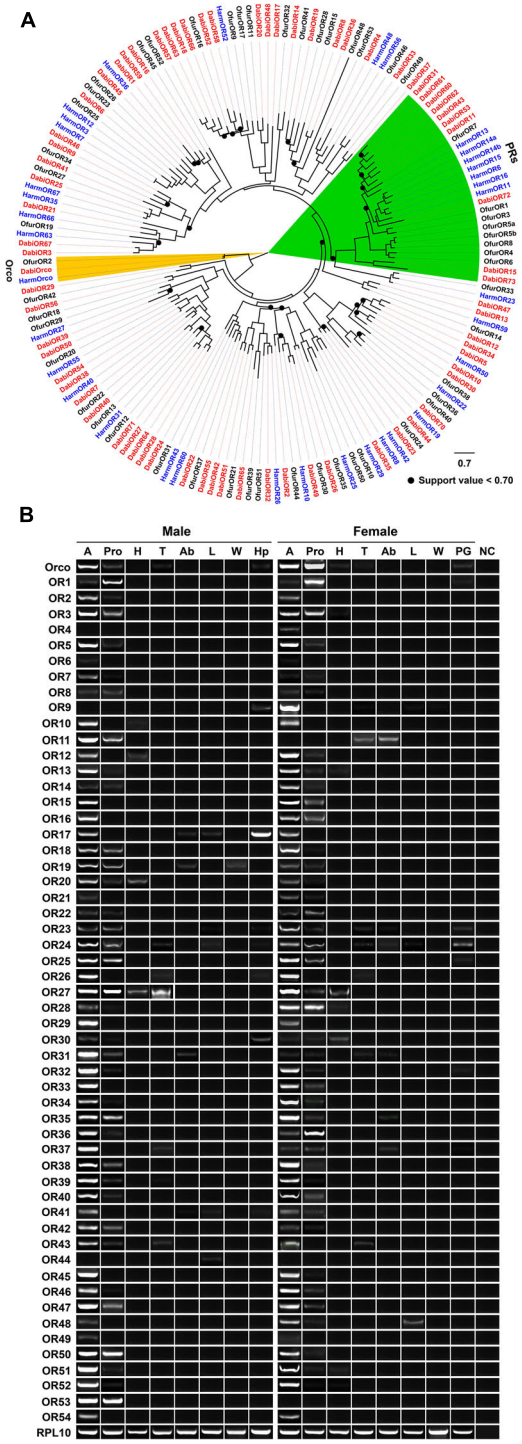

FIGURE 4 | The candidate OR gene family in *D. abietella*. **(A)** The phylogeny of ORs in *H. armigera* (Harm, blue), *D. abietella* (Dabi, red), and *O. furnacalis* (Ofur, black). For *H. armigera* ORs, we selected 35 representatives with known functional ligands. A candidate PR subfamily was highlighted and comprised 10 members in *D. abietella*. The tree was rooted with a conserved Orco lineage across insects. **(B)** Expression profiles of *DabiORs* in different tissues of both sexes from *D. abietella*. A reference gene, *DabiRPL10*, was used to check the quality and quantity of cDNA templates. NC, negative control using sterile water as the template. The full names of tissues are listed in **Table 1**.

antennae (FPKM>1), in agreement with their olfactory roles. Of these, 26 *DabiORs* had a relatively high transcription in the antennae (FPKM > 10), with *DabiOrco* showing the highest expression in males (FPKM=250.00) or females (FPKM=171.31). In addition, some genes appeared to have sex-biased expression in the antennae of both sexes. For instance, a candidate PR, *DabiOR4*, was expressed exclusively in female antennae, while *DabiOR11*, *OR53*, *OR60* and *OR72* expression was specific to male antennae (**Supplementary Table 4**). Interestingly, a certain number of *DabiORs* were transcribed in the proboscises (FPKM > 1) (**Supplementary Table 4**), a principle feeding and taste organ of adult Lepidoptera. As implied in previous studies, the ORs from other lepidopteran species also had proboscises-detectable expression (Briscoe et al., 2013; Guo et al., 2018; Koutroumpa et al., 2021). Considering the presence of olfactory sensilla on the proboscises such as sensilla styloconica, sensilla basiconica, and sensilla coeloconica (Faucheux, 2013; Guo et al., 2018; Li et al., 2020), it proposed a possibility that the proboscis may partly bear olfactory roles.

We further employed RT–PCR to examine the expression profiles of 55 full-length *DabiORs* in different tissues of both sexes. The results showed that the expression of almost all the genes was consistent with RNA–Seq data. Except for *DabiOR44* presenting extremely low expression only in male legs, the remaining 54 *DabiORs* were detected in female and/or male antennae. In addition, at least half of the 54 genes showed the expression in adult proboscises, although most of them were detected at a low level (**Figure 4B**). In comparison with OBPs and CSPs, most members of the OR gene family had more specific expression in the antennae (up to 65 in females and 68 in males). A considerable number of *DabiORs* were detected in female and male proboscises, representing 29 and 31 genes, respectively. None of *DabiORs* were found in female wings (**Table 2**).

Candidate Pheromone Receptors in *Dioryctria abietella*

In the phylogenetic analyses, 11 candidate *DabiPR* genes were found in *D. abietella*. To determine tissue- and sex-specific expression of these genes, we investigated their expression ratios in antennae and other tissues. qPCR analysis revealed that 9 of them were significantly expressed in the antennae relative to other tissues, in which 4 *DabiORs* (*DabiOR4*, *OR11*, *OR53,* and *OR62*) transcription was restricted to the antennae and other 5 genes (*DabiOR43*, *OR60*, *OR61*, *OR72,* and *OR73*) were detected in the antennal and non-antennal tissues. Of the 11 *DabiORs*, 4 genes (*DabiOR31*, *OR61*, *OR62,* and *OR73*) had almost equal expression between female and male antennae, while the remaining 7 genes showed a significantly sex-biased expression in the antennae. Among these, *DabiOR11*, *OR43*, *OR53,* and *OR60* were male-biased genes in the antennae, in which *DabiOR11* and *OR53* previously described as *DabiOR41* and *OR19*, respectively (Xing et al., 2021), had a similar male-biased expression. In contrast to that, *DabiOR4*, *OR15,* and *OR72* displayed a female-biased expression (**Figure 5**). It appeared that there were some consistencies between RNA–Seq and qPCR data, that is, similar sex-biased observations for some candidate PRs

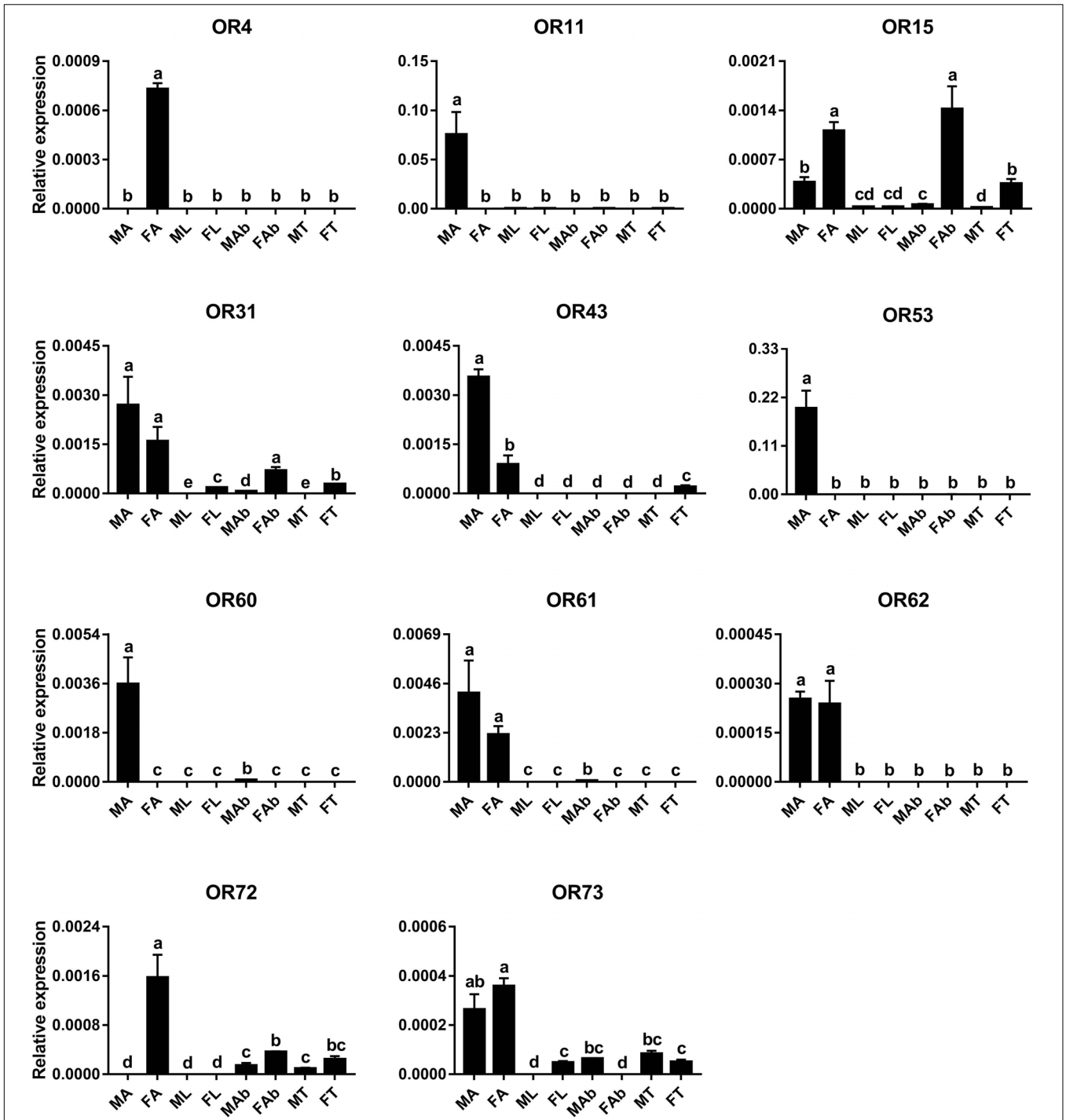

FIGURE 5 | Expression profile of candidate *DabiPRs* in different tissues of *D. abietella*. Significant difference of OR expression levels among tissues is indicated with different lowercase letters (ANOVA, LSD, $P < 0.05$). Relative expression of *DabiORs* was normalized relative to two reference genes, *DabiRPS4* and *DabiRPL8*. The full names of tissues are seen in **Table 1**.

with both FPKM and qPCR results, but some differences were also observed for some genes.

The diverse expression results of *DabiPRs* in the antennae were different from the PRs in noctuid species, in which the expression of most PR genes was significantly male-biased (Zhang and Löfstedt, 2015; Bastin-Héline et al., 2019). In comparison with other species in the Pyralidae family, 2 candidate *GmelPRs* from *G. mellonella*, *GmelOR13* and *OR50*, were evenly expressed in female and male antennae (Zhao et al., 2019; Jiang et al., 2021). In *P. interpunctella*, 4

candidate *PintPRs* exhibited different sex-biased expression patterns in the antennae, that is, *PintOR5* and *OR22* were significantly expressed in male antennae, whereas female-biased presence for *PintOR7* and *OR30* (Jia et al., 2018). In a closely related family Crambidae, a candidate *OscaPR* gene from *Ostrinia scapulalis*, *OscaOR7*, had roughly equal amounts of mRNA in female and male antennae, but failed to respond to female sex pheromones and pheromone orthologs (Miura et al., 2010). In *O. furnacalis*, *OfurOR1* identified as one of the PR members had no or extremely low expression in the antennae (Yang et al., 2015). Similar results about female-biased or equal expression of PRs in the antennae were also observed in *Loxostege sticticalis* (Wei et al., 2017), *Conogethes punctiferalis* (Jia et al., 2016), and *Chilo suppressalis* (Cao et al., 2014). All together, it was noticed that not all candidate *PRs* of the conserved PR clade in the Pyralidae and Crambidae families had a significantly high expression in male antennae.

Phylogenetic and Expression Profiling Analysis of *Dioryctria abietella* Gustatory Receptors

In the phylogenetic inference of GRs, we selected 29 DabiGRs showing more than 200 amino acids to construct the tree, together with 67 *B. mori* GRs and 15 *H. armigera* GRs. Following the classification system of GRs in Lepidoptera (Xu, 2020), DabiGRs partitioned into four subfamilies, i.e., CO_2, GR43a–like, sugar and bitter receptors comprising 2, 5, 7, and 15 relatives, respectively (**Figure 6A**). In the two subfamilies of GR43a–like and sugar GRs, the exact numbers of DabiGRs may be larger than we presented here in the tree, as some fragmented proteins (<200 amino acids) in *D. abietella* were not included. Similar events also occurred in the bitter GRs subfamily. In the CO_2 GR1 subclade, our transcriptome did not retrieve the ortholog of GR1 from *D. abietella*, but its partial sequences were presented in the previous antennal transcriptome (Xing et al., 2021). Given the high sequence identities of GR1 orthologs in this subclade (Xu and Anderson, 2015; Xu, 2020), we attempted to use all 62 DabiGRs to infer the phylogenetic relationships of GRs, coupled with the 67 and 15 GRs separately in *B. mori* (Guo et al., 2017) and *H. armigera* (Xu and Anderson, 2015). In agreement with the results of the originated tree, the orthologous gene of GR1 was not found from *D. abietella* (**Supplementary Figure 5**). This may be due to its extremely low expression in tissues of *D. abietella* or incorrect assembly.

Expression profiles revealed that all *DabiGRs* exhibited relatively low or no expression in the sequenced tissues ($0 \leq FPKM < 6$). Of notice, 10 *DabiGRs* were not identified from the unigene transcriptome and their FPKM values in tissues were unavailable. Fourteen out of the remaining 52 genes were considerably transcribed in the proboscises of both sexes, including 2 CO_2, 1 GR43a–like, 2 sugar, 5 bitter and 4 unclassified GRs ($1 < FPKM < 6$) (**Supplementary Table 4**). The proboscises-expressed *DabiGRs* were well consistent with their gustatory roles, as implied by the GRs in *Spodoptera littoralis* (Koutroumpa et al., 2021), *H. armigera* (Guo et al., 2018), and *H. melpomene* (Briscoe et al., 2013). Notably, FPKMs of only 3 *DabiGRs* (*DabiGR3*, *GR12,* and *GR42*) in the antennae were above 1 (**Supplementary Table 4**), largely supported by the observation that few or no GRs were identified in antennal transcriptomes (**Supplementary Table 1**). As expected, RT–PCR results further supported low expression of *DabiGRs* in tissues, as well as their extensive presence in the proboscises (**Figure 6B**). Using the data of FPKM and RT–PCR, we identified 19 and 15 *DabiGRs* in female and male proboscises, respectively, but no or a few genes were found in other tissues (0–6 relatives) (**Table 2**).

Phylogenetic and Expression Profiling Analysis of *Dioryctria abietella* Ionotropic Receptors

We constructed the maximum-likelihood tree of IRs from *B. mori*, *D. abietella*, *H. armigera,* and *O. furnacalis*, using DabiiGluRs as the outgroup. Phylogenetic analysis allowed us to distinguish the IRs into 29 IR orthologous clades, including one pseudogene group (IR2). Except for the IR31a clade, the remaining clades comprised at least one member of DabiIRs. Among the four moth species, most of the IR clades shared a one-to-one orthologous relationship. In particular, each of two clades possessed two gene copies, i.e., IR75q.1 and IR75p/p.2 (**Figure 7A**). Previously, IR31a genes were lost in several other species, including the Pyralidae family of *A. transitella* and *P. interpunctella*, the Cosmopterigidae family of *Hyposmocoma kahamanoa* and the Plutellidae family of *Plutella xylostella* (Yin et al., 2021). Therefore, it was possible that *D. abietella* had lost the ortholog of IR31a during the process of evolution.

In comparison, this divergent IRs (D–IRs) subfamily, especially the IR100 clade, had variable gene numbers ranging from 1 in *D. abietella* to 9 in *O. furnacalis* (**Figure 7A**). As implied in the previous studies, D–IRs members mainly contributed to the difference of IR numbers across the Lepidoptera, and moreover most of them were found to be lowly expressed genes in tissues. In contrast to this subfamily, antennal IRs (A–IRs) and Lepidoptera-specific IRs (LS–IRs) generally had high expression in at least one tissue, and high conservation with single-copy genes among different species (Liu et al., 2018; Zhu et al., 2018; Yin et al., 2021). Accordingly, our study may identify almost all the members of A–IRs and LS–IRs, but missed some D–IRs in *D. abietella*.

Based on RNA–Seq results, we found that almost all the A–IRs were expressed in the antennae at a considerable level ($FPKM > 1$), with the exception of *DabiIR40a* that appeared to be predominant in the antennae but remarkably low levels (females: 0.79 and males: 0.77). Three IR coreceptors, *DabiIR8a*, *IR25a* and *IR76b*, displayed higher expression in the antennae relative to other tissues. Apart from that, their expression was comparable in the proboscises ($FPKM > 1$) with that in non-antennal tissues, suggesting that they may be associated with taste function. The common presence of IR coreceptors in the antennae and proboscises was also observed in other A–IRs genes, such as *DabiIR21a*, *IR41a*, *IR64a*, *IR75p.1*, etc. (**Supplementary Table 4**), suggesting their dual functional roles in response to odorants and tastants. This expression

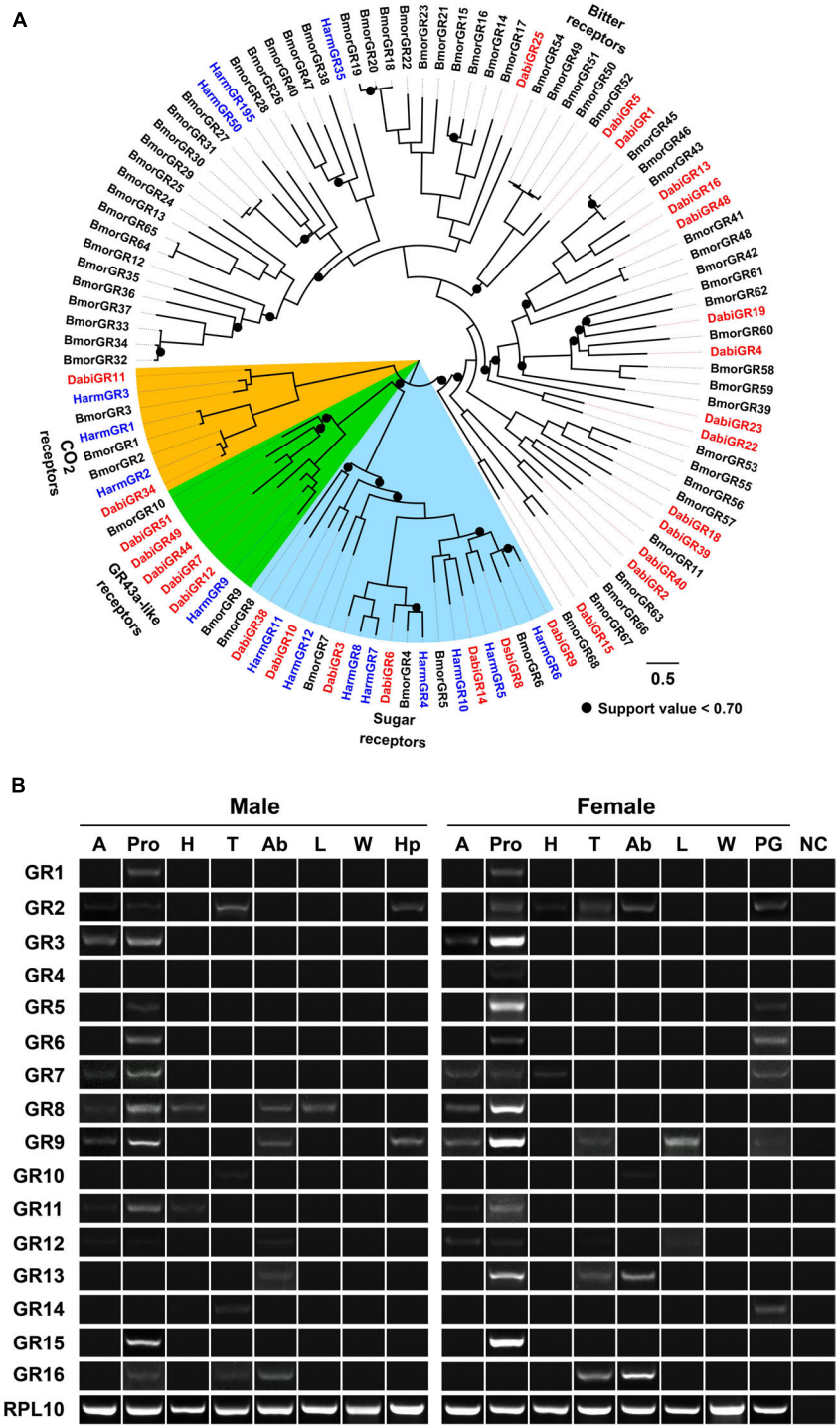

FIGURE 6 | The candidate GR gene family in *D. abietella*. **(A)** The phylogeny of GRs in *B. mori* (Bmor, black), *D. abietella* (Dabi, red), and *H. armigera* (Harm, blue). Fifteen HarmGRs were included, representing 3 CO_2, 1 GR43a–like and 8 sugar GRs, as well as 3 bitter GRs functionally characterized. **(B)** Expression profiles of candidate *DabiGRs* in different tissues of both sexes from *D. abietella*. A reference gene, *DabiPRL10*, was used to check the quality and quantity of cDNA templates. NC, negative control using sterile water as the template. The full names of tissues are seen in **Table 1**.

feature of A–IRs presented in olfactory and gustatory tissues was observed in *S. litura* (Zhu et al., 2018), *S. littoralis* (Koutroumpa et al., 2021), *Papilio xuthus* (Yin et al., 2021), and *H. armigera* (Liu et al., 2018). In addition, FPKM values of four LS–IRs in the antennae including a pseudogene of *DabiIR2* were above 1, but all of the D–IRs subfamily exhibited extremely low

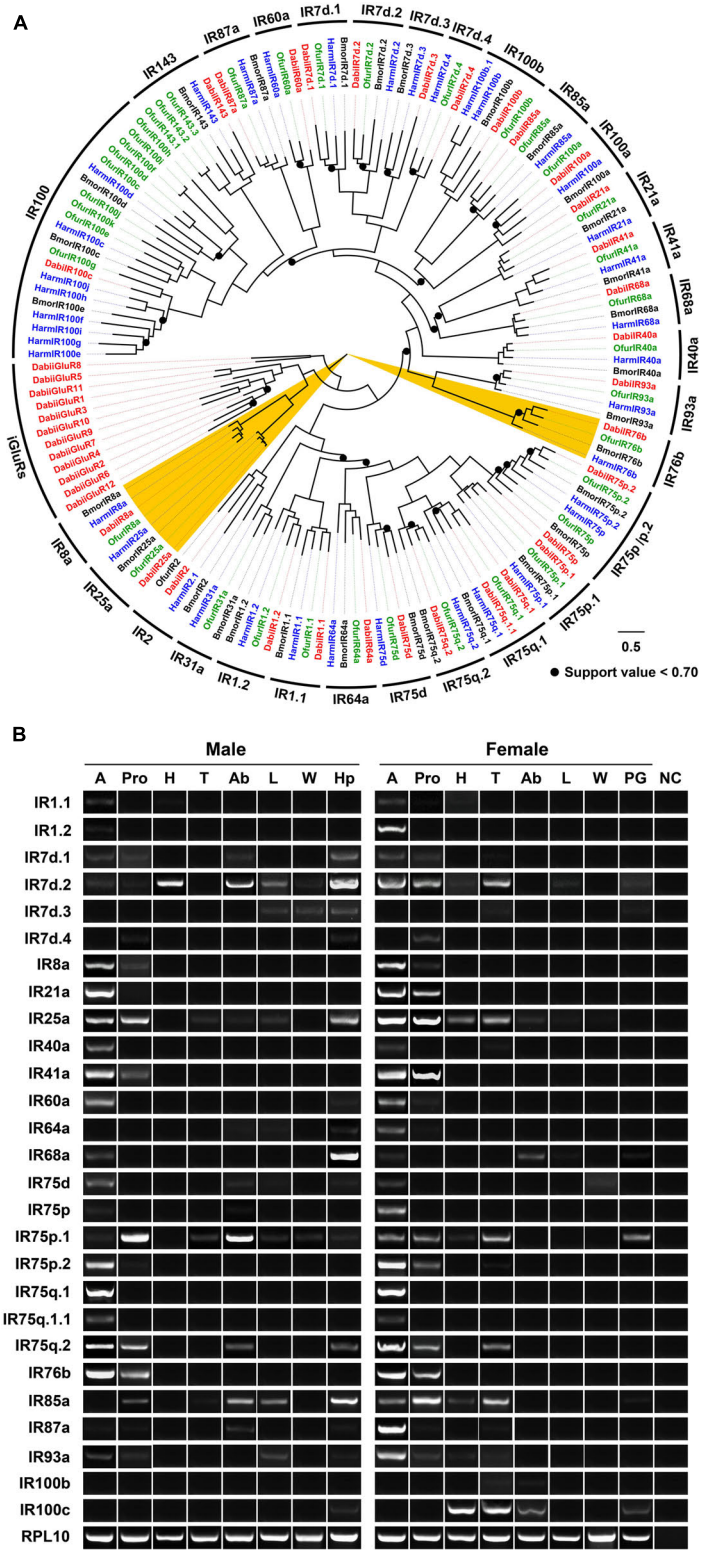

FIGURE 7 | The candidate IR gene family in *D. abietella*. **(A)** The phylogeny of IRs from *B. mori* (Bmor, black), *D. abietella* (Dabi, red), *H. armigera* (Harm, blue), and *O. furnacalis* (Ofur, green). According to sequence homology of IRs, members of the IR gene family were grouped into various orthologous clades, with three conserved IR coreceptors (IR8a, IR25a, and IR76b). The tree was rooted with *D. abietella* iGluRs. **(B)** Expression profiles of candidate *DabiIRs* in different tissues of both sexes from *D. abietella*. The quality and quantity of cDNA templates were measured by a reference gene, *DabiRPL10*. NC, negative control using sterile water as the template. The full names of tissues are listed in **Table 1**.

expression levels (FPKM < 1) in the antennae and other tissues (**Supplementary Table 4**).

Using the RT–PCR method, we further detected the expression profiling map of 31 *DabiIRs* in various tissues, except for *DabiIR2*. As a result, 29 out of 31 genes were expressed in at least one tissue, with the exception of *DabiIR100a* and *IR143* whose expression was not detected in all tested tissues. We tried to change the primers and annealing temperatures, but the products of *DabiIR100a* and *IR143* failed to be detected. This may reflect extremely low expression of the two genes in tissues. In line with RNA–Seq results, most of *DabiIRs* were transcribed in the antennae and proboscises. In particular, 2 D–IRs genes also showed a weak expression in the antennae, i.e., *DabiIR7d.1* and *IR7d.2* (**Figure 7B**), consistent with that of their orthologs from other moths (Liu et al., 2014, 2018; Yin et al., 2021). Some genes exhibited tissue-specific expression, for example, *DabiIR1.1*, *IR1.2*, *IR40a*, *IR60a*, *IR75q.1*, *IR75q.1.1*, and *IR87a* in the antennae, and *DabiIR85a* and *IR100d* in the proboscises (**Figure 7B**). All together, the antennae harbored the largest numbers of *DabiIRs* showing 24 in females and 18 in males, followed by the proboscises with 12 in females and 9 in males (**Table 2**).

Phylogenetic and Expression Profiling Analysis of *Dioryctria abietella* Sensory Neuron Membrane Proteins

Prior to this study, virtually all lepidopteran species with available genomic sequences possess 3 SNMPs, phylogenetically partitioned into three groups with single-copy genes in each

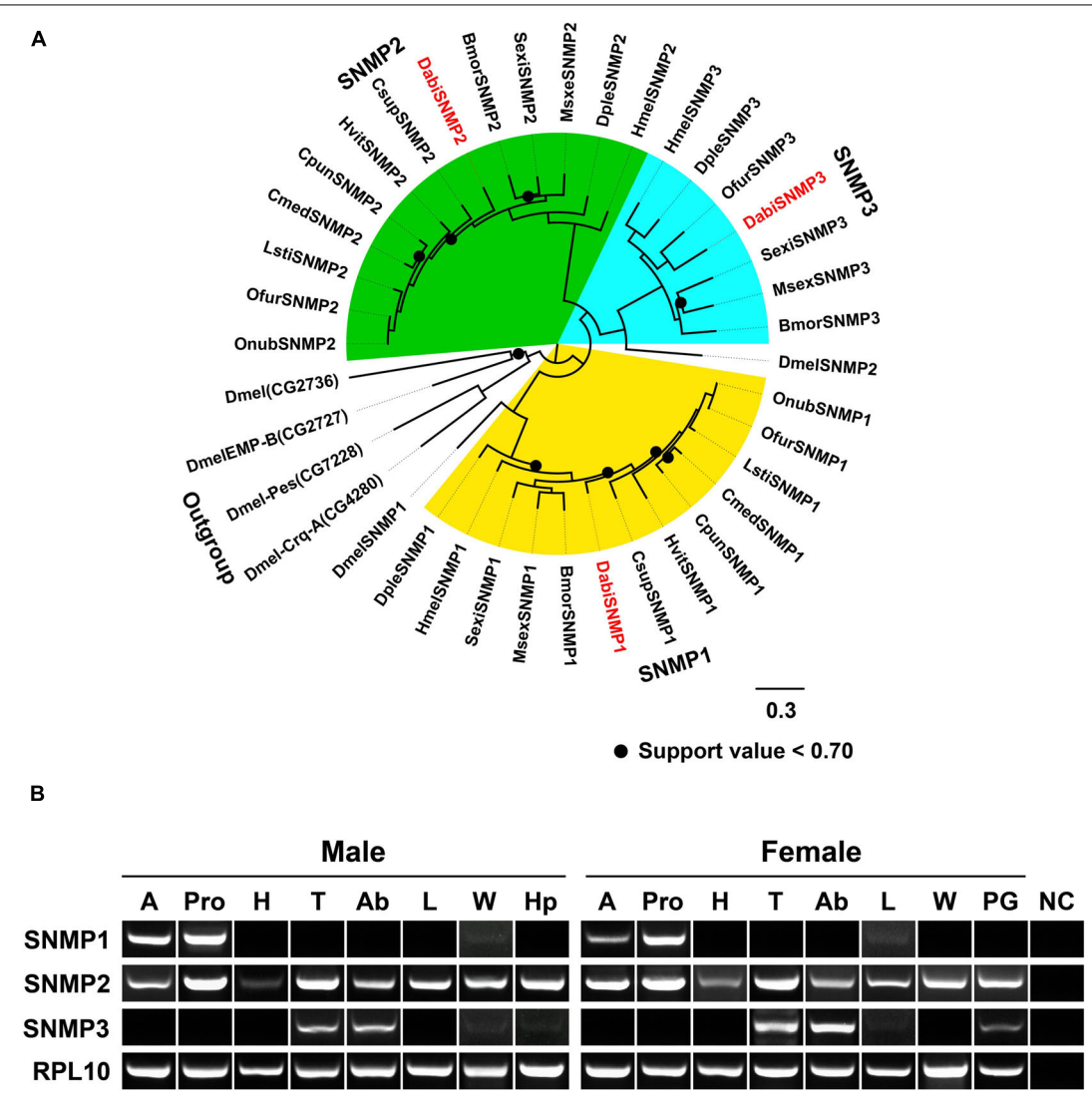

FIGURE 8 | The candidate SNMP gene family in *D. abietella*. **(A)** The phylogeny of SNMPs in Lepidoptera and *D. melanogaster*. DabiSNMPs newly identified in this study are indicated in red. Other members of the CD36 superfamily in *D. melanogaster* were used to root the tree. **(B)** Expression profiles of candidate *DabiSNMPs* in different tissues of *D. abietella*. The quality and quantity of cDNA templates were measured by a reference gene, *DabiRPL10*. NC, negative control using sterile water as the template. The full names of tissues are seen in **Table 1**.

group (Zhang et al., 2020). More recently, the SNMP gene repertoire in *A. yunnanensis* was composed of 4 members, representing 1 *AyunSNMP1*, 2 *AyunSNMP2s* (*AyunSNMP2.1* and *SNMP2.2*) and 1 *AyunSNMP3* (Li G. C. et al., 2021). In agreement with most lepidopteran species, here our transcriptome contained 3 SNMP orthologs that were split into three different clades (**Figure 8A**).

In the expression profiles of *DabiSNMPs*, *DabiSNMP1* was enriched in the antennae of both sexes, while *DabiSNMP2* was broadly presented in all tissues with particularly high expression levels in the antennae, proboscises and wings of female and male moths (FPKM > 500). In contrast to that of 2 *DabiSNMPs*, *DabiSNMP3* expression was not detected in chemosensory-related tissues like antennae, proboscises and legs, but presented primarily in adult abdomens and slightly in adult thoraxes (**Supplementary Table 4**). RT–PCR results further validated RNA–Seq data of 3 *DabiSNMPs*. Moreover, *DabiSNMP1* appeared to have a higher transcription in male antennae compared to females (**Figure 8B**), suggesting its putative olfactory roles in the sensing of female sex pheromones in *D. abietella*, coupled with SNMP1 functions in previous studies (Pregitzer et al., 2014; Liu S. et al., 2020). For the expression of *DabiSNMP3* in female and male abdomens (**Figure 8B**), we suggested that its transcripts may be restricted to midguts of both sexes, as its orthologous genes have been demonstrated to be midgut-specific relatives in *B. mori* and *H. armigera* (Zhang et al., 2020; Xu et al., 2021). Taken together, each tissue here studied included at least one SNMP transcript. In particular, 2 SNMP genes were found to have the expression in the antennae and proboscises (*DabiSNMP1* and *SNMP2*), as well as thoraxes and abdomens (*DabiSNMP2* and *SNMP3*) (**Table 2**).

CONCLUSION

In this study, we have characterized six chemosensory-related gene families from a destructive forest pest, *D. abietella*. First, a mixed transcriptome of 16 tissues, including key chemosensory organs of antennae, proboscises and legs, is sequenced and assembled, resulting in the generation of 952,658,466 clean reads and the accumulation of 142.90 G of data. Further, our transcriptome combined with RT–PCR and qPCR analyses identifies 235 genes involved in chemosensation (42 *OBPs*, 23 *CSPs*, 75 *ORs*, 62 *GRs*, 30 *IRs,* and 3 *SNMPs*, with an increase of 140 genes), 160 of which are expressed in the antennae (37 *OBPs*, 20 *CSPs*, 70 *ORs*, 6 *GRs*, 24 *IRs,* and 3 *SNMPs*), 137 in the proboscises (36 *OBPs*, 19 *CSPs*, 46 *ORs*, 21 *GRs*, 13 *IRs,* and 2 *SNMPs*), and 67 in the legs (32 *OBPs*, 21 *CSPs*, 4 *ORs*, 3 *GRs*, 6 *IRs,* and 1 *SNMP*). Phylogenetic analysis combined with sequence homology allows us to identify 11 candidate *DabiPR* genes, in which 4 relatives are significantly enriched in male antennae compared to females, and thus are regarded as candidates for the sensation of female sex pheromones in *D. abietella*. Together, our current work has identified potential molecular targets for sensing sex pheromones and plant-derived compounds that are applied to develop environmentally friendly alternatives for the control of this forest pest.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI SRA BioProject, accession no: PRJNA751144.

AUTHOR CONTRIBUTIONS

N-YL conceived this study and drafted the first manuscript. Z-QW, CW, G-CL, and S-MN performed the laboratory experiments. N-YL, Z-QW, and N-NY analyzed the data. N-YL and Z-QW revised the manuscript. All the authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.748199/full#supplementary-material

Supplementary Table 1 | Information on chemosensory genes expressed in antennae from 55 moth species.

Supplementary Table 2 | Primers used for expression profiling analyses of chemosensory genes in *D. abietella*.

Supplementary Table 3 | The CT values and primer amplification efficiencies of 11 *DabiORs* and 2 reference genes.

Supplementary Table 4 | Information on chemosensory genes from *D. abietella*.

Supplementary Figure 1 | Raw gel images of candidate chemosensory genes in RT–PCR analyses.

Supplementary Figure 2 | The melting curves of 11 *DabiORs* in qPCR analyses.

Supplementary Figure 3 | Transcriptome summary of 16 adult tissues in *D. abietella*. **(A)** Length distribution of unigenes and transcripts. **(B)** Numbers of unigenes annotated into different databases. **(C)** GO analyses of unigenes with the five most abundant categories for each term. **(D)** Homology of *D. abietella* unigenes with those of other species.

Supplementary Figure 4 | The phylogeny of ORs from *D. abietella* (Dabi) and other lepidopteran species. The tree was built based on the protein sequences derived from members of a novel PR clade of the Lepidoptera (Bastin-Héline et al., 2019) as well as *H. armigera* (Harm) and *S. littoralis* (Slitt) PRs, together with a conserved Orco lineage used to root the tree. *D. abietella* OR4 clustered into this novel PR clade and was identified as a novel PR candidate.

Supplementary Figure 5 | The phylogeny of 144 GRs in *B. mori* (Bmor, black), *D. abietella* (Dabi, red), and *H. armigera* (Harm, blue). Three conserved clades (CO_2, GR43a–like and sugar receptors) are highlighted with color patterns.

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