TcJAV3–TcWRKY26 Cascade Is a Missing Link in the Jasmonate-Activated Expression of Taxol Biosynthesis Gene DBAT in Taxus chinensis

Li Chen 1,†, Ling Wu 1,†, Liu Yang 1, Haiyang Yu 1, Pingliang Huang 1, Yuehua Wang 1, Ruifeng Yao 1,2 and Meng Zhang 1,*

1 State Key Laboratory of Chemo/Biosensing and Chemometrics, Hunan Provincial Key Laboratory of Plant Functional Genomics and Developmental Regulation, College of Biology, Hunan University, Changsha 410082, China
2 Shenzhen Research Institute of Hunan University, Shenzhen 518055, China
* Correspondence: zhangmeng2019@hnu.edu.cn; Tel./Fax: +86-731-85581765
† These authors contributed equally to this work.

Abstract: Jasmonates (JAs) are the most effective inducers for the biosynthesis of various secondary metabolites. Currently, jasmonate ZIM domain (JAZ) and its interactors, such as MYC2, constitute the main JA signal transduction cascade, and such a cascade fails to directly regulate all the taxol biosynthesis genes, especially the rate-limit gene, DBAT. Another JA signaling branch, JAV and WRKY, would probably fill the gap. Here, TcJAV3 was the closest VQ-motif-containing protein in Taxus chinensis to AtJAV1. Although TcJAV3 was overexpressed in AtJAV1 knockdown mutant, JAVRi17, the enhanced disease resistance to Botrytis cinerea caused by silencing AtJAV1 was completely recovered. The results indicated that TcJAV3 indeed transduced JA signal as AtJAV1. Subsequently, TcWRKY26 was screened out to physically interact with TcJAV3 by using a yeast two-hybrid system. Furthermore, bimolecular fluorescence complementation and luciferase complementary imaging also confirmed that TcJAV3 and TcWRKY26 could form a protein complex in vivo. Our previous reports showed that transient TcWRKY26 overexpression could remarkably increase DBAT expression. Yeast one-hybrid and luciferase activity assays revealed that TcWRKY26 could directly bind with the wa-box of the DBAT promoter to activate downstream reporter genes. All of these results indicated that TcJAV3–TcWRKY26 complex is actually another JA signal transduction mode that effectively regulates taxol biosynthesis in Taxus. Our results revealed that JAV–WRKY complexes directly regulated DBAT gene in response to JA stimuli, providing a novel model for JA-regulated secondary metabolism. Moreover, JAV could also transduce JA signal and function non-redundantly with JAZ during the regulation of secondary metabolisms.

Keywords: JAV; WRKY; JA signal transduction; DBAT; taxol biosynthesis

1. Introduction

Jasmonates (JAs), a kind of endogenous hormone that regulates plant growth and development, are considered one of the most effective inducers of secondary metabolite biosynthesis in various plants [1,2]. Moreover, as a hormone, JAs have been certified as a vital defense signal against herbivorous insects and necrotrophic pathogens [3]. Thus, the JA signaling transduction pathway has been deeply investigated in defense response [4], seed germination [5,6], seed size [7], regeneration [8], and others [9] over the past decades. Since JA is the most effective inducer of secondary metabolism, the JA-responding mechanisms of the biosynthesis genes of these secondary metabolites were deeply investigated [10–12].

JA regulates secondary metabolite biosynthesis dependent on jasmonate ZIM domain (JAZ) and its interactors, including MYCs, bHLHs, and MYBs [2]. For example, several investigations have elucidated a clear JA regulation mode of artemisinin biosynthesis in...
Artemisia annua. In brief, JA accumulation would promote the ubiquitination degradation of AaJAZs, resulting in the release of AaMYC2, which could directly activate CYP71AV1/DBR2 by binding with G-box-like elements [13]. The JA regulation mode is conserved in different secondary metabolisms, such as NtJAZs–NtMYC2 in nicotine biosynthesis and CrJAZs–CrMYC2 in vinblastine biosynthesis [14]. All of these studies showed that JAZ repressors are crucial components in transducing JA signals to the biosynthesis genes of secondary metabolites. Taxol, also called paclitaxel, is produced from Taxus spp. and used as the most broad-spectrum anticancer drug [15]. Similar to other secondary metabolites, JA is able to greatly promote taxol biosynthesis. However, different from most other secondary metabolites, such as artemisinin, whose biosynthesis only needs four or five enzymes, taxol has a highly complicated chemical structure and needs more than 19 enzymes to catalyze the diterpene universal precursor, geranylgeranyl pyrophosphate, to the final product [16]. Among 14 known enzymes, taxane synthase (TASY) and 10-deacetylbaccatin III-10β-O-acetyltransferase (DBAT) were functioned crucially for taxol biosynthesis; TASY decides the flux because it is the first enzyme catalyzing universal diterpene precursor and produces crucial skeleton; DBAT acts as a rate-limiting enzyme [17,18]. Additionally, TASY and DBAT are early JA-responsive genes (Figure 1a) [19]. However, only TASY had been clearly elucidated as the JA-responding mechanism. A similar JA transduction pathway to TASY consisting of TcJAZ3, TcMYC2a, and the downstream factor TcERF15 was found. TcMYC2a and TcERF15 regulate TASY by directly binding with the E-box and GCC-box, respectively [18,20,21]. However, no solid evidence showed that the two factors were direct regulators of other genes. That is, the current JA transduction mode mediated by TcJAZs was not sufficient to explain the JA-responsive mechanism of all genes. For instance, the core promoter region of DBAT had no E-/GCC-/MBS-box, which codes a key rate-limiting enzyme, or no JAZ-MYB complexes were found to regulate taxol biosynthesis [19,22,23]. These results implied that other ways exist to transduce JA signals to taxol biosynthesis in addition to mediation by JAZs.

JAV1 might be the missing link of JA signals and DBAT gene. Hu et al. found that, independent of JAZs, AtJAV1 could also transduce JA signals and function similarly as JAZs to activate defensive gene expression and elevate resistances against insects and pathogens [24]. AtJAV1 belongs to VQ-motif-containing protein (VQP), which is a class of plant-specific proteins with a conserved and single short FxxhVQxhTG amino acid sequence motif [25]. Importantly, VQP, including AtJAV1, could physically interact with several WRKY transcription factors, which belong to Groups I and Iic [26]. Interestingly, current effective regulators of the DBAT gene were all WRKYs, including TcWRKY1 (Group IIa), TcWRKY26 (Group I), and TcWRKY33 (Group I) [23,27,28]. The results gave us a clue that the JA regulation of the DBAT gene might be mediated by JAV–WRKY complexes. Moreover, the two Group I WRKYs, TcWRKY26 and TcWRKY33, might be the direct downstream factor of TcJAVs. Previously, TcWRKY26 was found to be a MeJA-responsive WRKY factor, and its overexpression increased DBAT expression in Taxus suspension cells [27], whereas TcWRKY33 was a SA-responsive factor (Chen et al. 2021). Thus, TcWRKY26 was the most potential downstream factor of TcJAVs.

In this study, we aimed to screen out the homolog of AtJAV1 in Taxus and verify whether the JAV–WRKY mode exists and effectively regulates the DBAT gene. TcJAV3 was identified to be closest to AtJAV1 through homolog search from multi-omics data. The TcJAV3 gene was complemented into the knockdown mutant JAV1Ri17, the Arabidopsis transgenic RNAi line of AtJAV1, to confirm the functions of TcJAV3 in JA signal transduction. We compared the disease resistance of complement lines, JAV1Ri17 and wild types, to evaluate the functions of TcJAV3. Then the interactions of reported TcWRKYs with TcJAV3 were conducted by using a yeast two-hybrid system (Y2H). The DBAT promoter was studied in detail. The interacting TcWRKY was checked to determine whether it is a direct regulator of the DBAT gene. Our results revealed that JAV–WRKY complexes take part in defense and play important roles in secondary metabolisms. In addition, two JA
transduction branches, JAV–WRKY and JAZ–MYC2, are equally important in transducing JA signals, although they might have different targets.

Figure 1. Characterization of TcJAV3 (a) Taxol biosynthesis pathway. GGPP (geranylgeranyl pyrophosphate) is the universal precursor of all diterpene, and TASY would cyclize it as the core structure of taxol. Then series of hydroxylation and acylation, including the 13′ side chain modification, take place on the core structure. DBAT is a rate-limiting enzyme in this pathway. (b) The aligned sequence of TcJAV3 and AtVQs. Besides the VQ-motif, the residues from 95 to 130 of TcJAV3 are highly similar with AtJAV1. (c) Phylogenetic tree of TcJAV3 and all AtVQPs. The gene expression levels of TcJAV3 and TcWRKY26 responding to JA are shown in (d,e). Statistics analysis was conducted as Student t-test; * represents p-value < 0.05, and ** represents p-value < 0.01.
2. Results

2.1. TcJAV3, a Typical VQP, Was Closest to AtJAV1

VQP was named after a 10 aa conserved VQ-motif in their proteins. Apart from the short VQ motif, VQP has a relatively low sequence homology. Thus, 34 Arabidopsis VQPs were quite divergent and probably functioned in various bioactivities [26]. Now, AtVQ22, also called AtJAV1, was confirmed to function in the JA signaling transduction pathway [24]. Therefore, TcJAV3 was sequence aligned and phylogenetically analyzed.

The alignment showed that the 10 aa VQ-motif and its N-terminal 25 residues, a total of 34 aa (95–128 aa), were highly conserved between TcJAV3 and AtJAV1 (Figure 1b). However, the other parts of these proteins had barely any similarity (Supplementary Figure S1). A comparison of TcJAV3 with other AtVQPs showed a similarity in 20 residues (about 109–128 aa), including the VQ-motif (Supplementary Figure S2). The results suggest that TcJAV3 was more similar to AtJAV1, and the 95–109 aa might have an unknown but important function for TcJAV3 and AtJAV1. Consistent with previous studies, a majority of the branches had low confidence values, including the branching containing TcJAV3 and AtJAV1 (Figure 1c and Supplementary Figure S3). Nonetheless, TcJAV3 was the closest to AtJAV1 and its homolog, indicating a similar function between them.

2.2. TcJAV3 Complemented Arabidopsis Knockdown Mutant JAV1Ri17

TcJAV3 was complemented to AtJAV1 knockdown Arabidopsis line, JAV1Ri17, under CaMV 35S promoter control for further study because of the presence of divergent sequences. In JAV1Ri17, AtJAV1 is silenced; mutant plants have enhanced resistance to necrotrophic pathogens and herbivorous insects [24]. Our results were similar with the previous report; that is, the knockdown JAV1Ri17 line was resistant to B. cinerea by phenotypic observations, including disease severity, lesion area, and plant-survival percentage. After B. cinerea spore suspension was sprayed for 5 days, coi1-2 barely survived, and all leaves were severely disease infected. About 84% of JAV1Ri17 lines survived; only 73% and 75% survived for the Col-0 and TcJAV3 complement lines, respectively; and no remarkable differences were found between the WT and TcJAV3 complement lines (Figure 2a–c).

Similarly, in the drop inoculation experiments, coi1-2 exhibited the most serious disease symptoms, with the largest lesion areas, but JAVRi17 showed a highly strong resistance. WT and TcJAV3 complement lines showed middle disease symptoms without difference (Figure 2d,e). The results demonstrate that the complementation of TcJAV3 indeed covered AtJAV1 silencing, and TcJAV3 performed the same function in the JA signal transduction pathway as AtJAV1. Thus, TcJAV3 was capable of transducing JA signals in Taxus.

2.3. TcWRKY26, a Typical Group I WRKY Factor, Physically Interacted with TcJAV3

According to previous reports, VQP commonly interacted with WRKY factors but only the members of Groups I and Iic [26]. Thus, we screened WRKYs from these two subtypes according to our previous reports [27].

TcWRKY26 contained two WRKY domains, WRKY_NT and WRKY_CT, which are characteristic of Group I WRKY proteins, whereas Groups II and III had only one WRKY domain each (Figure 3a and Supplementary Figure S3). The phylogenic analysis also showed that TcWRKY26 was clustered with known Group I WRKY factors with high values, and TcWRKY26 was the closest to AtWRKY20 (Figure 3b).
Figure 2. Disease-resistance phenotype in TcJAV3 complement Arabidopsis line. (a) The phenotypes of coi1-2, WT (col-0), AtJAV1 RNAi transgenic plants (JAVRi17), and TcJAV3 complement plants (35S-TcJAV3/JAVRi17) 7 days after spray inoculation with B. cinerea or water (CK). (b) Disease severity of the plants indicated in (a). The darker bars indicate the percentage of leaves with severe disease symptoms; the white or light gray bars indicate weak symptoms or no visible symptoms. (c) Survival percentage of the plants indicated in (a) (mean ± SEM; n = 12; statistics by one-way ANNOVA Duncan test). Bars with the different letter are significantly different from one another, p < 0.05. (d) Necrotic lesion area in each leaf described in (e) (mean ± SEM; n = 12; statistics by t test; * p < 0.01, ** p < 0.01, and **** p < 0.001). (e) The phenotypes of the leaves from WT, col1, JAVRi17, and 35S-TcJAV3/3/JAVRi17 at 48 h after drop inoculation with 5 µL spore suspension of B. cinerea or with water (CK).
TcWRKY26 contained two WRKY domains, WRKY_NT and WRKY_CT, which are characteristic of Group I WRKY proteins, whereas Groups II and III had only one WRKY domain each (Figure 3a and Supplementary Figure S3). The phylogenetic analysis also showed that TcWRKY26 was clustered with known Group I WRKY factors with high values, and TcWRKY26 was the closest to AtWRKY20 (Figure 3b).

**Figure 3.** TcWRKY26, classical Group I WRKY, physically interacted with TcJAV3 (a) The two WRKY domains of TcWRKY26; only Group I WRKY factors have two WRKY domains. In the WRKY_domain_CT, the two D residues (labeled as *) and Cx2C motif are essential for the interaction with VQPs. (b) Phylogenetic tree of TcWRKY26 and reported Group I WRKYs. (c) Y2H results of TcWRKY26 and TcJAV3. SD/-TL: SD/-Trp-Leu; SD/-TLH: SD/-Trp-Leu-His; SD/-TLHA: SD/-Trp-Leu-His-Ade. (d) BiFC results of TcWRKY26 and TcJAV3. (e) LCI results of TcWRKY26 and TcJAV3. Bar = 50 µm.

Only four amino acid residues were found between the two conserved Cys residues (Cx4C) in the zinc-finger structure of the C- and N-terminal WRKY domains of TcWRKY26 (Figure 3b and Figure S4). Additionally, two Asp residues, D491 and D494, which immediately precede the WRKYGQK signature sequence in the WRKY-CT of TcWRKY26, were the same with the D359 and D362 of AtWRKY33. Cx4C and two D residues are critical for interaction with VQPs [26].

Using Y2H, the yeast cells containing TcJAV3 and TcWRKY26 could grow in SD/-TLHA-deficient medium, indicating that TcJAV3 could bind with TcWRKY26 in yeast (Figure 3c). Moreover, TcJAV3 and TcWRKY26 were fused with the C- and N-terminus EYFP, respectively. Then fluorescence microscopy revealed a clear yellow fluorescence in tobacco leaves (Figure 3d). Luciferase complementary imaging (LCI) also verified that TcJAV3 could physically interact with TcWRKY26 in tobacco (Figure 3e).
2.4. TcWRKY26 Was a Direct Regulator of DBAT by Binding with W-Box in Its Promoter

The overexpression of the transient cell lines of TcWRKY26 could upregulate the DBAT gene by two folds [27]. However, we do not know whether TcWRKY26 directly regulates the DBAT gene. Many studies reported that two w-boxes are crucial cis-elements in the DBAT promoter [23,28] (Figure 4a). Using Y2H, the yeast cells containing TcJAV3 and TcWRKY26 could grow in SD/-TL H A- deficient medium, indicating that TcJAV3 could bind with TcWRKY26 in yeast (Figure 3c). Moreover, TcJAV3 and TcWRKY26 were fused with the C- and N-terminus EYFP, respectively. Then fluorescence microscopy revealed a clear yellow fluorescence in tobacco leaves (Figure 3d). Luciferase complement imaging (LCI) also verified that TcJAV3 could physically interact with TcWRKY26 in tobacco (Figure 3e).

Thus, we first tested the binding affinity of TcWRKY26 and the two w-boxes by using the yeast one-hybrid system (Y1H). The wa-box and wb-box were triplicated and ligated into pHIS2.1 to generate bait vectors. Then TcWRKY26-AD was co-transformed into yeast Y187 cells with two bait vectors (Figure 4b). The positive transformers containing TcWRKY26-AD with wa-box or wb-box could grow in all mediums by selecting the deficient medium, and these transformers with the w-box were inhibited in SD/-TLA with more than 100 mM 3-AT (Figure 4c). Such results indicated that TcWRKY26 could bind with wa-box or wb-box and that wa-box had a higher affinity than wb-box.

Subsequently, the original DBAT promoter and progressive deletion fragments were separately cloned to the 5′ terminus of luciferase to generate three reporters (Figure 4d). Co-transformation with the effector 35S-TcWRKY26 showed that only complete DBATp
had an increased LUC activity in tobacco leaves. The fragments containing wa-box only or no w-box did not show any difference when co-transformed with TcWRKY26. The result indicated that TcWRKY26 directly upregulated the DBAT gene by binding with wa-box.

3. Discussion

AtJAV1 belongs to VQPs and is characterized by a 10 aa peptide (labeled as FxxhVQx-hTG). However, excluding the VQ-motif, no similarity exists between VQPs in the same species [25]. Thus, screening out the homolog of AtJAV1 in Taxus was crucial in our work. According to high-throughput data, TcJAV3 was found to be the most relative with AtJAV1 from total 24 full-length TcVQ proteins in T. chinensis. Only the residue F of the VQ-motif in TcJAV3 was different with the Y residue of the VQ-motif in AtJAV1 (Figure 1a). Moreover, an N-terminus with a 25 aa peptide, which immediately precedes the VQ-motif, was also highly conserved with AtJAV1, suggesting that TcJAV3 and AtJAV1 evolved more strictly. Moreover, TcJAV3 was grouped with AtJAV1 and its homologs but not other Arabidopsis VQPs, although the branch value was low. All of these results indicated that TcJAV3 is a potential AtJAV1 homolog in Taxus. Therefore, we complemented TcJAV3 into the Arabidopsis AtJAV1 knockdown line, JAV1Ri17, to test whether TcJAV3 could recover the phenotype change caused by AtJAV1 knockdown. JAV1Ri17 exhibited a stronger resistance to pathogen infection, such as B. cinerea infection [24]. AtJAV1 functions as a negative regulator to control plant defense. Whole-plant spray inoculation and drop inoculation tests indicated that the complement transgenic lines of TcJAV3 were more susceptible to B. cinerea infection than JAV1Ri17 (Figure 2). Complement transgenic lines displayed similar disease symptoms with Col-0, indicating that TcJAV3 actually played similar roles as AtJAV1. Otherwise, TcJAV3 could transduce JA signals in Taxus with high possibilities. Similar to JAZ proteins, JAVs also recruit several transcription factors to form a complex. Moreover, JAV proteins were found to physically interact with WRKY factors [26]. Interestingly, we and co-workers had found that there were two w-boxes that specially bind with WRKY, localized in the key site of DBAT promoter region previously. Although these two cis-elements were first identified as salicylic acid (SA)-responsive elements, many JA-responsive WRKY factors bind with them, such as TcWRKY1 [23]. More WRKY factors were found to bind with the two w-boxes, suggesting that the two w-boxes are crucial for DBAT gene expression [28]. Interestingly, JAV1 transduces JA signals by recruiting WRKY factors. Such information notified us that JAV1 and some WRKYs might be the missing link for DBAT to respond to JA signals. Cheng et al. found that two structural features of WRKY domains are critical for interaction with VQPs. D359 and D362, which precede the WRKYGQK-motif in AtWRKY33 (Group I), are critical residues for interaction with VQPs. The other feature is that only four amino acid residues are present between the two conserved Cys residues (Cx4C) in the zinc-finger structure of the C- and N-terminal WRKY domains. An insertion mutant of AtWRKY33 lost the ability to bind with VQ10 [26]. Therefore, we screened these WRKYs from reported functional TcWRKY factors. Through Y2H screening, a Group I factor, TcWRKY26, was validated to interact with TcJAV3 in yeast. We also performed BiFC and LCI experiments to further confirm the interaction of TcJAV3 and TcWRKY26. Previously, TcWRKY26 was found to be a MeJA-responsive WRKY factor, and its overexpression increased DBAT expression in Taxus suspension cells [27]. However, no proof has shown that TcWRKY26 is a direct regulator of the DBAT gene, and how TcWRKY26 responds and transduces JA signals are not clear. W-boxes (wa- and wb-box) are the key cis-elements in the DBAT promoter [23]. Thus, TcWRKY26 could directly bind with these w-boxes and then regulate DBAT gene expression. In this study, we verified that TcWRKY26, indeed, directly binds with the two w-boxes, especially wa-box, to regulate DBAT, as determined by Y1H and LUC activity assays (Figure 4). All of these results concluded that the TcJAV3–TcWRKY26 protein complex is the key component to transduce the JA signals to the DBAT gene. Moreover, our results indicated that the JAV–WRKY
complex plays important roles in the biosynthesis of secondary metabolites as important biotic defenses.

JAV1 is a freshly identified component of the JA signal transduction pathway; thus, not much is known about its functions in the regulation of secondary metabolite biosynthesis [24,29]. Hu et al., who first found JAV1, thought that JAV1 is a master controller that regulates JA-mediated plant defense but does not play a detectable role in plant development in Arabidopsis. However, according to our results, the JA signaling transduction pathway mediated by JAV also has multiple roles but not in plant defense. Indeed, components related to JAZ, including COI1, bHLH transcription factors (MYC2, MYC3, and MYC4), R2R3-MYB transcription factors (MYB21 and MYB24), and the WD-repeat/bHLH/MYB complexes, all play dual roles in regulating defense responses and diverse developmental processes [30–33]. Therefore, JAZ and JAV made JA a comprehensive and master hormone in regulating various plant bioactivities.

Current studies reported two ways to respond to JA signals for JAV. Ali et al. found that the ring-type E3 ubiquitin ligase JUL1 targets AtJAV1 and functions like COI1, which targets and mediates JAZ degradation [29]. The other way was that JAV–JAZ–WRKY forms a triple complex (JJW complex), and injury would cause the disintegration of the JJW complex by JAV1 phosphorylation, resulting in degradation [34]. Further studies were needed to clarify the detail of the response mechanism mediated by TcJAV3 (Figure 5).

Figure 5. JA regulation mechanism of taxol biosynthesis. Without JA molecules, the TcWRKY26 and TcMYC2a were physically interacted with and inhibited by TcJAV3 and TcJAZ3, respectively. However, when plants suffered attacks of insects and pathogens, TcJAV3 and TcJAZ3 were ubiquitination degraded, TcWRKY26 and TcMYC2a were released to activate DBAT and TASY genes. However, according to current reports, there is another way: the triplet of JAZ–JAV–WRKY was destructed when JAV was phosphorylated, and then WRKY was released. Further studies were needed to clarify the details of the mechanisms.

In conclusion, our work clarified another JA transduction pathway mediated by TcJAV3 and TcWRKY26 in Taxus that was different from the classical transduction system mediated by JAZ and its interactors (Figure 5). Moreover, the transducing complex con-
sisting of TcJAV3 and TcWRKY26 was the key and direct reason for the response of DBAT to JA signals. The DBAT gene encodes the step-limited enzyme in taxol biosynthesis and was discovered to quickly respond to JA signals early. However, how DBAT responds to JA signals was unclear. According to our results, TcJAV3 and TcWRKY26 were the missing links between JA signals and the DBAT gene. These results completed the regulation mechanism of JA signals on taxol biosynthesis and are valuable for related research on the functions of JAV in the biosynthesis of various secondary metabolites.

4. Materials and Methods

4.1. Plant and Pathogen Materials

Three-year-old *Taxus media* plants were cottage propagated from one mother plant, which was bought from Muyang Xiangwen Landscaping Co., Ltd, Suqian, China. The *Arabidopsis thiana* wild-type Col-0 and *Nicotiana benthamiana* were preserved and bred in our lab. The knock-down mutant *JAVRi17* and T-DNA insertion mutant *coi1-2* were provided by Prof. Xie’s lab, Tsinghua University. Both *Taxus media* and *Arabidopsis thiana* grew in the greenhouses at 21 °C, with a 16 h/8 h light/dark photoperiod. Moreover, *Nicotiana benthamiana* grew at 25 °C with the same photoperiod. For the *A. thiana* used in whole-plant spray incubation, the plants were under a short-day period with 14 h/10 h light/dark (short day, SD) for 5 weeks.

*Botrytis cinerea* 05.10 was preserved in PDB (Potato Dextrose Broth) medium by our lab.

4.2. Obtaining, Sequence Alignment, and Phylogenetic Analysis of TcJAV3 and TcWRKY26

Fresh *Taxus* leaves were harvested and quickly frozen by liquid N2. After crushing, the total RNA was isolated by using HiPure HP Plant RNA Mini Kit (Magen). The first-strand cDNA templates were generated according to the protocol of TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgene, Beijing, China). According to the previous report, primers were designed to amplify *TcWRKY26* [27]. Finally, we used PCR to amplify the two genes.

To identify TcJAV3, several transcriptome data and *Taxus* genome data were obtained from public databases. We used local blast tools (NCBI blast+ v2.8) to search the homologues of AtJAV1, and then these fragments with the FxxhVQxhTG were further investigated and labeled TcJAVs. All of these TcJAVs and AtJAV1-32 were aligned by ClustalW with Protein weight matrix GONNET (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=NPSA/npsa_clustalw.html, accessed at 22 May 2022).

The sequence alignment figures were all colored by Espript3.0 (https://espript.ibcp.fr/ESPr ipt/cgi-bin/ESPr ipt.cgi, accessed at 22 May 2022). Then the alignment files were submitted to MEGA-X for phylogenetic analysis to generate a neighbor-joining tree with 1000 resampling.

4.3. Complement Vector Construction and Arabidopsis Transforming

The TcJAV3 fragments were ligated into plant overexpression plasmid pBinGlyDsRED-35S with EcoRI and XhoI restriction sites [35], and then the complement vector 35S:TcJAV3OE was obtained. The alignment of TcJAV3 gene and AtJAV1 gene are shown in Figure S5. Moreover, the plasmid was transformed into *Arabidopsis* (Col-0 ecotype) with *Agrobacterium tumefaciens* GV3101, using the floral dip method [36].

4.4. Botrytis Cinerea Growth and Plant Inoculation

*B. cinerea* was grown on PDB for 7–10 days at 20 °C under a 12 h photoperiod. Spores were collected and suspended in water containing 0.1% Triton X-100. In this study, both whole-plant spray incubation and drop incubation were used to evaluate the disease resistance, and *coi1-2* mutants were the positive control.

For drop incubation, the *Arabidopsis* leaves were plated on 1/2 MS with 1–1.5% agar, and then 5 µL of *B. cinerea* spore suspension (2.5 × 104 spores/mL) was dropped onto the
central part of leaves. Then these plates were incubated under high humidity at 22 °C with a 12 h photoperiod. Each genotype had 12 leaves at least.

At 48 h post-inoculation, the lesion area (cm²) on each leaf was measured by using ImageJ (National Institutes of Health). All experiments were repeated three times. Error bars denote ±SEM. According to the one-way ANNOVA Duncan test, asterisks indicate statistically significant differences compared with WT (** p < 0.01).

For whole-plant spray incubation, 5-week-old plants cultured under SD conditions were sprayed with 2.5 × 10^4 spores/mL of B. cinerea spore suspension (~2 mL/plant). The plants sprayed with water were the negative control. Then these plants were grown under consistent humid conditions for 7 days; the first 2 days should be dark, but the remaining 5 days consisted of a normal photoperiod. The standard of disease severity was determined according to reference Hu et al. [24]. The survival plants were judged according to the disease severities of all leaves in each plant. The plants were thought to survive if less than 50% of the leaves had lesser disease severity (≤50%). At least 12 plants from each genotype were used in each experiment, and all experiments were repeated three times. The survival ratio was statistical according to Student’s t-test.

4.5. Yeast Two-Hybrid

TcJAV3 and TcWRKY26 were cloned into pGBK7 via EcoRI/XhoI sites and pGADT7 via EcoRI/SalI sites to generate TcJAV3-BD- and TcWRKY26-AD-fused protein, respectively. Particularly, the TcJAV3 fragments were added with EcoRI and XhoI restriction sites, while pGBK7 was linearized with EcoRI and SalI. Since XhoI and SalI have the same cohesive end, the fragments and linear plasmid can be ligated by T4 ligase. The primers used are listed in Supplementary Table S1.

The yeast AH109 competent cells were obtained by 1.1 × TE/LiAc treatment, and the AD and BD vectors were co-transformed into AH109 cells by PEG/LiAc method. Then the transformers were plated on solid selective medium (SD/-Trp/-Leu, SD-TL) and cultured at 30 °C for 2–4 days. Then the cultures of positive transformers were gradient diluted (originally OD600 = 2.0), and they were dropped on SD-TL, SD-Trp/-Leu/-His (SD-TLH), and SD-Trp/-Leu/-His/-Ade (SD-TLHA) plates. The plates were grown at 30 °C for 3–5 days and then observed.

4.6. Bimolecular Fluorescence Complementation (BiFC)

TcJAV3 and TcWRKY26 were cloned into pSPYCE(M) via BamHI/SalI sites and pSPYNE(R)173 via BamHI/XhoI sites to generate TcJAV3-CE- and NE-TcWRKY26-fused protein, respectively. TcJAV3-CE and NE-TcWRKY26 vectors were transformed into GV3101, respectively. The positive transformers were harvested, resuspended, mixed by 10 mM MgCl₂ with 10 mM MES (pH = 5.6) and 40 µM AS, and then stewed for 2 h. Then the mixtures were injected into leaf blade abaxially, and the injected plants were dark cultured for 12 h; they were then cultured under normal light conditions for 48 h. Finally, fluorescence was observed by Laser confocal microscope ZEISS 880 (ZEISS, Germany). The leaves containing TcJAV3-CE and empty pSPYNE(R)173 vector (NE), empty pSPYCE(M) (CE), and NE-TcWRKY26 were used as negative controls.

4.7. Luciferase Complementary Imaging

TcJAV3 and TcWRKY26 were ligated into JW771N-LUC and JW771C-LUC to generate CLUC-TcJAV3- and TcWRKY26-NLUC-fused protein, respectively. After Agrobacterium infiltration of indicated combinations in N. benthamiana leaves for 2 days, the luciferase substrate (luciferin) was sprayed onto the surface of the leaves, and luminescence was detected with an Imaging System (NEWTON 7.0Bio, Vilber, France).
4.8. Yeast One-Hybrid

The two w-boxes, \( w_a \)- and \( w_b \)-box, were respectively ligated into pHis2.1 plasmid to generate two baits. The triplication fragments of \( w_a \)- and \( w_b \)-box were artificially synthesized. In order to ligate with linear plasmid, the sense and antisense sequence were added the cohesive end of \( \text{EcoRI} \) at 5′ end and \( \text{SpeI} \) at 3′ end, so that after annealing, two single chains would form a double chain with cohesive end of specific restriction site. Then the double chain could be ligated with the linear pHis2.1, which was digested by \( \text{EcoRI} \) and \( \text{SpeI} \).

The method of preparation of yeast Y187 competent cells was the same as that of AH109. The co-transforming pH was 2.1–3 × \( w_a \)-box or pH is 2.1–3 × \( w_b \)-box with \( \text{TcWRKY26-AD} \) into Y187; the transforming and selecting were both the same as previously described. Finally, the positive transformers were plated on SD-TLH medium with different concentrations of 3-AT.

4.9. LUC Activity Assay

\( \text{TcWRKY26} \) was cloned into pCAMBIA3302Y with \( \text{EcoRI} \) and \( \text{SmaI} \), finally obtained the effector vector, \( \text{pTcWRKY26-OE} \). For the reporter vectors, progressive deletion fragments of DBAT promoter were ligated into pGreenII0800-LUC and generated pGreenII0800LUC-DBAT, pGreenII0800LUC-DBAT-1, and pGreenII0800LUC-DBAT-de-Wbox. The reporter pGreenII0800LUC-DBAT-1 lacked \( w_a \)-box, and pGreenII0800LUC-DBAT-de-Wbox lost the two boxes.

Differently, effector vector \( \text{pTcWRKY26-OE} \) was transformed into GV3101, while three reporter vectors were transformed into GV3101 containing pSOUP plasmid. Then the effector and each reporter were co-transformed into \( \text{N. benthamiana} \) leaves, respectively, according to the previous method. After 48 h, LUC activities were determined by Dual Luciferase Reporter Gene Assay Kit (YEASEN, Shanghai, China). All experiments were repeated three times. The survival ratio was statistic according to the Student’s \( t \)-test.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms232113194/s1.

Author Contributions: Conceptualization, M.Z. and L.C.; methodology, L.Y.; software, M.Z and L.Y.; validation, L.W., L.Y., H.Y. and P.H.; writing—original draft preparation, M.Z.; writing—review and editing, L.C.; supervision, R.Y.; project administration, Y.W.; funding acquisition, M.Z. and R.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Natural Science Foundation of China (grant number: 32000230); National Key Research and Development Program of China (grant number: 2021YFA1300400); Natural Science Foundation of Hunan Province (grant number: 2020JJ5050) and Shenzhen Science and Technology Innovation Commission (grant number: 2021Szvup037). And The APC was funded by Shenzhen Science and Technology Innovation Commission.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The sequence of \( \text{TcJAV3} \) was submitted to GenBank under accession number ON759763.

Acknowledgments: We thank Daoxin Xie (Tsinghua Univ., China) for providing the knockdown mutant Ri17. This work is financially supported by grants from the National Key Research and Development Program of China (2021YFA1300400), the National Natural Science Foundation of China (32000230), Natural Science Foundation of Hunan Province (2020JJ5050), and Shenzhen Science and Technology Innovation Commission (No. 2021Szvup037).

Conflicts of Interest: The authors declare that they have no conflict of interest.
References

1. Wasternack, C.; Strnad, M. Jasmonates are signals in the biosynthesis of secondary metabolites—Pathways, transcription factors and applied aspects—A brief review. New Biotechnol. 2019, 48, 1–11. [CrossRef] [PubMed]

2. De Geyter, N.; Gholami, A.; Goormachtig, S.; Goossens, A. Transcriptional machineries in jasmonate-elicited plant secondary metabolism. Trends. Plant Sci. 2012, 17, 349–359. [CrossRef] [PubMed]

3. Huang, H.; Liu, B.; Liu, L.; Song, S. Jasmonate action in plant growth and development. J. Exp. Bot. 2017, 68, 1349–1359. [CrossRef] [PubMed]

4. Wang, J.; Wu, D.; Wang, Y.; Xie, D. Jasmonate action in plant defense against insects. J. Exp. Bot. 2019, 70, 3391–3400. [CrossRef] [PubMed]

5. Wang, Y.; Hou, Y.; Qu, J.; Wang, H.; Wang, S.; Tang, L.; Tong, X.; Zhang, J. Abscisic acid promotes jasmonic acid biosynthesis via a ‘SAPK10-bZIP72-AOC’ pathway to synergistically inhibit seed germination in rice (Oryza sativa). New Phytol. 2020, 228, 1336–1353. [CrossRef]

6. Pan, J.; Hu, Y.; Wang, H.; Guo, Q.; Chen, Y.; Howe, G.A.; Yu, D. Molecular Mechanism Underlying the Synergetic Effect of Jasmonate on Abscisic Acid Signaling during Seed Germination in Arabidopsis. Plant Cell 2020, 32, 3846–3865. [CrossRef]

7. Hu, S.; Yang, H.; Gao, H.; Yan, J.; Xie, D. Control of seed size by jasmonate. Sci. China. Life. Sci. 2021, 64, 1215–1226. [CrossRef]

8. Zhou, W.; Lozano-Torres, J.L.; Biliou, I.; Zhang, X.; Zhai, Q.; Smant, G.; Li, C.; Scheres, B. A Jasmonate Signaling Network Activates Root Stem Cells and Promotes Regeneration. Cell 2019, 177, 942–956. [CrossRef]

9. Chen, X.; Marszalkowska, M.; Reinhold-Hurek, B. Jasmonic Acid, Not Salicylic Acid Restricts Endophytic Root Colonization of Rice. Front. Plant Sci. 2019, 10, 1758. [CrossRef]

10. van der Fits, L.; Memelink, J. ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 2000, 289, 295–297. [CrossRef]

11. Fu, X.; Peng, B.; Hassani, D.; Xie, L.; Liu, H.; Li, Y.; Chen, T.; Liu, P.; Tang, Y.; Li, L.; et al. AaWRKY9 contributes to light- and jasmonate-mediated to regulate the biosynthesis of artemisinin in Artemisia annua. New Phytol. 2021, 231, 1858–1874. [CrossRef] [PubMed]

12. Yukimune, Y.; Tabata, H.; Higashi, Y.; Harai, Y. Methyl jasmonate-induced overproduction of paclitaxel and baccatin III in Taxus cell suspension cultures. Nat. Biotechnol. 1996, 14, 1129–1132. [CrossRef] [PubMed]

13. Shen, Q.; Lu, X.; Yan, T.; Fu, X.; Lv, Z.; Zhang, F.; Pan, Q.; Wang, G.; Sun, X.; Tang, K. The jasmonate-responsive AaMYC2 transcription factor positively regulates artemisinin biosynthesis in Artemisia annua. New Phytol. 2016, 210, 1269–1281. [CrossRef] [PubMed]

14. Patra, B.; Pattanaik, S.; Schultenhofer, C.; Yuan, L. A network of jasmonate-responsive bHLH factors modulate monoterpeneoid indole alkaloid biosynthesis in Catharanthus roseus. New Phytol. 2018, 217, 1566–1581. [CrossRef] [PubMed]

15. Weaver, B.A. How Taxol/paclitaxel kills cancer cells. Mol. Biol. Cell 2014, 25, 2677–2681. [CrossRef] [PubMed]

16. McElroy, C.; Jennewein, S. Fermenters. In Biotechnology of Natural Products; Schwab, W., Lange, B.M., Wüst, M., Eds.; Springer International Publishing: Cham, Switzerland, 2018. [CrossRef]

17. Li, B.J.; Wang, H.; Geng, T.; Chen, J.J.; Chen, T.J.; Yang, J.L.; Zhu, P. Improving 10-deacetylbaccatin III-10-beta-O-acetyltransferase catalytic fitness for Taxol production. Nat. Commun. 2017, 8, 15544. [CrossRef] [PubMed]

18. Zhang, M.; Li, S.T.; Nie, L.; Chen, Q.P.; Xu, X.P.; Yu, L.J.; Fu, C.H. Two jasmonate-responsive factors, TcERF12 and TcERF15, respectively act as repressor and activator of taxol gene of taxol biosynthesis in Taxus chinensis. Plant Mol. Biol. 2015, 89, 463–473. [CrossRef] [PubMed]

19. Onrubia, M.; Moyano, E.; Bonfill, M.; Cusido, R.M.; Goossens, A.; Palazon, J. Coronatine, a more powerful elicitor for inducing taxane biosynthesis in Taxus media cell cultures than methyl jasmonate. J. Plant Physiol. 2013, 170, 211–219. [CrossRef]

20. Zhang, M.; Jin, X.; Chen, Y.; Wei, M.; Liao, W.; Zhao, S.; Fu, C.; Yu, L. TcMYC2a, a Basic Helix-Loop-Helix Transcription Factor, Transduces JA-Signals and Regulates Taxol Biosynthesis in Taxus chinensis. Front. Plant Sci. 2018, 9, 863. [CrossRef] [PubMed]

21. Zhang, M.; Chen, Y.; Nie, L.; Jin, X.; Fu, C.; Yu, L. Molecular, structural, and phylogenetic analyses of Taxus chinensis JAZs. Gene 2017, 620, 66–74. [CrossRef]

22. Li, S.T.; Zhang, P.; Zhang, M.; Fu, C.H.; Zhao, C.F.; Dong, Y.S.; Guo, A.Y.; Yu, L.J. Transcriptional profile of Taxus chinensis cells in response to methyl jasmonate. BMC Genom. 2012, 13, 295. [CrossRef] [PubMed]

23. Li, S.; Zhang, P.; Zhang, M.; Fu, C.; Yu, L. Functional analysis of a WRKY transcription factor involved in transcriptional activation of the DBAT gene in Taxus chinensis. Plant Biol. 2013, 15, 19–26. [CrossRef] [PubMed]

24. Hu, P.; Zhou, W.; Cheng, Z.; Fan, M.; Wang, L.; Xie, D. JAV1 controls jasmonate-regulated plant defense. Mol. Cell 2013, 50, 504–515. [CrossRef] [PubMed]

25. Jing, Y.; Lin, R. The VQ Motif-Containing Protein Family of Plant-Specific Transcriptional Regulators. Plant Physiol. 2015, 169, 371–378. [CrossRef] [PubMed]

26. Cheng, Y.; Zhou, Y.; Yang, Y.; Chi, Y.J.; Zhou, J.; Chen, J.Y.; Wang, F.; Fan, B.; Shi, K.; Zhou, Y.H.; et al. Structural and functional analysis of VQ motif-containing proteins in Arabidopsis as interacting proteins of WRKY transcription factors. Plant Physiol. 2012, 159, 810–825. [CrossRef] [PubMed]

27. Zhang, M.; Chen, Y.; Nie, L.; Jin, X.; Liao, W.; Zhao, S.; Fu, C.; Yu, L. Transcriptome-wide identification and screening of WRKY factors involved in the regulation of taxol biosynthesis in Taxus Chinensis. Sci. Rep. 2018, 8, 5197. [CrossRef] [PubMed]
28. Chen, Y.; Zhang, H.; Zhang, M.; Zhang, W.; Ou, Z.; Peng, Z.; Fu, C.; Zhao, C.; Yu, L. Salicylic Acid-Responsive Factor TcWRKY33 Positively Regulates Taxol Biosynthesis in Taxus chinensis in Direct and Indirect Ways. *Front. Plant Sci.* 2021, 12, 697476. [CrossRef]

29. Ali, M.R.M.; Uemura, T.; Ramadan, A.; Adachi, K.; Nemoto, K.; Nozawa, A.; Hoshino, R.; Abe, H.; Sawasaki, T.; Arimura, G.I. The Ring-Type E3 Ubiquitin Ligase JUL1 Targets the VQ-Motif Protein JAV1 to Coordinate Jasmonate Signaling. *Plant Physiol.* 2019, 179, 1273–1284. [CrossRef]

30. Hong, G.J.; Xue, X.Y.; Mao, Y.B.; Wang, L.J.; Chen, X.Y. Arabidopsis MYC2 interacts with DELLA proteins in regulating sesquiterpene synthase gene expression. *Plant Cell* 2012, 24, 2635–2648. [CrossRef]

31. Xie, D.X.; Feys, B.F.; James, S.; Nieto-Rostro, M.; Turner, J.G. COII: An Arabidopsis gene required for jasmonate-regulated defense and fertility. *Science* 1998, 280, 1091–1094. [CrossRef]

32. Song, S.; Qi, T.; Huang, H.; Ren, Q.; Wu, D.; Chang, C.; Peng, W.; Liu, Y.; Peng, J.; Xie, D. The Jasmonate-ZIM domain proteins interact with the R2R3-MYB transcription factors MYB21 and MYB24 to affect Jasmonate-regulated stamen development in Arabidopsis. *Plant Cell* 2011, 23, 1000–1013. [CrossRef] [PubMed]

33. Qi, T.; Song, S.; Ren, Q.; Wu, D.; Huang, H.; Chen, Y.; Fan, M.; Peng, W.; Ren, C.; Xie, D. The Jasmonate-ZIM-domain proteins interact with the WD-Repeat/bHLH/MYB complexes to regulate Jasmonate-mediated anthocyanin accumulation and trichome initiation in Arabidopsis italiana. *Plant Cell* 2011, 23, 1795–1814. [CrossRef] [PubMed]

34. Yan, C.; Fan, M.; Yang, M.; Zhao, J.; Zhang, W.; Su, Y.; Xiao, L.; Deng, H.; Xie, D. Injury Activates Ca(2+)/Calmodulin-Dependent Phosphorylation of JAV1-JAZ8-WRKY51 Complex for Jasmonate Biosynthesis. *Mol. Cell* 2018, 70, 136–149. [CrossRef] [PubMed]

35. Yin, Y.; Guo, Z.; Chen, K.; Tian, T.; Tan, J.; Chen, X.; Chen, J.; Yang, B.; Tang, S.; Peng, K.; et al. Ultra-high alpha-linolenic acid accumulating developmental defective embryo was rescued by lysophosphatidic acid acyltransferase 2. *Plant J.* 2020, 103, 2151–2167. [CrossRef] [PubMed]

36. Clough, S.J. Floral dip: Agrobacterium-mediated germ line transformation. *Methods Mol. Biol.* 2005, 286, 91–102. [CrossRef]