An Integrative Volatile Terpenoid Profiling and Transcriptomics Analysis for Gene Mining and Functional Characterization of AvBPPS and AvPS Involved in the Monoterpenoid Biosynthesis in Amomum villosum

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Amomum villosum, also known as Fructus Amomi, has been used to treat digestive diseases such as abdominal pain, vomiting, and dysentery. Volatile terpenoids are the active metabolites in the essential oil of Fructus Amomi. Nevertheless, downstream genes responsible for activating metabolites biosynthesis in A. villosum still remain unclear. Here, we report the use of an integrative volatile terpenoid profiling and transcriptomics analysis for mining the corresponding genes involved in volatile terpenoid biosynthesis. Ten terpene synthase (TPS) genes were discovered, and two of them were cloned and functionally characterized. AvTPS1 (AvPS: pinene synthase) catalyzed GPP to form α-pinene and β-pinene; AvTPS3 (AvBPPS: bornyl diphosphate synthase) produced bornyl diphosphate as major product and the other three monoterpenoids as minor products. Metabolite accumulation and gene expression pattern combined with AvPS biochemical characterization suggested that AvPS might play a role in biotic defense. On the other hand, the most active ingredient, bornyl acetate, was highly accumulated in seeds and was consistent with the high expression of AvBPPS, which further indicated that AvBPPS is responsible for the biosynthesis of bornyl acetate, the final metabolite of bornyl diphosphate in A. villosum. This study can be used to improve the quality of A. villosum through metabolic engineering, and for the sustainable production of bornyl acetate in heterologous hosts.

Keywords: Amomum villosum, volatile terpenoids, transcriptomics, bornyl diphosphate synthase, pinene synthase, bornyl acetate
INTRODUCTION

Amomum villosum is a plant of the ginger family which is grown in Southeast Asia and especially in southern China. A. villosum is also a Chinese traditional medicinal herb, cultivated for its fruits, which contain highly aromatic seeds (Li et al., 2010). The dried fruit, including seeds, is also known as Fructus Amomi (Chinese medicine name: Sharen), which has been used to treat digestive diseases such as abdominal pain, vomiting and dysentery (Chinese Materia Medica, 1999; Pharmacopoeia, 2015). In addition, Fructus Amomi has been approved by China Food and Drug Administration and due to its aroma and flavor characteristics, it has been widely used in Chinese cuisine for preparation of food, liquors, and tea.

The desired characteristics of Fructus Amomi in medicine and cuisine are mainly attributed to its essential oil, in which three potentially bioactive components were identified: bornyl acetate, borneol, and camphor (Xue et al., 2015). Its most active ingredient, bornyl acetate, has been reported to reduce 5-fluorouracil-induced intestinal mucositis (Zhang et al., 2017). Bornyl acetate is the quality standard of Fructus Amomi according to the Chinese Pharmacopoeia (Pharmacopoeia, 2015). Moreover, it has been reported that bornyl acetate had antioxidant, anti-inflammatory, antiabortion and anticancer activities (Wang et al., 2011; Kim et al., 2013; Chen et al., 2014; Yang et al., 2014; Li and Wang, 2016).

Terpenoids represent the largest and most diverse class of natural products among the myriad compounds produced by plants (Tholl, 2015). Plant terpenoids are used extensively for their aromatic qualities and have a role in traditional herbal remedies. In plants, there are two metabolic pathways that produce terpenoids: the mevalonate (MVA) pathway in the cytoplasm and the 2-C-methyl-d-erythritol-4-phosphate (MEP) pathway in the plastid (Tholl, 2015). The upstream steps of these two pathways have been well understood. IPP and DMAPP are the intermediates which produce the monoterpene precursor geranyl diphosphate (GPP) and sesquiterpenoid precursor farnesyl diphosphate (FPP) in these pathways (Figure 1A).

The complexity and diversity of terpenoids are mainly derived from the highly variable cycled and/or rearranged nature of the observed hydrocarbon skeletal structures catalyzed by terpene synthases (TPS) (Aubourg et al., 2002; Gao et al., 2012). These synthases convert the acyclic prenyl diphosphates into a multitude of cyclic and acyclic forms. Moreover, terpene diversity arises from the ability of these catalysts to form multiple products from a single substrate (Degenhardt et al., 2009). In addition to the main product, nearly half of all characterized monoterpene and sesquiterpene synthases form significant amounts of additional products (Degenhardt et al., 2009). In A. villosum, the bioactive components bornyl acetate, borneol, and camphor belong to monoterpene. To our knowledge, TPSs responsible for their biosynthesis in A. villosum remain unknown.

In recent years, integration of metabolomics and transcriptomics has been developed to explore the biosynthesis pathway of the active ingredients, such as artemisinin, tanshinone, and salidroside in medicinal plants (Gao et al., 2014; Ma et al., 2015; Wei et al., 2016; Torrens-Spence et al., 2018). Over the recent years, dried fruit has been used for the metabolic profiling of A. villosum (Deng et al., 2005; Kang et al., 2013; Xue et al., 2015; Zhang et al., 2017). Little is known about the metabolic profiling of fresh tissues (e.g., roots, stems, leaves, seeds, and pericarp) of A. villosum growing in its native habitat, Yangchun. This city has been well known for producing genuine and high quality Fructus Amomi (Lai et al., 2016). It has been reported that the contents of essential oil and bornyl acetate change with fruit development (Chen and Xu, 2007). In the present study, fruit from two stages, 45 DAF (days after flowering) and 75 DAF, were used for comprehensive metabolomics and transcriptomics to elucidate the complexity of monoterpene and sesquiterpene (essential oil), and to further explore the genes involved in their biosynthesis. Fifteen monoterpenes (Figure 1B) were observed by metabolomics of seven fresh tissues of A. villosum. In addition, our data revealed higher concentrations of bornyl acetate, borneol, and camphor in nearly mature fruit (75 DAF) seeds compared to young fruit (45 DAF) seeds. The bioinformatic analysis of transcriptomics mined 10 TPS candidate genes (AvTPS1-AvTPS10) including five monoterpene synthase genes, three sesquiterpene synthase genes, and two diterpene synthase genes.

Two monoterpene synthase genes with full coding sequences were further cloned and characterized. AvTPS1 catalyzed GPP to produce α-pinene and β-pinene, while AvTPS3 produced bornyl diphosphate as major product, and camphene, β-myrcene, and limonene as minor products. Furthermore, AvTPS3 was highly expressed in the seeds collected at both stages, which was consistent with the accumulation of bornyl acetate, and which suggested that AvTPS3 has an important role in bornyl acetate biosynthesis in A. villosum. This study provides a good reference for engineering sustainable production of bornyl diphosphate and the resultant bornyl acetate in heterologous hosts.

MATERIALS AND METHODS

Plant Materials

A. villosum was planted in Yangchun, Guangdong Province, China. The leaves, stems, roots, creeping stems, and fruits from healthy plants were collected and frozen at −80°C. The fruit was separated into pericarp and seeds. Fruits at different stages were used: YF (young fruit) collected at 45 DAF (days after flowering), with pericarp labeled as PYF (pericarp of young fruit) and seeds labeled as SYF (seeds of young fruit); F (nearly mature fruit) collected at 75 DAF, with pericarp labeled as PF, and seeds labeled as SF (Figure 2A).

Volatile Terpenoid Extraction and Analysis

Approximately, 0.2 g of leaves, roots, creeping stems, and fruits at different stages (separated into pericarp and seeds) were ground frizzed in liquid nitrogen and extracted with 1.5 mL hexane using an ultrasonic cleaner for 30 min, and then incubated at 56°C for 1 h. Samples were then centrifuged at 10,000 rpm for 5 min and the resulting supernatants were pipetted into new
FIGURE 1 | Monoterpene and sesquiterpene biosynthesis pathway (A) and the structures of the monoterpenes in A. villosum (B). AACT, acetoacetyl-CoA thiolase; HMGS, 3-hydroxy-3-methyl glutaryl coenzyme A synthase; HMGR, 3-hydroxy-3-methyl glutaryl coenzyme A reductase; MVK, mevalonate kinase; PMK, 5-phosphomevalonate kinase; MVD, mevalonate diphosphate decarboxylase; IDI, isopentenyl diphosphate isomerase; FPPS, farnesyl diphosphate synthase; TPS, terpene synthase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; MCT, 2-C-methyl-D-erythritol-4-(cytidyl-5-diphosphate) transferase; CMK, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase; MDS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase; HDR, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase; GPPS, geranyl diphosphate synthase.

2 mL tubes. One milliliter of hexane extract was pipetted into 1.5 mL vials for GC-MS analysis. The extraction was analyzed using Agilent 7890B Gas Chromatograph with 5977A inert Mass Selective Detector (Agilent, United States). Helium was used as the carrier gas (1 mL/min), and then separated on the HP-5MS column (30 m × 250 µm × 0.25 µm film thickness). GC oven temperature was programmed at an initial temperature of 35°C for 5 min with an increase of 12°C/min to 300°C. Temperature was then kept at 300°C for 5 min. NIST14/Wiley275 Mass Spectral Library was used for metabolite identification. The compounds were identified by the mass spectral library. The predominant monoterpenoids in this research, including α-pinene, β-pinene, camphene, β-myrcene, D-limonene, linalool, camphor, borneol, and bornyl acetate, were further identified using their authentic standards. The contents of bornyl acetate, camphor, borneol, camphene, and limonene were quantified based on their respective standard curves, and the contents of other volatile terpenes were quantified using α-pinene as an external standard. There were three biological replicates and three technical replicates for each tissue.

RNA Extraction
The total RNA of each sample was isolated using OminiPlant RNA kit (CWbiotech, China), following the manufacturer’s protocol. RNA quality was verified using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and checked using RNase free agarose gel electrophoresis. RNA with OD_{260}/OD_{280} at 1.8-2.2 was used for further analysis.

Library Construction, Sequencing, de Novo Assembly, and Annotation
Equal amounts of RNA from each sample (leaves, stems, roots, and two stages of pericarp and seeds) were mixed to obtain a single large pool (total amount > 20 µg). Next, poly (A) mRNA was isolated and reverse-transcribed to first-strand cDNA. Then, the second-strand cDNA was synthesized and the cDNA fragments were purified and enriched to construct the final cDNA library. The cDNA library was constructed and sequenced on the Illumina sequencing platform (IlluminaHiSeq™ 2000) using the paired-end technology by Gene Denovo Co. (Guangzhou, China). De novo assembly and annotation were performed by Gene Denovo Co. as described (He et al., 2018).

Screening of the Candidate TPS Genes Involved in Volatile Terpenoid Biosynthesis
The first group of candidate unigenes were identified directly by their KEGG annotation and selected by their length and RPKM (reads per kb per million reads) expression value. Besides the
FIGURE 2 | Volatile terpene analysis in different tissues of A. villosum. (A) The materials diagrams. PYF, pericarp of young fruit (45 DAF); SYF, seeds of young fruit; PF, pericarp of 75-DAF fruit; SF, seeds of 75-DAF fruit; L, leaf; R, root; S, creeping stem. Scale bars = 0.5 cm. (B) The ratios of monoterpene to sesquiterpene in different tissues of A. villosum. (C) The contents of total monoterpenes in different tissues of A. villosum. (D) Percentage of monoterpenes in the pericarp of 75-DAF fruit. (E) Percentage of monoterpenes in the seeds of 75-DAF fruit.
Correlation Analysis of Candidate Mono-TPS Genes and Monoterpenes Based on the Transcriptome Data and the Volatile Terpenoid Profiling

In order to analyze the correlation of candidate genes and volatile terpenoid, the second transcriptome sequencing with three biological replicates for each tissue was performed by Gene Denovo Co using pericarp, seeds and creeping stems collected at DE3 cells and the positive colonies were inoculated in the LB media containing 25 µg/mL carbenicillin and 17 µg/mL chloramphenicol at 37 °C till OD600 reached 0.4–0.6. The protein was induced by 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and 0.2% arabinose at 16°C for 16 h. The recombinant proteins were purified with Ni-NTA resin, following manual recommendations (Qiagen, Hilden, Germany). The purified proteins were dialyzed in PD-10 Desalting Columns (GE Healthcare).

Subcellular Localization

Vector pAN580, which has enhanced green fluorescent protein (EGFP), was chosen to perform the subcellular localization. Based on the cloning vector pLB-AvTPS1 and pLB-AvTPS3, the complete ORFs of AvTPS1 and AvTPS3 without termination codon were inserted into pAN580 vectors to get pAN580-AvTPS1 and pAN580-AvTPS3 vectors, respectively. The primers for the construction of pAN580-AvTPS1 and pAN580-AvTPS3 were described in Supplementary Table S4. QRT-PCR of AvTPS1 and AvTPS3 was performed as previously reported (Nanjareddy et al., 2016). ZEISS LSM 800 with Airyscan (ZEISS, Germany) was used for subcellular localization analysis in tobacco protoplast cells, Nicotiana tabacum was grown in a greenhouse for 4–6 weeks. The tobacco leaves were then collected and cut into approximately 0.5 mm strips using small sharp razors. Protoplast isolation and vector transfection were performed as previously reported (Nanjareddy et al., 2016). ZEISS LSM 800 with Airyscan (ZEISS, Germany) was used for subcellular localization; EGFP fluorescent signals were visualized with an excitation wave length of 488 nm and an emission wavelength of 509 nm. The red autofluorescence resulting from chlorophylls was captured by an emission wavelength of 650 nm. The empty vector pAN580, which has an EGFP-Fusion tag, and the empty protoplast were used as negative controls.

Quantitative Real-Time PCR

For quantifying two gene transcriptional expressions, the quantitative real-time PCR (qRT-PCR) was performed. RNA extraction and reverse transcription were performed as described above. The primers (qRT-TPS1F/R and qRT-TPS3F/R) for qRT-PCR were designed by Primer Premier 5.0 and were described in Supplementary Table S4. QRT-PCR of AvTPS1 and AvTPS3

Prokaryotic Expression and Purification of AvTPS1 and AvTPS3 Protein

The ORF of AvTPS1 and AvTPS3, excluding the N-terminal transit peptide, were ligated into pET32a(+) expression vector using the In-Fusion Cloning Kit (Takara, China). The primers were described in Supplementary Table S4. The positive constructs were then transformed into the competent Rosetta
were carried out with EvaGreen 2× qPCR Master Mix (ABM, Canada) on CFX96 Real-Time PCR Detection System (Bio-Rad, United States) following the manufacturers’ instructions. Next, the target gene transcript levels were monitored with double reference genes β-actin and TUA as controls for normalization and calculated using the $2^{-\Delta \Delta C T}$ method. All these experiments were performed with three biological replicates and two technical replicates.

**RESULTS**

**The Volatile Terpenoids in Different Tissues of A. villosum**

The volatile terpenoid profiling of roots, creeping stems, leaves, fruit, seeds and pericarp collected from their native habitat, Yangchun, was examined to improve our understanding of volatile oil biosynthesis and accumulation. The volatile terpenoids among the different tissues and developmental stages of *A. villosum* were detected and analyzed by GC-MS (Figure 2A, Supplementary Figure S1 and Supplementary Table S5). In total, 15 monoterpenes and nine sesquiterpenes were found. Monoterpenes (92.25%) dominated the identified components of the essential oil, followed by a lesser portion of sesquiterpenoids (7.75%). Most of the metabolites were detected in the seeds collected at different developmental stages (young and nearly mature stage); 12 monoterpenes and 6 sesquiterpenes, accounting for 4/5 and 2/3 of total monoterpane and sesquiterpene, were found in the SF (seeds collected at 75 DAF). Alpha-pinene and caryophyllene were the most abundant metabolites throughout all the tissues. Monoterpenoids were the dominant volatile terpenoid in all the tissues (Figure 2B). The seeds at both stages had higher monoterpene content compared to other tissues. In addition, higher contents of total monoterpane and bornyl acetate were observed in SF (seeds collected at 75 DAF) compared to SYF (seeds collected at 45 DAF) (Figure 2C and Supplementary Table S5). β-pinen (27.93%), α-pinene (18.17%), and linalool (18.16%) were the first three principal components in pericarp (Figure 2D); yet, bornyl acetate found in seeds had the highest relative content (50.35%) compared to the eleven other monoterpenes (Figure 2E).

**Transcriptome Sequencing, Assembly, Annotation, and Functional Classification**

Transcriptome sequencing was performed to gain insight into terpenoid biosynthesis. High-throughput sequencing and *de novo* assembly yielded 157,474 contigs and 144,020 unigenes (Supplementary Table S6). The transcriptome data has been submitted to NCBI with the SRA accession number SRP148009. After aligning to the major public databases Nr, COG, Swiss-Prot and KEGG, 72,002 (49.99%) unigenes were totally annotated (Table 1). For functional annotation and classification against the COG database, 15,041 unigenes were grouped into 25 classifications (Supplementary Figure S2). Among them, 1,376 unigenes were assigned to the cluster "secondary metabolites biosynthesis, transport and catabolism". Gene ontology (GO) has three main categories: biological processes, molecular function, and cellular component. In our transcriptomic data, 31,978 unigenes were grouped into 44 GO classifications with 14,456 unigenes assigned to the metabolic processes.

**Candidate TPS Genes Involved in Volatile Terpenoid Biosynthesis**

In order to mine more candidate TPS genes in *A. villosum*, transcriptome data from this research, named AvD, and transcriptome data from MeJA-treated *A. villosum* (He et al., 2018), named AvM, were analyzed. After analysis of unigenes was directly annotated by KEGG, seven mono-TPS unigenes with longer length and higher expression (RPKM) level were selected (Supplementary Table S8). Four of them were annotated to the myrcene or ocimene synthase (MS/OS) gene, while three to the linalool synthase (LIS) gene. Furthermore, the relevant mono-TPS and sesqui-TPS gene sequences from other plants were re-annotated to obtain candidate TPS genes; consequently, unigenes with longer length and higher expression level were selected (Supplementary Table S9). Both annotated and re-annotated unigenes were analyzed and the repetitive unigenes with high identity (> 99.6%) between the two sets of transcriptome data were merged. Finally, 10 candidate TPS genes from *AvTPS1* to *AvTPS10* were screened out (Table 2). The deduced amino acid sequences of *AvTPS1* to *AvTPS10* were shown in Supplementary Table S10.

Due to the diversity and specificity of terpenoids as 'specialized' metabolites in different plants, TPSs are defined as...
a mid-size family in the plant kingdom, which include eight subfamilies, from TPS-a to TPS-h; the TPSs responsible for the production of different and special terpenoids are diverse and specific as well (Chen et al., 2011). In this study, the deduced amino acid sequences of 10 AvTPSs were aligned with sequences of already known TPSs from subfamily a-h (Supplementary Table S3) (Chen et al., 2011). In addition, cluster analysis was performed to analyze the relationship between them (Figure 4). AvTPS1, AvTPS2 and AvTPS3 were classified into the TPS-b subfamily, which is also specific to angiosperms and mono-TPSs or isoprene synthases, and many of the enzymes of the TPS-b group produce cyclic monoterpenes (Chen et al., 2011). AvTPS4 and AvTPS5 were classified into TPS-g and TPS-d sub-family for mono-TPSs, respectively. AvTPS6, AvTPS7, and AvTPS8 were classified into TPS-a sub-family for sesqui-TPSs. Yet, AvTPS9 and AvTPS10 were classified into TPS-c sub-family for diterpene synthase.

Monoterpenoids were the dominant volatile terpenoids in A. villosum; bioactive components bornyl acetate, borneol and camphor are monoterpenoids. The fruit, which is the primary medicinal part of A. villosum, is abundant with monoterpenes, and both the seeds and creeping stems are rich with bornyl acetate (Figure 2B and Supplementary Figure S1). Therefore, two developmental stages of pericarp, seeds, and creeping stems were selected to perform the second transcriptome sequencing with three biological replicates for each tissue to quantify the gene expression. The transcriptome data of five candidate mono-TPS genes and monoterpenoid data were integrated. According to the correlation networks for AvTPS1-AvTPS5 and 13 monoterpenoids (Figure 5 and Supplementary Table S11), AvTPS3 was positively correlated with nine monoterpenoids, such as bornyl acetate, borneol, camphor, etc., suggesting that AvTPS3 was likely responsible

### TABLE 2 | Ten candidate TPS genes.

| Name | Unigene From AvD | Unigene From AvM | Predicted function |
|------|------------------|------------------|-------------------|
| AvTPS1 | Unigene0137026   | Unigene0137502   | Monoterpene synthase |
| AvTPS2 | Unigene0107445   | Unigene0134139   |                   |
| AvTPS3 | Unigene0115960   |                  |                   |
| AvTPS4 |                  | Unigene0138809   |                   |
| AvTPS5 | Unigene0047574   | Unigene0056791   |                   |
| AvTPS6 | Unigene0132051   |                  | Sesquiterpene synthase |
| AvTPS7 |                  | Unigene0106615   |                   |
| AvTPS8 | Unigene0116438   | Unigene0106619   |                   |
| AvTPS9 | Unigene0093054   | Unigene0083374   | Diterpene synthase |
| AvTPS10 | Unigene0135312  |                  |                   |

AvD, the transcriptome data of this paper; AvM, the transcriptome data of MeJA-treated A. villosum (He et al., 2018).
FIGURE 4 | Phylogenetic analysis of AvTPSs from A. villosum and the amino acid sequences of other terpene synthases. The terpene synthases from other plants in this analysis were downloaded from NCBI. The detailed information of sequences was show in Supplementary Table S3. The phylogenetic analysis were performed using the neighbor-joining method by the tool MEGA and iTOL (http://itol.embl.de/).

for the biosynthesis of these monoterpenoids. In addition, AvTPS1 and AvTPS5 were positively correlated with β-pinene and β-phellandrene.

Because AvTPS1 (unigene 0137026) and AvTPS3 (unigene 0115960) have the complete ORFs, they were selected for further cloning and functional characterization primarily. The gene and deduced amino acid sequences of AvTPS1 and AvTPS3 have been submitted to GenBank with the accession number MG431984 and MG763230, respectively. Based on the alignment of amino acid sequences, AvTPS1 had the highest identity (87%) with TPS7 (AHJ57305.1) from Hedychium coronarium, while AvTPS3 showed highest identity (65%) with a mono-TPS (AH146572.1) from Zingiber montanum; both of them had conserved mono-TPS domains, RRX₈W, RXR, DDXXD, and (N,D)D(L,I,V)X(S,T)XXXE(NSE/DTE) (Supplementary Figure S3). The motifs DDXXD and NSE/DTE are responsible for metal
and S-tag was about 84.7 kDa as predicted (Figure 5, the red rectangular part). The transit peptide of AvTPS1 fused with His and S-tag was approximately 85.9 kDa (Figure 7A).

**AvTPS3 Produced Bornyl Diphosphate as Major Product and Was Renamed as AvBPPS**

The complete ORFs of AvTPS3 was 1791 bp, which encoded 596 amino acids with a predicted 25 amino acid transit peptide on its N-terminal (Figure 5, the red rectangular part). The transit peptide was truncated and the left coding region was sub-cloned into the pET32a expression vector. The AvTPS3 recombinant protein fused with His and S-tag was approximately 85.9 kDa (Figure 7A).

By using the similar reaction condition and GC-MS method as AvTPS1, AvTPS3 was found to catalyze GPP to produce three monoterpenic compounds, camphene, limonene, and β-myrcene (Figures 7B–D). After treatment with alkaline phosphatase, bornyl diphosphate transforms to borneol, which can be detected by GC-MS (Despinasse et al., 2017; Hurd et al., 2017). In order to investigate if AvTPS3 could catalyze GPP to bornyl diphosphate, the reacting products of AvTPS3 with GPP and its negative control were treated with alkaline phosphatase and then analyzed by GC-MS. Four monoterpenes were detected after dephosphorylation; borneol was the major product (65.91%), along with camphene (17.31%), limonene (13.14%), and β-myrcene (3.63%) (Figures 7C–E). Therefore, we identified AvTPS3 as bornyl diphosphate synthase (AvBPPS) according to its most abundant product. Like AvPS, AvBPPS was not able to catalyze FPP to form any products. Furthermore, we found that AvBPPS had highest enzymatic activity under pH6 and that it exhibited higher activity with the metal ion Mg$^{2+}$ than with Mn$^{2+}$ (Supplementary Figure S4C). AvBPPS significantly lost its enzymatic activity when the pH value was higher than 7 (Supplementary Figure S4B).

**AvPS (AvTPS1) and AvBPPS (AvTPS3) Localized in Chloroplast**

ChlorP, WoLF PSORT, Predotar, and TargetP were used to predict AvTPS1 and AvTPS3's subcellular localization. Briefly, our data showed that they were both localized in plastids with high probability (Supplementary Table S12). To confirm the subcellular localization of AvTPS1 and AvTPS3, pAN580-AvTPS1 and pAN580-AvTPS3 containing the complete ORFs and eGFP-tags were constructed and then transformed into protoplasts of *Nicotiana tabacum* using a PEG-mediated method. The images of protoplast cells were obtained by confocal laser scanning microscopy. The results showed that the green fluorescence of AvTPS1-eGFP and AvTPS3-eGFP were localized in plastid (Figure 8), indicating that both AvPS and AvBPPS performed their catalyzing functions in plastid like other monoterpenes from plants.
Correlation Between Gene Expression Levels of AvPS and AvBPPS With Metabolite Accumulation in A. villosum

To investigate the correlation between the expression levels of AvPS (AvTPS1) and AvBPPS (AvTPS3) with monoterpane accumulation, qRT-PCR of AvPS and AvBPPS in seven tissues of A. villosum was performed. AvPS was highly expressed in the pericarp of both developmental stages (PYF and PF) with the highest expression level in the pericarp of young fruit, but not expressed in the seeds (Figure 9A). Interestingly, α-pinene existed in every tissue whereas β-pinene was not found in seeds (Figure 9B). Thus, we hypothesized that other pinene synthase(s) might be responsible for the α-pinene biosynthesis in the seeds of A. villosum. The ratios of β-pinene to α-pinene in leaves (1.78) and in pericarp of both stages (1.56 in PYF and 1.54 in PF, respectively) were similar to the ratio of β-pinene to α-pinene in the in vitro enzyme assay of AvPS (1.73). Nonetheless, among the seven tissues, both α-pinene and β-pinene contents were highest in the leaves, along with a lower expression of AvPS (Figure 9B). There might be other TPS that also produce pinene in the leaves of A. villosum.

AvBPPS was highly expressed in the seeds of both stages (SYF and SF). Interestingly, the expression levels in SYF were approximately 400 times higher than that in leaves (Figure 9C). Moreover, the transcript levels in SYF were approximately 20 times higher compared to SF (Figure 9C). Borneol is the direct precursor of camphor and bornyl acetate (Adam and Croteau, 1998; Despinasse et al., 2017; Hurd et al., 2017). Therefore, in
FIGURE 7 | Analysis of reaction products generated by recombinant protein AvTPS3 from GPP. (A) Expression and purification of AvTPS3 recombinant protein in E. coli Rosetta (DE3) harboring pET32a(+)-AvTPS3 1, total protein before induction; 2, total protein after induction; 3, soluble protein; 4, no-binding protein; 5–7, purified AvTPS3 recombinant protein from the 1st collected tube to the 3rd collected tube. (B) The GC-MS chromatogram of the monoterpene products by AvTPS3 protein and the camphene, β-myrcene and limonene standards. (C) The GC-MS chromatogram of the monoterpene products generated by AvTPS3 protein after dephosphorylation and the borneol standard. The compounds of peaks: 1, camphene; 2, β-myrcene; 3, limonene; 4, borneol; 5, geraniol. (D) Mass spectra of peak 1, peak 2, peak 3 and peak 4 in the NIST14/Wiley275 library compared with the mass spectra of the camphene, β-myrcene, limonene and borneol standards. (E) Product percentage of AvTPS3.
order to further explore the metabolite accumulation related with AvBPPS expression, the contents of borneol, camphor, and bornyl acetate were analyzed as well as the contents of camphene, limonene, and β-myrcene in different tissues. The results show that the higher expression levels of AvBPPS in SYF and SF were in accord with the higher accumulation levels of camphene, limonene, β-myrcene, borneol, camphor, and bornyl acetate (Figures 9D,E). Furthermore, bornyl acetate was much more abundant among the six monoterpenes compounds (Figure 9E). The higher transcript levels of AvBPPS were positive with the higher accumulation levels of camphene, limonene, β-myrcene, borneol, and borneol’s direct products camphor and bornyl acetate in seeds, which was consistent with the in vitro enzyme function of AvBPPS.

**DISCUSSION**

Metabolomics is usually referred to as “comprehensive metabolite profiling” and is usually used to identify potential bioactive plant metabolites (Liu et al., 2016; Zhong et al., 2016; Barbosa et al., 2017; Zhu et al., 2017). In *A. villosum*, terpenoid profiling of roots, creeping stems, leaves and two stages of pericarp and seeds were performed to depict the scenario of metabolites among the different tissues and developmental stages. In total, 24 terpenes, including 15 monoterpenes and 9 sesquiterpenes, were found to reveal a tissue specificity trend. This data suggests that the active ingredient production in *A. villosum* is associated with the tissue and its development. Metabolite-related genes were mined through analyzing a combination of metabolomics and transcriptomics data. Ten TPSs genes were screened out; based on the correlation analysis and the integrity of ORF, *AvTPS1* and *AvTPS3* were further selected for cloning and their biochemical functions were investigated. *AvTPS1* (AvPS) produced α-pinene and β-pinene, while *AvTPS3* (AvBPPS) formed bornyl diphosphate (major product) and camphene, limonene, β-myrcene (minor products). Since bornyl diphosphate is also the precursor of borneol, camphor and bornyl acetate, *AvTPS3* (AvBPPS) is responsible for the six main monoterpenes, bornyl acetate,
FIGURE 9 | Expression levels of AvPS and AvBPPS and relative monoterpenes in different tissues of A. villosum. (A,B) The relative expression level of AvPS (A) and AvBPPS (B). (C) The contents of α-pinene and β-pinene in different tissues of A. villosum. (D) The contents of camphene, β-myrcene and limonene in different tissues of A. villosum. (E) The contents of camphor, borneol and bornyl acetate in different tissues of A. villosum.

camphor, camphene, borneol, limonene, and β-myrcene in seeds of A. villosum. AvPS and AvBPPS were observed to be localized in plastid, which was consistent with their role in monoterpene biosynthesis. The direct and subsequent products of AvPS and AvBPPS and their downstream pathway were shown in Figure 10.

According to our terpenoid profiling, monoterpenes and sesquiterpenes were the main volatile components of A. villosum. Moreover, monoterpenes were more prominent than sesquiterpenes, with monoterpenes accounting for over 76.59 % in pericarp and 93.39 % in seeds of the total terpenoids detected in our samples (Figure 2B). Higher monoterpene content in fruit (compared to sesquiterpene) was already reported by previous few studies (Xue et al., 2015; Lai et al., 2016; Zhang et al., 2017). However, in these studies, dried fruit was used for terpene analysis. In contrast, we used fresh samples to depict the real temporal metabolome in A. villosum. Our results indicated that the seeds are the main accumulation organ of monoterpene, especially bornyl acetate. There were slight differences between our results and previous reports, where the intact dried fruit was used for measurement (Deng et al., 2005; Kang et al., 2013; Zhang et al., 2017). In our experiment, fruit was divided to pericarp and
FIGURE 10 | The direct and subsequent products of AvPS and AvBPPS and their downstream pathway. The pathway was adapted from Bohlmann et al. (1997) and Wise et al. (1998). The AvPS products were marked with blue frame and AvBPPS products were marked with red frame. Both main products were marked with full line and the sub-products were marked with dotted line.

seeds. Bornyl acetate, borneol, and camphor, the three potentially bioactive components which showed high occurrences in both developmental stages of seeds, were not detected in the pericarp, indicating that these monoterpenes are mainly biosynthesized in the seeds of *A. villosum*.

In our monoterpenic profiling, \(\alpha\)-pinene was widely distributed throughout almost the whole plant, i.e. roots, creeping stems, leaves, pericarp, and seeds. Interestingly, \(\beta\)-pinene was found in all the tissues, except seeds. Pinene is a naturally occurring constituent of the essential oils in many plant species that has a relevant role in insect repellency and allelopathy (inhibiting root growth of the tested weed species) (Bohlmann et al., 1997; Phillips et al., 1999; McKay et al., 2003; Hassanpouraghdam, 2011; Chowhan et al., 2013; Huang et al., 2013; Pajaro-Castro et al., 2017). *AvPS* (*AvTPS1*) catalyzed GPP to form \(\alpha\)-pinene and \(\beta\)-pinene, producing 63% \(\beta\)-pinene as a major product. Combined with the fact that *AvPS* was highly expressed in pericarp, the exterior tissue protecting the fruits against biotic stress, we supposed that *AvPS* might be the gene involved in biotic defense in *A. villosum*.

*AvPS* (*AvTPS1*) was not responsible for pinene biosynthesis in the seeds of *A. villosum*: no expression of this gene was detected in the seeds, and the expression of *AvPS* was not in accordance with the accumulation pattern of pinene in the leaves; therefore, it was speculated that other pinene synthases were present and were responsible for the pinene biosynthesis of the seeds and leaves, or that other TPSs could produce pinene as one of their multi-products in *A. villosum*. In this study, ten candidate genes were screened. Among them, *AvTPS1* to *AvTPS5* were classified into subfamilies that have mono-TPS function. *AvTPS1* and *AvTPS3* were cloned and characterized first since they had complete ORF in the transcriptome data. Nevertheless, whether *AvTPS2* and *AvTPS5* are involved in pinene biosynthesis needs to be further investigated. The TPS family size in angiosperm plant ranges from approximately 40 to 150 (Chen et al., 2011; Falara et al., 2011; Keilwagen et al., 2017). The TPS members in *A. villosum* should be considerably more than 10 as well. Considering this, besides these 10 candidate TPS genes, other TPS genes in *A. villosum* are likely to be discovered by further research.

Bornyl acetate was also found in other plant species, including liverwort (*Adam and Croteau*, 1998; *Ab Ghani* et al., 2016), *Korean fir* and *Larix decidua* (Pinaceae family) (Jeong et al., 2007; *Garcia* et al., 2017), *Sabina chinensis* (Cupressaceae family) (Gu et al., 2018), *Lippia sidoides* (Verbenaceae family) (de *Morais* et al., 2016), *Artemisia montana*, and *Chrysanthemum indicum* (Asteraceae family) (Stoianova-Ivanova et al., 1983; *Kunihiro* et al., 2017). Bornyl acetate was distributed in the leaves of most of the plants mentioned above, but was especially rich in the seeds of *A. villosum*. *AvBPPS* (*AvTPS3*) catalyzed GPP to form camphene, \(\beta\)-myrcene, and limonene, and produced bornyl diphosphate as a major product, which is the direct precursor of borneol. It is thought that borneol is converted to camphor by dehydrogenation (Despinasse et al., 2017; *Hurd* et al., 2017), and to bornyl acetate by acetyl transferation (Adam and *Croteau*, 1998). Our correlation analysis indicated that *AvBPPS* (*AvTPS3*) was positively correlated with bornyl acetate, borneol, camphor, camphene, \(\beta\)-myrcene, and limonene (Supplementary Figure S3). Combined with its high expression in seeds, which is consistent with the high content of bornyl acetate, *AvBPPS* is the key enzyme involved in the bornyl acetate biosynthesis in *A. villosum*. Until now, only three BPPSs have been cloned from *Lippia dulcis*, *Lavandula angustifolia*, and *Salvia officinalis* (Wise et al., 1998; Despinasse et al., 2017; *Hurd* et al., 2017). Alignment of *AvBPPS*
with these three BPSSs showed the relatively lower identity (40% with LdBPPS, 42% with LaBPPS and 41% with SoBPPS). Nevertheless, their mono-TPS domains (RRX₈W, RXR, DDXXD and NSE/DTE) were highly conserved (Supplementary Figure S6). All four BPSSs were clustered into the TPS-b subfamily similarly with the previous report (Despinasse et al., 2017), and AvBPSS (AvTPS3) rooted the TPS-b clade (Figure 4), suggesting that AvBPSS might retain more ancestral features than the other three BPSSs.

The biosynthesis of bornyl diphosphate, the direct precursor of borneol and bornyl acetate, is the key step for future metabolic engineering and plant breeding. In A. villosum, the upstream enzymes HMGR (MVA pathway), DXR and DXS (MEP pathway) in terpenoid biosynthesis have been cloned and characterized (Yang et al., 2012; Wang et al., 2014). Combinational expression of AvBPSSs with upstream genes can increase bornyl diphosphate production. An understanding of the gene-terpene network and the key enzymes involved in the biosynthesis of the bioactive components is important for improving the quality of A. villosum through metabolic engineering and breeding. The future discovery of the downstream enzymes after AvBPSSs, such as dehydrogenase catalyzing bornyl diphosphate to form borneol and acyltransferase catalyzing borneol to form bornyl acetate, could illuminate the complete biosynthesis pathway of bornyl acetate which then could be utilized to synthesize bornyl acetate in heterologous hosts.

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AUTHOR CONTRIBUTIONS

JY, DM, and HW designed the experiments. HW, KD, ML, XJ, LZ, and HZ performed the experiments and analyzed the data. JY, HW, and DM wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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