Analysis of Rac/Rop Small GTPase Family Expression in Santalum album L. and Their Potential Roles in Drought Stress and Hormone Treatments

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Abstract: Plant-specific Rac/Rop small GTPases, also known as Rop, belong to the Rho subfamily. Rac proteins can be divided into two types according to their C-terminal motifs: Type I Rac proteins have a typical CaaL motif at the C-terminal, whereas type II Rac proteins lack this motif but retain a cysteine-containing element for membrane anchoring. The Rac gene family participates in diverse signal transduction events, cytoskeleton morphogenesis, reactive oxygen species (ROS) production and hormone responses in plants as molecular switches. S. album is a popular semiparasitic plant that absorbs nutrients from the host plant through the haustoria to meet its own growth and development needs. Because the whole plant has a high use value, due to the high production value of its perfume oils, it is known as the “tree of gold”. Based on the full-length transcriptome data of S. album, nine Rac gene members were named SaRac1-9, and we analyzed their physicochemical properties. Evolutionary analysis showed that SaRac1-7, AtRac1-6, AtRac9 and AtRac11 and OsRac5, OsRacB and OsRacD belong to the typical plant type I Rac/Rop protein, while SaRac8-9, AtRac7, AtRac8, AtRac10 and OsRac1-4 belong to the type II Rac/ROP protein. Tissue-specific expression analysis showed that nine genes were expressed in roots, stems, leaves and haustoria, and SaRac7/8/9 expression in stems, haustoria and roots was significantly higher than that in leaves. The expression levels of SaRac1, SaRac4 and SaRac6 in stems were very low, and the expression levels of SaRac2 and SaRac5 in roots and SaRac2/3/7 in haustoria were very high, which indicated that these genes were closely related to the formation of S. album haustoria. To further analyze the function of SaRac, nine Rac genes in sandalwood were subjected to drought stress and hormone treatments. These results establish a preliminary foundation for the regulation of growth and development in S. album by SaRac.

Keywords: Santalum album; Rac gene family; haustorium; tissue-specific expression; drought stress; hormone treatments

1. Introduction

S. album is a small semiparasitic tree belonging to the genus Santalum in the family Santalaceae. This family is composed of 29 genera with approximately 400 species, 19 of which are specific to the Santalum genus [1–3]. It is distributed in India, Malaysia, Australia and Indonesia and is also cultivated in Guangdong and Taiwan [4]. S. album is known as the “tree of gold”, and its heartwood is widely used in high-end craft sculpture and high-end furniture manufacturing [5,6]. The essential oil extracted from its heartwood is mainly used in the cosmetic and pharmaceutical industries due to its special aroma [7,8]. Sandalwood usually yields 3–7% essential oil depending on the region and hemisphere [9]. The value of a sandalwood tree depends on three important characteristics: the volume of heartwood and the concentration and quality of its heartwood oil [10]. S. album grows for a long time; generally, it begins to form heartwood after 7 or 8 years of growth in
natural environments. Unsustainable exploitation of wild trees, combined with increasing global demand for sandalwood products has threatened native sandalwood populations in some places, such as southern India. Because of the contradiction between the increasing market demands for its heartwood and the shortage of *S. album* heartwood on the market, the shortage of heartwood with its destruction of natural resources has become increasingly complicated [11].

*S. album* is a semiparasitic plant; its roots have a special organ, the haustorium, which, through the haustorium’s contact with the host plant root, absorbs its own water and nutrients [12]. Therefore, the growth and development of haustoria play a vital role in the growth and development of *S. album* and the formation of heartwood. As far as the current scientific progress is concerned, the study of the regulatory genes related to haustoria in *S. album* is still limited.

ROS can regulate plant growth and development, hormone transduction, root hair development and so on. In sandalwood, Rboh is significantly induced by the haustorial inducer DMBQ, and the ROS signal produced by Rboh protein is necessary for the development of sandalwood haustoria [13]. At present, the growth and development of sandalwood is related to the size, quantity and quality of haustoria. According to the available literature, Rac and Rboh can regulate ROS concentrations through interactions and then promote the formation of sandalwood haustoria. In conclusion, the interaction between Rac and Rboh is an indispensable link in the formation of ROS for haustorium development.

Plant-specific Rac small GTPases belong to the plant Rho subfamily [14]. In animals, Rho is divided into several subfamilies, including Rho, Cdc42 and Rac. Rop is also called Rop in plants [15]. Rac is the sole plant subfamily of the conserved Rho family of small GTPases [16–19]. They are soluble proteins that localize and function at the plasma membrane by way of posttranslational lipid modifications [20–22]. Rac proteins can be divided into two types according to their C-terminal motifs: Type I Rac proteins, which have a typical Caal motif at the C-terminal, and Type II Rac proteins, which have a truncated and functional motif. Whereas type-I Racs probably undergo prenylation, type-II Racs undergo S-acylation but not prenylation [23]. All type-II Racs have an additional exon at the 3′ end of the gene, probably resulting from the insertion of an intron into an ancestral Rac [24,25]. Most if not all plant Racs act at the plasma membrane [26], and they are molecular switches that regulate diverse signaling cascades [27,28]. They are widely involved in plant signal transduction [29–31], cytoskeleton morphogenesis, polarized cell growth [32,33], cell morphogenesis, defense, responses and reactive oxygen species (ROS) production [34–36]. Taken together, Rac small GTPases act as a simple binary switch capable of receiving a wide variety of inputs and accordingly generating a multitude of specific outputs. Some members of the plant Rac gene family have been shown to regulate the growth and development of *S. album* through ROS production. Based on previous studies, nine Rac genes were obtained from the transcriptome and genomic data of *S. album* and compared with eleven Rac family members in *A. thaliana* and seven Rac family members in rice. The bioinformatics data of these sequences and tissue-specific expression were analyzed and these results will provide a basis for further analysis of the related functions of *S. album* Rac genes and its relationship with haustorium formation. Additionally, the expression levels of *SaRac1-9* under drought stress and hormone treatments were studied, which play an important role in the growth and development of sandalwood.

2. Materials and Methods

2.1. Plant Materials and Treatments

The materials used in the experiment are stored in this laboratory, Institute of Tropical Forestry, Chinese Academy of Forestry Sciences (Guangzhou, 23°11′ N, 113°23′ E). Four tissues were collected: root, stem, leaf and primary haustorium (after collection, they were immediately immersed in liquid nitrogen), each with three biological replicates, and stored in a −80 °C refrigerator. Sandalwood seedlings grew in a greenhouse in the soil for
approximately 2 months, until they reached 15 cm. Saplings displaying similar growth were subsequently used for analysis. The soil water was withheld to drought for 0, 3 and 9 d, in which the soil relative water content was reduced from 70% to 32%. The soil relative water content of control plants was maintained at 80%, which represents a suitable soil environment for sandalwood growth. For hormone treatments (ABA, IAA, ethephon), the plants were divided into four zones (ABA, IAA, ethephon, CK), which were treated with different hormones at a concentration of 100µM/L. Three biological replicates were included. After 48 h, samples were collected at the same time and immediately immersed in liquid nitrogen.

2.2. Genome-Wide Identification and Sequence Analysis of Rac Genes in S. album

According to the gene annotation information of the sandalwood transcriptome, the coding sequence of Rac was obtained, and the DNA sequence and promoter sequence of Rac were obtained from S. album genome data by homologous alignment. The nucleotide sequence homology of Rac in sandalwood was analyzed using TBtools.

The conserved domains of nine SaRac proteins were predicted using NCBI-Conserved Domain Search, and the structure of SaRac was analyzed using TBtools. Novel putative motifs were explored using the MEME server (https://meme-suite.org/meme/, accessed on 13 July 2022). By selecting a motif of full length, eight conserved motifs located in the SaRac domain were identified. To analyze the cis-regulatory element in the putative SaRac promoter, we performed a cis-regulatory element analysis of the 2000 BP promoter sequence of the nine SaRac genes using TBtools and the Plant CARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 1 August 2022), and their functions and numbers were predicted.

2.3. Analysis of Physicochemical Properties of SaRac

The basic physical and chemical properties of the protein, including molecular weight (MW), isoelectric point (pI) hydrophilicity and number of amino acids, were analyzed using ProtParam online software (https://web.expasy.org/protparam/, accessed on 25 August 2022). Protein subcellular localization was predicted by using the WoLF PSORT website (https://wolfsort.hgc.jp/, accessed on 27 August 2022).

2.4. Phylogenetic Analysis

A multiple sequence alignment of 27 Rac proteins from S. album and other species, including A. thaliana and rice, was performed. The 11 Rac members of A. thaliana were obtained from TAIR (https://www.arabidopsis.org/, accessed on 12 July 2022), and the 7 Rac members of rice were obtained from NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 12 July 2022) using the MUSCLE method. A phylogenetic tree was constructed by using the NJ method implemented in MEGA-X. The parameters for tree construction were as follows. Phylogenetic analysis parameters: bootstrap (1000 replicates); gaps/missing data: pairwise deletion; model: Dayhoff model; pattern among lineages: same (homogeneous) and rates among sites: uniform rates. Finally, the phylogenetic tree was constructed.

2.5. Chromosome Location and Gene Structure Analysis

Positional information and gene structures of SaRac genes on chromosomes of S. album were obtained from the gene annotation information of the S. album transcriptome. The chromosomal locations were displayed with TBtools (github.com/CJ-Chen/TBtools, accessed on 30 August 2022). The numbers and organization of introns, exons and gene structures were drawn and displayed using TBtools.

2.6. Collinearity of the SaRac Gene

The chromosomal locations of SaRac genes were obtained by TBtools software. Using TBtools software, the synteny relationships of orthologous Rac genes among S. album, A. thaliana and rice were evaluated. The parameters for collinearity of the SaRac gene were
as follows: the genome file was used to construct the chromosome skeleton to obtain the gene density file, and then TBtools was used to prepare the collinearity file, extract the location of the SaRac gene, highlight the collinearity region of the target gene and finally, visualize it.

2.7. Expression Profiles of SaRac Genes in Different Tissues and under Drought and Hormone Treatments

According to the instructions of the plant RNA extraction kit (R6827, Omega Bio-tek, Norcross, GA, USA), total RNA was extracted from S. album. The SaRac1-9 gene sequence was obtained by reverse transcription of cDNA with a reverse transcription kit (DRR037S, Takara, Dalian, China), and quantitative primers were designed according to the whole-field sequence of the SaRac1-9 gene (Table S1). Real-time quantitative PCR primers were designed using Primer 3.0 software with S. album. Actin was used as an internal reference gene. Using cDNA as a template, real-time quantitative PCR was performed according to the instructions of the SYBR Green Premix Ex Taq II Kit (Qiagen, Dusseldorf, Germany). The qPCR mixture were 0.5 µL of primers, 1 µL of cDNA, 10 µL of 2 × Mix and 8 µL of dd H2O. The reaction procedure was 95 °C for 10 s, 60 °C for 10 s and 72 °C for 20 s, and 40 cycles were performed. Three biological replicates and 3 technical replicates were carried out. SPSS 25 software was used to analyze the significance of the data, and OriginPro 2019b software was used to draw the graph.

3. Results

3.1. Sequence Analysis of the SaRac Gene Family in S. album

Nine cDNA and DNA sequences of SaRac were isolated from the transcriptome and genome of S. album and named SaRac1-9. The basic physical and chemical properties of the protein were analyzed by ProtParam (Table 1). The nine predicted full-length Rac proteins varied from 196 (SaRac5) to 211 (SaRac7) amino acid residues, and the relative molecular mass ranged from 21.45 (SaRac5) to 23.28 (SaRac7) kDa, with isoelectric points in the range of 9.18–9.62.

Table 1. Chemical properties of proteins in the SaRac gene family.

| Gene Name | Name   | Number of Amino Acids | Molecular Weight (kDa) | PI     | Instability Index | Total Number of Negatively Charged Residues (Asp + Glu) | Total Number of Positively Charged Residues (Arg + Lys) | Grand Average of Hydrophobicity (GRAVY) | In Silico Prediction WOLF PSORT |
|-----------|--------|-----------------------|------------------------|--------|-------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------|-----------------|
| Sal10G07090.1 | SaRac1 | 197                   | 21.58                  | 9.20   | 38.58             | 18                                              | 25                                              | −0.075                       | plas            |
| Sal10G04200.1 | SaRac2 | 198                   | 21.83                  | 9.43   | 34.09             | 16                                              | 27                                              | −0.129                       | chlo            |
| Sal8G02490.1  | SaRac3 | 198                   | 21.81                  | 9.38   | 40.23             | 18                                              | 25                                              | −0.042                       | chlo            |
| Sal7G09100.1  | SaRac4 | 197                   | 21.55                  | 9.32   | 41.48             | 18                                              | 27                                              | −0.106                       | chlo            |
| Sal9G31620.1  | SaRac5 | 196                   | 21.45                  | 9.25   | 39.09             | 16                                              | 28                                              | −0.047                       | chlo            |
| Sal9G09920.1  | SaRac6 | 196                   | 21.74                  | 9.62   | 38.96             | 17                                              | 25                                              | −0.083                       | chlo            |
| Sal6G04230.1  | SaRac7 | 211                   | 23.28                  | 9.18   | 44.44             | 19                                              | 28                                              | −0.069                       | chlo            |
| Sal9G07020.1  | SaRac8 | 209                   | 22.94                  | 9.27   | 36.73             | 19                                              | 27                                              | −0.141                       | plas            |
| Sal9G04490.1  | SaRac9 | 196                   | 21.63                  | 9.55   | 40.28             | 18                                              | 27                                              | −0.120                       | cyto            |

3.2. Analysis of SaRac Protein Homology

The conserved domains of the nine SaRac proteins were predicted using NCBI-Conserved Domain Search, and the results showed that all nine Racs contained Rop-like domains (Figure 1A) belonging to the Rop gene family. The structure of the SaRac gene family was analyzed using TBtools (Figure 1B). To investigate the structural diversity of Racs, a total of 10 conserved motifs in the Racs were captured by the MEME website, and we obtained five conserved motifs located in the Rac domain. We further analyzed the sequence structures of nine SaRac genes and aligned them to eleven Rac members in A. thaliana [37] and seven Rac members in rice [38] (Figure 1C,D). It is worth noting that...
the type and distribution of the C-terminal domains of most Rac are similar. The results showed that the nine SaRac proteins all had five conserved motifs, which were the same as the Rac proteins in Oryza sativa and A. thaliana.

Figure 1. The gene structures and conserved motifs of Rac family members in S. album based on evolutionary relationships. (A) Rop-like domains of SaRac proteins. (B) The exon-intron structure of SaRac proteins. (C) Conserved motifs of SaRac proteins. (D) Tree of evolutionary relatives: rice, A. thaliana and sandalwood.

3.3. Sequence Analysis of the SaRac Gene Family

Novel putative motifs were explored using the MEME server. By selecting a motif of full length, we identified eight conserved motifs located in the SaRac domain (Figure 2A,B).

To analyze cis-acting elements in putative SaRac promoters, the 2000 BP promoter sequences of nine SaRac genes were identified as cis-regulatory elements by TBtools and the Plant CARE website, and their functions and numbers were predicted (Figure 2C). There were differences in the types and numbers of regulatory elements in the promoters of the nine SaRac genes, but all of them had multiple hormone response elements and stress response elements. For example, SaRac1 and SaRac4 have two hormone response elements, while SaRac2 and SaRac3 have three hormone response elements, SaRac6-8 have four hormone response elements and SaRac5 and SaRac9 have five hormone response elements. In addition, SaRac3/6/8 have one stress response element, SaRac4/7/9 have two stress response elements and SaRac5 and SaRac2 have three and four stress response elements, respectively. Among them, SaRac6 has ten CGTCA motifs, and we can further speculate that this gene may be related to the chemical defense response of S. album.

3.4. Chromosome Distribution of the SaRac Gene Family

To clarify the distribution of SaRac on the chromosomes of S. album, we used TBtools software to map the location of SaRac family members (Figure 3A). The SaRac family members of S. album showed irregular distribution on the chromosome and did not form a large number of gene clusters. The graph shows that nine SaRac genes are located on chr6, chr7, chr8, chr9 and chr10 of S. album, and more than half of them have one SaRac gene member on the chromosome. The remaining two chromosomes have four and one SaRac gene members. Notably, in S. album linkage groups6/7/8/9/10, the eight SaRac genes were classified into five segmental duplication events (SaRac4/SaRac1, SaRac4/SaRac3, SaRac3/SaRac1, SaRac9/SaRac1 and SaRac6/SaRac1) (Figure 3A,B) (Table S2).
Figure 2. Sequence analysis of the SaRac gene family. (A) Motif locations of SaRac. (B) Discovered motifs. (C) Cis-acting Elements in Putative SaRac Promoters.

Figure 3. Interchromosomal relationships of SaRac genes. (A) The location of SaRac genes on the S. album chromosome. (B) Interchromosomal relationships of Rac genes in S. album. Gray lines indicate all synteny blocks in the S. album genome, and red lines indicate segmental duplication events.

To further infer the phylogenetic relationship between sandalwood, A. thaliana and rice, we constructed two syntenic maps of sandalwood with A. thaliana and rice. A total of three SaRac genes showed syntenic relationships with Racs in O. sativa (Figure 4A). In A. thaliana, there are many SaRac genes that are in common with Racs (Figure 4B) (Table S3).
Figure 3. Interchromosomal relationships of SaRac genes. (A) The location of SaRac genes on the S. album chromosome. (B) Interchromosomal relationships of Rac genes in S. album. Gray lines indicate all synteny blocks in the S. album genome, and red lines indicate segmental duplication events.

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3.5. Phylogenetic Comparison of Rac in Different Species

To further evaluate the phylogenetic relationship between SaRac and other plants, 11 and 7 Rac sequences from A. thaliana and rice were compared with 9 Rac sequences from S. album, and the phylogenetic tree of the whole protein sequence alignment was constructed by MEGA-X. The results showed that the Rac genes of these three species can be divided into five subgroups: I, II, III, IV and V (Figure 5). From the available literature, we further obtained the role of Rac in A. thaliana and rice. For instance, in A. thaliana, AtRac1/6/11 are highly expressed in mature pollen and play an important role in pollen tube growth [37]. AtRac2 overexpression inhibits the growth of root tips. AtRac3/8 inhibit ABA-induced responses, including actin recombination in guard cells, stomatal closure, seed germination, root elongation and gene expression [39,40]. AtRac4 is a positive regulator of root hair initiation and apical growth [41]. AtRac5 acts on actin dynamics, polar growth, root hair growth and so on; finally, AtRac10 participates in the regulation of membrane transport. On the other hand, in rice, OsRac1 is a resistance and grain size gene [42,43]. OsRac4/5 are negative regulators of blast resistance, and OsRacB is a direct effector of OsRopGEF2/3/6. It is a potential downstream target of OsRopGEF2/3/6/8 and confers salt tolerance, a negative regulator of disease resistance [44]. In addition, we can more accurately estimate the functions likely to be contained in the members of the nine SaRac gene families.

3.6. Analysis of Rac/Rop Multibase Regions in S. album, A. thaliana and O. sativa

The members of the Rac gene family are divided into type I Rac/Rop protein and type II Rac/Rop protein. Type I proteins have a conserved CaaL motif at the C-terminus. However, type II has a truncated, but functionally modified, posttranscriptional motif. Nine SaRac, eleven AtRac and seven OsRac proteins were sequenced, and the results showed that SaRac1-7 and AtRac1-6, AtRac9 and AtRac11 as well as OsRac5, OsRacB and OsRacD belong to the typical type I, while SaRac8-9 and AtRac7, AtRac8 and AtRac10 and OsRac1-4 belong to type II (Figure 6).

The conserved G domains in the N-terminal region of these proteins were GTPase active domains (G1, G3), Mg\textsuperscript{2+} binding domains (G2) and GTP binding sites (G4, G5). The G2 and G3 domains are also called switch I and II loops, and the C21 and C156 positions are the conserved L-cysteine residues of the G domain (Figure 6).
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Further obtained the role of Rac in A. thaliana and rice. For instance, in sandalwood, in contrast, a high expression of SaRac2 may have a strong positive correlation with the growth and development of haustoria in different species. The expression levels of SaRac1-7 and AtRac1-6, AtRac9 and AtRac11 as well as OsRac5, OsRacB and OsRacD were sequenced, and the results showed that SaRac, eleven AtRac and seven OsRac proteins were highly expressed in mature pollen and play an important role in pollen tube growth. Conserved motifs are highlighted by blue boxes.

Figure 5. Phylogenetic analysis of Racs from three species (A. thaliana, O. sativa, S. album). Full-length polypeptide sequences were used to make the interspecific phylogenetic tree.

Figure 6. Protein sequence multialignment and domain structure of Racs from S. album, O. sativa and A. thaliana. Conserved motifs are highlighted by blue boxes.
3.7. Tissue Specificity of Rac Expression in S. album

We first investigated the tissue-specific expression of nine Rac gene family members in roots, stems, leaves and primary haustoria of S. album by green fluorescent quantitative PCR with gene-specific primers. Nine genes (SaRac1-9) were expressed in roots, stems, leaves and primary haustoria, but there was a difference in their expression levels. The expression of SaRac7/8/9 in stems, haustoria and roots was higher than that in leaves, but the expression of SaRac1, SaRac4 and SaRac6 in stems was lower in leaves. The expression levels of SaRac2 in stems and primary haustoria were high. It is worth noting that the expression levels of SaRac2 and SaRac5 in roots were approximately 6 and 13, respectively, relative to leaves (Figure 7). Therefore, we can further speculate that SaRac2 and SaRac5 may have a strong positive correlation with the growth and development of haustoria in sandalwood. In contrast, a high expression of SaRac2 and SaRac5 may inhibit the expression of SaRac2 and SaRac5 in leaves. The tissue-specific expression of Rac showed that different Rac members play different roles in different signaling pathways of S. album.

![Figure 7. Expression profiles of SaRac genes in S. album across different organs. The expression level of SaRac genes in S. album in four organs (leaf, stem, haustoria, root). The relative expression level was calculated by setting the expression value of SaRac genes in the leaves of S. album at 1. * indicates significant difference by t test at p < 0.05.](image)

3.8. Expression of SaRac Genes under Drought and Hormones Treatments

To better understand the function of SaRac in response to abiotic stress, nine SaRac genes were selected for further analysis. Under drought stress, the expression of more than a third of the genes, including SaRac1, SaRac3, SaRac4 and SaRac7 were increased. The general trend of SaRac1, SaRac3, SaRac4 and SaRac7 expression was first increased and finally decreased at 9 d. However, the expression levels of SaRac2 and SaRac9 were downregulated. Only two genes, SaRac6 and SaRac8, showed higher expression under long-term drought treatment than those under control conditions (Figure 8A).
The expression levels of SaRac1, SaRac6, SaRac7 and SaRac8 increased after 48 h of IAA treatments, indicating that these genes were responsive to IAA. On the contrary, the expression levels of SaRac2, SaRac4, SaRac5 and SaRac9 were lower under IAA treatments than those under control conditions. Furthermore, more than half of the genes, including SaRac1, SaRac3, SaRac5 and SaRac6, showed higher expression under ethephon treatments. It is worth mentioning that the expression of SaRac5 under ethephon-treated conditions was 8 times higher than that under control conditions. Moreover, ABA treatment significantly induced the expression of SaRac1, SaRac3, SaRac7 and SaRac8. However, SaRac2, SaRac4 and SaRac9 expression were decreased in response to both ethephon and ABA treatments.

4. Discussion

Rac small GTPases are members of the plant-specific Rho subfamily and are involved in many signaling events, such as defense responses, pollen tube growth, root hair development, reactive oxygen species (ROS) production and phytohormone response, and play a very important role in the abovementioned events [45,46]. Rac protein is a soluble protein that localizes in the plasma membrane and functions through posttranslational lipid modification [23,47,48]. For example, 11 Rac genes have been identified in A. thaliana, and there are eight type I Rac genes: AtRac1-6, AtRac9, and AtRac11. Seven Rac gene family members were identified in rice, while seven Rac family members were identified in H. vulgare, indicating that the copy number of Rac genes is not the same in different species. Studies have shown that AtRac1/6/11 are highly expressed in mature pollen, and AtRac4/2 are a pair of positive and negative regulators of root hair tips. At present, most of the research on Rac genes focuses on medicine and animals, while research on plants focuses on model plants, such as A. thaliana, O. sativa and Hordeum vulgare. Little is known about the mechanisms by which SaRac impacts the growth of S. album.

Reactive oxygen species (ROS) produced by NADPH oxidase have been shown to play many important roles in signaling and development in plants, such as in plant defense response, cell death, abiotic stress, stomatal closure, and root hair development [49–53]. In sandalwood, they control the development and formation of haustoria [13]. At present, studies have shown that the interaction between Rac GTPases and the N-terminal extension is ubiquitous and that a substantial part of the N-terminal region of Rboh, including the two EF-hand motifs, is required for the interaction [54]. The interaction between Rac and Rboh also provides further theoretical help for the study of the mechanism of Rac. At the same time, the regulation of Rboh ROS production by Rac provides a theoretical basis for the development of sandalwood haustoria.
Auxin, abscisic acid (ABA) and ethephon play key roles in the development of many plants. These three hormones are often used as the main substances in plant hormone response experiments. In this study, we treated nine Rac genes with drought and hormones. Only two genes, SaRac6 and SaRac8, showed higher expression under long-term drought stress than those under control conditions.

The results of hormone treatments indicated that the expression of SaRac1, SaRac3, SaRac7 and SaRac8 were higher under ABA treatments. Moreover, more than half of the genes, including SaRac1, SaRac3, SaRac5 and SaRac6, showed higher expression under ethephon treatments. Furthermore, the expression levels of SaRac1, SaRac6, SaRac7 and SaRac8 increased after 48 h of IAA treatments, indicating these genes are responsive to IAA. Previous studies had demonstrated that auxin biosynthesis is essential for haustorium in haustorium formation in the root-parasitic plants [55,56]. Thus, these genes may be involved in the ontogeny of the S. album haustorium and further influence the growth and development of sandalwood.

In our study, to better understand the evolution of the Rac gene family in sandalwood, the structure, conserved motifs, phylogenetic relationships and collinearity of SaRac genes were characterized. One conserved motif was located in the Rac domain, suggesting that the Rac domain is conserved among A. thaliana, rice and sandalwood. Most of the SaRac genes exhibited similar numbers of exons. Phylogenetic analysis revealed that SaRac, AtRac and OsRac proteins could be classified into five subgroups. Groups IV and V contain more Racs that come from all three species, and groups I and II also contain Racs from all three species. Moreover, group III contains one Rac in A. thaliana. For instance, SaRac5 to SaRac9 in group IV and SaRac4 to AtRac1 in group V could have expansive functions surrounded by AtRac, SaRac or OsRac proteins with different functions.

Additionally, synteny maps between two representative species and sandalwood were constructed to better understand the phylogenetic relationships. More than 10 pairs were detected in A. thaliana, and three pairs were detected in rice, indicating a strong homologous relationship between sandalwood and A. thaliana, and a weak homologous relationship between sandalwood and rice.

Gene duplication is a major mechanism underlying the evolution of novel protein functions. We detected five SaRac genes that were assigned to segmental duplication events, implying high segmental duplication. These results indicated that some SaRac genes were possibly generated by gene duplication.

Most Rac proteins identified to date have been functionally characterized in A. thaliana and rice, and their roles include the regulation of root hair initiation and apical growth, hormonal responses, stress responses and so on. Among them, the best-described Rac proteins are the members of group IV (Figure 5). These Rac genes are involved in root hair formation and disease resistance. In the phylogenetic tree, we can more accurately estimate the functions likely to be contained in the members of the SaRac gene family. For instance, we can predict the role of SaRac gene by the AtRac members of group IV. Similarly, we can make a preliminary prediction of the role of SaRac members in groups I/II/V.

Sandalwood is considered one of the most valuable trees in the world. Its heartwood is often used in carving crafts, cosmetics, medicine and other industries, but its main value lies in the essential oils extracted from the heartwood [57]. Therefore, it is important to investigate whether SaRac may be related to their accumulation in sandalwood haustorium tissue, thereby affecting the growth and development of the heartwood of sandalwood and, in turn, the quality of heartwood essential oils [58].

In gene expression, promoters play an important role in regulation, through which gene expression can be changed to change the characteristics of plants. Therefore, the study of promoters is a key step in genetic engineering and gene expression research [59]. In addition to the typical core promoter, there are many regulatory elements controlling the functional expression of genes. Analysis of cis-acting elements revealed that most of the SaRac promoters contained a large number of elements related to hormones and stress response. The abscisic acid response element (ABRE) was found in the promoters of
SaRac2-9, and the gibberellin response element (P-box) was found in the promoters of SaRac1/2/4/5/6/7/8/9. However, SaRac2/3/5/8/9 all had TC-rich repeats related to defense and stress responses, suggesting that these Rac genes may be involved in disease resistance and stress resistance of *S. album*.

To understand the function of nine *SaRac* members more accurately, based on the functional prediction of gene promoters, in this study, the expression levels of nine genes at four sites were detected by green fluorescent quantitative PCR. The results showed that, compared with the expression levels of leaves in each gene, the expression levels of *SaRac1*, *SaRac4* and *SaRac6* in stems were very low, while the expression levels of *SaRac2* in stems and primary haustoria were very high, and the expression levels of *SaRac2* and *SaRac5* in roots were approximately 6 and 13, respectively. In general, the level of protein expression in specific tissues is closely related to its function. The high expression of *SaRac2* and *SaRac5* in roots and *SaRac2/3/7* in haustoria may indicate that these genes are closely related to the formation of haustoria in *S. album*.

### 5. Conclusions

Because of the lack of research on the molecular mechanism of growth and development in *S. album* at present, in this study, the biological information and expression pattern of 9 Rac genes in *S. album* were analyzed (Figure 9). These results lay the physical and chemical foundation for further studies of the Rac family genes involved in the growth and development of *S. album* and regulation its functions. The perspectives of research on the semiparasitic sandalwood will develop towards the Rac-dependent generation of ROS in promoting haustorium development, which more effectively provides a data base for the growth mechanism of sandalwood.

**Figure 9.** Framework figure. Protein and gene sequences of *SaRacs* were obtained from genome. Bioinformatic analyses were conducted, and the expression profiles of *SaRac* genes in different tissues and under drought and hormones treatments were obtained. These results established a preliminary foundation for the functional study of *SaRac* genes.

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/life12121980/s1](https://www.mdpi.com/article/10.3390/life12121980/s1), Table S1: Primer design; Table S2: Segmental replication events in sandalwood; Table S3: Synteny analysis of Rac genes between sandalwood, *A. thaliana* and rice.

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