Characterization of Terpene synthase variation in flowers of wild Aquilegia species from Northeastern Asia

Song Yang1, Ning Wang1, Shadrack Kimani1,4, Yueqing Li4, Tingting Bao1, Guogui Ning2, Linfeng Li4, Bao Liu1, Li Wang1,* and Xiang Gao1,*

1 Key Laboratory of Molecular Epigenetics of MOE and Institute of Genetics & Cytology, Northeast Normal University, Changchun 130024, China
2 Key Laboratory of Horticultural Plant Biology, Ministry of Education, College of Horticulture and Forestry Sciences, Huazhong Agricultural University, Wuhan 430070, China
3 Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, Institute of Biodiversity Science, School of Life Sciences, Fudan University, Shanghai 200438, China
4 School of Pure and Applied Sciences, Karatina University, Karatina, Kenya
*Corresponding authors. E-mail: gaoxiang424@163.com; wanglee57@163.com

Abstract

There are several causes for the great diversity in floral terpenes. The terpene products are determined by the catalytic fidelity, efficiency and plasticity of the active sites of terpene synthases (TPSs). However, the molecular mechanism of TPS in catalyzing terpene biosynthesis and its evolutionary fate in wild plant species remain largely unknown. In this study, the functionality of terpene synthases and their natural variants were assessed in two Northeastern Asia endemic columbine species and their natural hybrid. Synoptically, TPS7, TPS8, and TPS9 were highly expressed in these Aquilegia species from the Zuojia population. The in vitro and in vivo enzymatic assays revealed that TPS7 and TPS8 mainly produced (+)-limonene and β-sesquiphellandrene, respectively, whereas TPS9 produced pinene, similar to the major components released from Aquilegia species. Multiple sequence alignment of Aquilegia TPS7 and TPS8 in the Zuojia population revealed amino acid polymorphisms. Domain swapping and amino acid substitution assays demonstrated that 413A, 503I and 529D had impacts on TPS7 catalytic activity, whereas 420G, 538F and 545 L affected the ratio of β-sesquiphellandrene to β-bisabolene in TPS8. Moreover, these key polymorphic amino acid residues were found in Aquilegia species from the Changbai Mountain population. Interestingly, amino acid polymorphisms in TPSs were present in individuals with low expression levels, and nonsynonymous mutations could impact the catalytic activity or product specificity of these genes. The results of this study will shed new light on the function and evolution of TPS genes in wild plant species and are beneficial to the modification of plant fragrances.

Introduction

Volatile terpenes, mainly consisting of monoterpenes and sesquiterpenes, are among the most abundant components of floral scent and play crucial roles in plant development and chemical ecology, especially in the interactions between plants and stresses [1–6]. The structurally diverse terpenes found in nature result from the mid-sized terpene synthase (TPS) family [7, 8], which directly converts geranyl diphenophosphate (GPP), neryl diphenophosphate (NPP) or farnesyl diphenophosphate (FPP) into versatile mono-/sesqui-terpenes [1–6]. In evolutionary history, TPS genes have undergone species-specific duplication and divergence, which contributes to the plasticity (the ability of TPS to use a broad range of substrates to generate multiple products) and adaptation of terpene metabolism [7, 9, 10]. Generally, a landmark feature of TPS genes is that they often lie in clusters of tandemly duplicated genes, including both functional genes and pseudogenes, evidently demonstrates that duplicated TPS genes have possibly led to phenotypic variations, although instances of convergent evolution have been observed in maize TPS23 [7, 11–14]. Chen et al. 2011 concluded that the TPSs from angiosperms and gymnosperms could be cataloged into seven clades, effectively supporting the evident evolutionary plasticity of the TPS family [7].

Recent studies on the functional characterization of several plant TPS genes have revealed the existence of remarkable plasticity in terpenoid biosynthesis in higher plants [5, 7, 12, 13, 15–22]; thus, the prediction of enzymatic products solely based on the protein similarity of terpene synthases is often difficult or impossible. Broadly, typical TPSs possess conserved structural features, such as RR(X)6W in the N-terminus and a metal cofactor binding domain, such as DDXXD or NSE/DTE in the C-terminus [7, 12, 15, 23]. However, these conserved motifs are necessary only for their catalytic features and cannot fully explain the diversity or infidelity (the ability of a TPS to generate multiple...
products) of the enzymatic products of TPS proteins. In contrast, other amino acids within or adjacent to the active site might play even more important roles in determining product diversity or infidelity, as even a single amino acid variation in or near the active site was found to have dramatic effects on catalytic activity and product specificity [24]. Previously, domain exchange and point mutation assays have been utilized to identify the roles of residues that affect product profiles in specific regions [25–29], suggesting that TPS evolution is likely centered on the recombination of functional domains or substitution of key amino acids. Despite those attempts to understand the product specificity or infidelity driven by key amino acids in TPSs, little is known about how TPSs catalytically function in closely related wild plant species [14, 24].

Aquilegia (Columbine) is a perennial Ranunculus genus comprising over 70 closely related species that occupy a wide range of ecosystems across Asia, Europe and North America. Aquilegia is best known for its floral spurred petals [30], and the genus is widely cultivated as an ornamental plant. The genus consists of very closely related taxa with small and almost identical genomes [31, 32]; thus, the expansion of genomic resources for a single genus lays a strong foundation for understanding the biochemistry and genetics from numerous aspects. Aquilegia has been employed in countless ecological and evolutionary studies, including those on herkogamy, radiation speciation due to pollinator shifts, mating system evolution, floral development, and floral color evolution [32–38]. In addition to floral color and floral shape, floral scents acting as olfactory signals also play important roles in attracting pollinators and are beneficial for long-distance attraction [39, 40]. However, no effort has been devoted to understanding the molecular mechanisms that contribute to floral volatile diversification within Aquilegia species.

Aquilegia oxysepala and Aquilegia japonica have been identified as geographically representative Aquilegia species in Northeastern Asia [41]. In this study, two Aquilegia species and their natural hybrid from Changbai Mountain (42.4°N, 128.15°E) and Zuojia District (43.87°N, 126.57°E) were employed to functionally characterize TPS genes and evaluate the basic amino acids driving the biosynthesis of diverse floral volatile terpenes. The expression levels and enzymatic products of three TPS proteins perfectly mirrored the major volatile terpenes released from Aquilegia flowers. Conceivably, nonsynonymous amino acid mutations were observed when the expression levels of the TPS genes changed, and several key amino acids were identified to account for the catalytic activity and product specificity of Aquilegia TPS proteins. Our findings provide a foundation for further exploration of the functional characterization and evolution of TPS genes in wild plant species and pave the way to deciphering the roles of floral volatile terpenes in the fitness and radiation speciation of Aquilegia species.

results

Isolation of TPSs involved in floral volatile terpene synthesis of Aquilegia species endemic to Northeastern Asia

According to Chen et al. 2011, TPSs responsible for volatile terpenes usually cluster into the TPS-a subclade composed of sesquiterpene synthases, the TPS-b subclade mainly responsible for monoterpene synthesis and the TPS-g family, including TPSs that function in both monoterpene and sesquiterpene synthesis in angiosperms [7]. A total of forty-two (42) TPSs were identified from the genome of Aquilegia coerulea. In detail, ten (10), twenty-seven (27) and five (5) fell into the TPS-a, TPS-b and TPS-g subclades, respectively (Fig. 1a). To identify the floral TPSs expressed in Aquilegia species endemic to Northeastern Asia, flowers of A. oxysepala, A. japonica and their hybrid (Fig. S1) were collected at different developmental stages (S1–S4) from Zuojia (43.87°N, 126.57°E) and subjected to RNA-Seq. As a result, most TPSs had relatively similar but low transcript levels, suggesting that they hold minor roles in floral volatile terpene biosynthesis (Fig. S2). In addition, quantitative real-time PCR (qPCR) was carried out to confirm the TPS expression patterns in the fully opened flowers (S4). Consistent with the RNA-Seq results, TPS7, TPS8, TPS9 and TPS27 showed remarkably high expression levels, indicating that their encoded proteins might have pivotal roles in determining the specific volatile terpenes detected in Aquilegia flowers (Fig. 1b, S3).

To further characterize the TPSs, the full-length cDNA sequences of the most highly expressed TPS7 and TPS8 were taken as representatives and isolated from seven (7) individuals of A. oxysepala or A. japonica and one (1) natural hybrid. Amino acid sequence alignment of TPS7 and TPS8 revealed conserved DDXX(D/E) and (N,D)XX(S,T,G) XXXE (NSE/DTE), which functioned in binding Mg2+ or Mn2+ cofactors, and terpene cyclization related RR(X)sW (Figs. S4, S5). Interestingly, residue polymorphisms were observed in the TPSs, indicating that the polymorphisms might result in functional differences in these Aquilegia proteins. The biosynthesis of monoterpenes and sesquiterpenes is thought to be compartmentalized, with monoterpenes being produced in the plastids where GPP or NPP is synthesized and sesquiterpenes being formed in the cytosol where FPP is generated [7, 42]. Consistent with this regularity, AoTPS7 and AoTPS9 were confined to plastids, similar to most known monoterpene synthases, while AjTPS8 and AoTPS27 were localized in the cytoplasm, suggesting their different roles in plant terpene synthesis (Fig. 1c).

The expression levels of TPSs are consistent with the volatile terpene emissions of Aquilegia flowers

Intricate floral-scent bouquets occur in many plant species. In the A. oxysepala population from the Zuojia location, the leading floral volatile terpenes identified
Figure 1. Phylogenetic analysis, gene expression detection and subcellular localization of Aquilegia TPS genes. a Phylogenetic analysis of Aquilegia coerulea TPS sequences. The TPS sequences were processed by Clustal Omega and input into MEGA-X to construct the neighbor-joining tree. Bootstrap values represent 1000 replicates. The TPS-a, TPS-b, TPS-c, TPS-e/f, and TPS-g clades are highlighted with colored lines. b The relative expression levels of 42 TPSs in Aquilegia flowers collected from Zuojia, Jilin Province, China. All the transcripts were normalized to β-actin and compared with A. oxysepala TPS1. c Subcellular localization of the highly expressed TPS7, TPS8, TPS9 and TPS27 genes in Arabidopsis protoplasts. Green, GFP fluorescence detected in the green channel; red, chlorophyll autofluorescence detected in the red channel; merged, merged green and red channel images; and bright light, bright field image. Scale bar =25 μm. d Representative flowers of A. oxysepala, A. japonica and their hybrid. Scale bar =1 cm.

from fully opened flowers (S4) were composed of (+)-limonene (average 42%), α-caryophyllene (average 19%), (1R)-(1S)-α-pinene (average 18%) and (1S)-(−)-β-pinene (average 9%), while in A. japonica, β-sesquiphellandrene (average 62%) and β-bisabolene (average 17%) were the two major volatile terpenes (Fig. 2a, 2b; Table S1). Interestingly, the aforementioned volatile terpenes could also be detected in their natural hybrid, with (+)-limonene as the most abundant component (80%) (Fig. 2a, 2b; Table S1). To quantitatively describe the volatile terpenes, the standard curve of (+)-limonene, which was the most abundant monoterpene detected, was calculated and employed to define the terpenes released from Aquilegia flowers at different developmental stages (Fig. S6). Generally, monoterpenes or sesquiterpenes could only be detected from S2 to S4 in A. oxysepala, A. japonica and their natural hybrid and had different release patterns (Fig. 2c; Table S2). For instance, the emission of (1R)-(+)−α-pinene peaked in S2 flowers of A. oxysepala, while (+)-limonene was most abundant in flowers at S4. In A. japonica, neither monoterpenes nor sesquiterpenes were detected at the first two developmental stages, whereas β-sesquiphellandrene constituted a large portion of the total terpenes in flowers at S3 and S4. In their natural hybrid, (1S)-(−)-β-pinene was dominant in S2 flowers, which was surpassed by (+)-limonene at S3 and S4 (Fig. 2c; Table S2). Furthermore, we checked the quantitative results by making another standard curve of β-caryophyllene (Fig. S7). Similar results as those obtained from the (+)-limonene standard curve were obtained (Table S3).

To further determine the possible contributions of the highly expressed TPS7, TPS8, TPS9 and TPS27 genes to the total volatile terpene release, their transcripts were normalized by AqIPP2 (GenBank KC854337) and measured in the fully opened flowers (S4) of different Aquilegia individuals (Fig. 2d). The results showed that TPS7 and TPS8 were highly expressed in A. oxysepala and A. japonica, respectively, implying their possible roles in synthesizing the dominant (+)-limonene or β-sesquiphellandrene. The transcript levels of TPS7 and TPS8 paralleled the abundant amounts of (+)-limonene and β-sesquiphellandrene released from the hybrid flowers, further strengthening this assumption.
Figure 2. Volatile terpene emissions and TPS expression of Aquilegia individuals from the Zuojia population. a. Floral volatile terpene profiles of representative Aquilegia individuals. b. Volatile terpenes emitted from fully blooming flowers of Aquilegia individuals. c. Volatile terpenes released from flowers at different developmental stages of representative A. oxysepala 7#, A. japonica 7#, and their hybrid. Data represent the relative content calculated by the standard curve of (+)-limonene. d. Relative expression levels of TPS7, TPS8, TPS9 and TPS27 in flowers of Aquilegia individuals. e. Relative expression levels of TPS7, TPS8, TPS9 and TPS27 in flowers at different developmental stages of representative A. oxysepala 7#, A. japonica 7#, and their hybrid. The transcripts were normalized by AqIPP2 (GenBank KC854337) and compared with the lowest expression level of TPS in specific species. All the data were calculated as log2. Red and blue boxes indicate high and low expression levels, respectively. The detailed terpene contents are listed in Table S1 and Table S2. Here, 1 to 29 represent different volatiles: 1, (1R)-(−)-α-pinene; 2, (1S)-(−)-β-pinene; 3, α-phellandrene; 4, β-phellandrene; 5, β-pinene; 6, myrcene; 7, (+)-limonene; 8, 3-carene; 9, (E)-β-ocimene; 10, linalool; 11, α-terpinene; 12, an unidentified sesquiterpene; 13, (−)-α-copaene; 14, α-bergamotene; 15, an unidentified sesquiterpene; 16, (2)-β-farnesene; 17, an unidentified sesquiterpene; 18, α-curcumene; 19, (E)-β-farnesene; 20, himachalene; 21, β-caryophyllene; 22, zingiberene; 23, β-bisabolene; 24, β-sesquiphellandrene; 25, α-caryophyllene; 26, an unidentified sesquiterpene; 27, cubebene; 28, α-muurolene; and 29, an unidentified sesquiterpene.

Comparatively, the relatively lower transcript levels of TPS9 and TPS27 might correlate with the relatively lower pinene or caryophyllene levels (Fig. 2d). Moreover, the transcripts of the four Aquilegia TPS genes increased with flower development (Fig. 2e). To validate the results, transcripts of the highly expressed TPS7, TPS8 and TPS9 were also normalized by β-actin, and similar expression patterns were observed (Fig. S8). Together, the results indicated that the four Aquilegia TPSs were mainly responsible for the main volatile terpenes released from Aquilegia flowers.

The in vitro enzymatic products of AoTPS7, AjTPS8 and AoTPS9 mirror the major volatile terpenes released from Aquilegia flowers

It is usually challenging to define the direct involvement of each TPS gene in the detected floral volatile blend unless TPS-encoded enzymes are functionally evaluated in detail. To elucidate the functionality of TPS proteins and their roles in the biosynthesis of the emitted floral volatile terpenes in Aquilegia species, the highly expressed AoTPS7 (A. oxysepala TPS7), AjTPS8 (A. japonica TPS8), AoTPS9 (A. oxysepala TPS9) and AoTPS27 (A. oxysepala TPS27) from A. oxysepala or A. japonica were cloned and subjected to further biochemical analysis. To prepare recombinant proteins, the four TPS genes were expressed...
in the E. coli BL21(DE3) strain. Recombinant AoTPS7, AjTPS8, and AoTPS9 were successfully purified, whereas AoTPS27 failed to be expressed as a soluble protein (Fig. 3a). Enzymatic assays revealed that the recombinant proteins could convert GPP, NPP, (E,E)-FPP or (Z,Z)-FPP into a variety of products, suggesting that these terpene synthases were generally versatile enzymes (Fig. 3b–3d; Table S4). In detail, AoTPS7 could utilize GPP and NPP as substrates to generate (+)-limonene, accounting for 97% (15238.96 ng) and 99% (8759.98 ng) of the total products, respectively. In addition, AoTPS7 could transform (E,E)-FPP rather than (Z,Z)-FPP into (+)-α-longipinene (4.62 ng, 20%), α-bergamotene (3.10 ng, 14%) and other sesquiterpenes (Fig. 3b). AjTPS8 could catalyze GPP into (+)-limonene (3767.41 ng, 40%), β-pinene (1310.7 ng, 14%), (1S)-(−)-β-pinene (940.74 ng, 10%) and (1R)-(−)-α-pinene (818.95 ng, 9%) in order of amount. When NPP was used as the substrate, (+)-limonone (2272.94 ng, 62%), (1S)-(−)-β-pinene (391.75 ng, 11%) and (1R)-(−)-α-pinene (303.35 ng, 8%) were among the main products. Using (E,E)-FPP as a substrate, β-sesquiphellandrene (3192.4 ng, 52%) was identified as the most abundant sesquiterpene, followed by β-bisabolene (1231.19 ng, 20%), an unidentified sesquiterpene (792.38 ng, 13%) and zingiberene (359.64 ng, 6%). When (Z,Z)-FPP was used as the substrate, β-bisabolene (341.78 ng, 27%), an unidentified sesquiterpene (241.33 ng, 19%), α-himachalene (177.72 ng, 14%), β-sesquiphellandrene (199.15 ng, 16%) and α-caryophyllene (122.6 ng, 10%) were produced (Fig. 3c). AoTPS9 could convert GPP to (1S)-(−)-β-pinene derivatives (4923.49 ng, 55%), (1R)-(−)-α-pinene (1356.23 ng, 15%) and other monoterpenes. Relatively higher quantities of (1S)-(−)-β-pinene (2237.77 ng, 60%) and an unidentified sesquiterpene (36.43 ng, 72%) were produced by AoTPS9 when using NPP and (E,E)-FPP as substrates, respectively (Fig. 3d). Similar to AoTPS7, AoTPS9 could not catalyze (Z,Z)-FPP into any detected sesquiterpenes (Fig. 3d). In contrast, proteins extracted from E. coli BL21(DE3) expressing the empty pET32a vector could not catalyze GPP, NPP, (E,E)-FPP or (Z,Z)-FPP (Fig. S9).

In summary, the results indicated that the recombinant Aquilegia TPS proteins displayed different catalytic features, and the enzymatic products were basically consistent with major volatile compounds emitted by Aquilegia flowers. Notably, TPS7, TPS8 and TPS9 might mainly control the synthesis of (+)-limonene, β-sesquiphellandrene and pinene isomers, respectively, considering their subcellular localizations and available substrates in Aquilegia species.

The catalytic activity of the Aquilegia TPS7 protein is largely affected by polymorphic amino acids

As mentioned above, amino acid polymorphisms were found among TPS proteins from different Aquilegia individuals, which might affect the catalytic properties of TPSs. For example, sequences of the highly expressed AoTPS7 and HTTPS7 (hybrid TPS7) were almost identical (only one residue difference), whereas the less expressed AjTPS7 had 7 amino acid substitutions when compared to AoTPS7 (Fig. 4a). To verify the function of the TPS7 proteins from different Aquilegia species, AoTPS7, HTTPS7 and AjTPS7 were transformed into Nicotiana tabacum (cv. K326) leaves and several transgenic lines were obtained (Fig. S10a). Headspace solid-phase microextraction gas chromatography–mass spectrometry (HS-SPME-GC–MS) analysis showed that transgenic tobacco overexpressing AoTPS7 and HTTPS7 released more (+)-limonene than those overexpressing AjTPS7 (Figs. 4b, S10b). Furthermore, in vitro enzymatic assays were carried out to explore whether the polymorphisms of TPS7 affected the production of (+)-limonene. Consistent with the overexpression assays in tobacco, recombinant AoTPS7 ((+)-limonene, 3060.60 ± 948.44 ng) catalyzed GPP to produce increased amounts of (+)-limonene compared to AjTPS7 ((+)-limonene, 4.97 ± 3.63 ng). To minimize the key amino acids, AoTPS7 and AjTPS7 were split at residue 308 into N (containing 4 mutant residues) and C (containing 3 mutant residues) termini, respectively. Two kinds of chimeric proteins, N(Ao)C(Aj) and N(Aj)C(Ao), were purified from E. coli BL21(DE3) and employed in enzymatic assays against GPP. The chimeric protein
N(Aj)C(Ao) had higher efficiency in producing (+)-limonene than AjTPS7, suggesting that the three (3) amino acid residues in the C-terminus of AoTPS7 contributed significantly to its increased catalytic activity (Fig. 4c, 4d). Additionally, the 3 residues in the C-terminus of AjTPS7 were back-mutated to V413A, L503I and G529D by different combinations. Enzymatic assays showed that the V413A and L503I mutants scarcely affected the catalytic activity of TPS7, while the G529D mutant marginally increased the activity compared to the parental counterpart (Fig. 4c, 4d).

Subsequently, a bioassay of the variant AjTPS7 protein harboring double mutations (V413AL503I) produced a very low amount of (+)-limonene, while the AjTPS7 protein carrying the V413AG529D and L503IG529D mutations yielded higher amounts of (+)-limonene, at 1944.73 ± 241.80 ng and 1763.71 ± 6.12 ng, respectively, than the parent AjTPS7 (4.97 ± 3.63 ng) (Fig. 4c, 4d), indicating that 413A, 503I and 529D were vital for the catalytic activity of the TPS7 protein. However, the triple mutant V413AL503IG529D, corresponding to N(Aj)C(Ao), resulted in less (+)-limonene than the double mutants V413AG529D and L503IG529D (Fig. 4c, 4d), indicating that the 4 mutant residue in the N-terminus should not be overlooked in determining the catalytic activity of TPS7. Moreover, we tried to obtain the kinetic parameters of
Aquilegia TPS7 proteins. For example, the $k_m$, $V_{max}$, and $K_{cat}$ of AoTPS7 were 4.77 μM, 0.007 mM s$^{-1}$ and 0.02 s$^{-1}$, respectively (Fig. S11). However, AjTPS7 produced too little (+)-limonene to calculate the kinetic parameters. Considering that the results presented may be enough to compare the differences in these enzymes, we tentatively abandoned the pursuit of accurate kinetic parameters for all the proteins. Overall, the results suggested that the catalytic activity of AjTPS7 could be significantly affected by several residue variations.

Three amino acids of TPS8 likely determine the ratio of $\beta$-sesquiphellandrene to $\beta$-bisabolene

AoTPS8 and HTPS8 shared identical sequences (Fig. S5), whereas six variant residues were observed between AoTPS8 and AjTPS8 (Figs. S5, 5a). To verify the function of the TPS8 proteins, AoTPS8 and AjTPS8 were first transformed into N. tabacum (cv. K326), but no obvious terpene changes were detected. Alternatively, AoTPS8 and AjTPS8 were transiently expressed in Nicotiana benthamiana leaves, which resulted in the generation of diverse sesquiterpenes mainly composed of $\beta$-sesquiphellandrene and $\beta$-bisabolene (Figs. 5b, S12). Interestingly, the ratio of $\beta$-sesquiphellandrene to $\beta$-bisabolene produced by AoTPS8 was ~7% to ~34%, whereas the ratio became ~52% to ~18% for AjTPS8 (Fig. 5d). Moreover, the six residue variations made TPS8 produce mostly $\beta$-sesquiphellandrene and $\beta$-bisabolene. $\beta$-sesquiphellandrene and $\beta$-bisabolene constituted ~41% of the total products generated by AoTPS8, while these main products of AjTPS8 increased to ~69% (Fig. 5d; Table S5). To further investigate the key amino acids responsible for the change in this ratio, site-directed mutation was conducted on AoTPS8 sequentially. Using (E, E)-FPP as a substrate, the A420G, V538F and F545L mutants of AoTPS8 were found to significantly influence the yield of $\beta$-sesquiphellandrene and $\beta$-bisabolene, whereas Q14R, L444F and V556I variations were not (Fig. 5d; Table S5). Furthermore, AoTPS8 mutant proteins carrying double mutations and triple mutations resulted in significant yield alterations of $\beta$-sesquiphellandrene to $\beta$-bisabolene, further validating that these residues were crucial for $\beta$-bisabolene and $\beta$-sesquiphellandrene biosynthesis (Fig. 5d; Table S5). Notably, the triple mutation (A420GV538FF545L) was enough to produce a similar amount or ratio of $\beta$-bisabolene and $\beta$-sesquiphellandrene as AjTPS8, suggesting that the residues at positions 420, 538 and 545 played vital roles in the biosynthesis of $\beta$-sesquiphellandrene and $\beta$-bisabolene in AjTPS8.

Correlation analysis between volatiles of A. oxysepala and A. japonica from the Changbai Mountain population and TPS gene expression

As Aquilegia is widely distributed, we are quite interested in the correlation between volatile release and the underlying TPSs among different populations. Consequently, the volatile terpenes of A. oxysepala and A. japonica from Changbai Mountain (42.4°N, 128.15°E) were detected. A. oxysepala from Changbai Mountain released relatively more (+)-limonene (~65% versus ~42%) than that from Zuojia. Comparatively, A. oxysepala from both areas had similar terpene release patterns (Figs. 2, 6a; Tables S1, S6). In contrast, A. japonica from Changbai Mountain released ~96% (+)-limonene, while the sesquiterpenes $\beta$-sesquiphellandrene and $\beta$-bisabolene constituted ~79% of the total volatile terpenes of A. japonica from the Zuojia population (Figs. 2, 6a; Tables S1, S6). Therefore, it was reasonable to conclude that the volatile terpenes exhibited geography-specific variations among different Aquilegia populations. To verify the molecular basis of the geography-specific volatile terpene emissions, the transcripts of TPS7 and TPS8 were measured in different Aquilegia individuals from the Changbai Mountain population. TPS7 was found to be highly expressed, which corresponded well with the (+)-limonene release from both A. oxysepala and A. japonica in this geographic area (Fig. 6b). As the key amino acid residues found in Aquilegia TPS7 and TPS8 were determinants of their catalytic properties, variations in these sites were detected in different Aquilegia individuals from both Zuojia and the Changbai Mountain
population. As shown in Fig. 6c, the mutations in TPS7 were species dependent instead of geography related, as all the mutations only appeared in A. japonica (Fig. 6c). For TPS8, only A. oxysepala from Zuojia changed from AVF to GFL (Fig. 6c). Considering the expression levels of TPS7 and TPS8 in different populations, mutations were often present in individuals with low expression levels in specific species, suggesting that there might be some correlations between amino acid mutations and gene expression.

Together, these results suggest that TPS orthologs have divergent roles within Aquilegia populations endemic to Northeast Asia, and they are likely to be subfunctionalized due to nonsynonymous amino acid mutations when their expression levels become low.

**Discussion**

The quality and quantity of volatile terpenes have been extensively studied in both angiosperms and gymnosperms. Although previous studies have attempted to examine genus-specific floral volatile constituents in anthers, pollen and stamens of the Ranunculaceae family [43], little is known about Aquilegia-specific floral chemistry. Previous studies have demonstrated that floral volatile chemistry is species-specific with the possibility for great variation among related species or different selection events of similar species/varieties [44]. Abundant terpenes are synthesized solely in specific taxa and probably evolved to cope with different ecological habitats, thus forming crucial cues...
Figure 5. Possible amino acid residues responsible for the product plasticity in Aquilegia TPS8. a Amino acid sequence alignment between AoTPS8 (from A. oxysepala #) and AjTPS8 (from A. japonica #). The conserved TPS motifs are indicated by lines, and polymorphic amino acids are marked with red boxes. The numbers in each line indicate the positions of the last residue. b Volatile compounds detected in the control and transgenic Nicotiana benthamiana transiently overexpressing TPS8. AtFPS2, which encodes Arabidopsis thaliana farnesyl diphosphate synthase in FPP synthesis, was coinfiltrated into tobacco leaves. The tobacco leaves were sampled and analyzed by GC–MS analysis. Tobacco leaves infiltrated with AtFPS2 alone were used as controls. c Enzymatic assays of AoTPS8, AjTPS8, and a series of mutant TPS8 proteins based on the AoTPS8 backbone. (E, E)-FPP was employed as the substrate in these assays. The enzymatic products are detailed in Table S5. d Relative contents of β-sesquiphellandrene and β-bisabolene catalyzed by AoTPS8, AjTPS8 and mutant TPS proteins. Data represent the mean ± SD of two replicates calculated from relative peak areas. One-way ANOVA was carried out to compare significant differences (Duncan, P < 0.05).

for attracting pollinators, repelling enemies such as insects, mollusks and mammals or becoming toxic to insects, bacteria and fungi [45, 46]. The Aquilegia species show obvious differences in vegetative and floral traits with specializations for different ecological niches and pollinators [37, 47–49]. Therefore, evaluation of divergent floral volatiles in A. oxysepala and A. japonica and their hybrid may provide insight into pollinator shift and speciation. For example, studies found that Aquilegia pubescens and Aquilegia formosa have adapted to different pollinators. Bees and hummingbirds visited A. formosa, whereas hawkmoths exclusively visited A. pubescens [33]. Presently, A. oxysepala and A. japonica from Northeastern Asia were also found to have quite different terpene release patterns (Figs. 2, 6). However, whether the different floral volatiles released by A. oxysepala and A. japonica affect their pollinators or benefit them in specific ecological niches remains to be further investigated.

The enormous architectural diversity of floral volatile terpenes is driven by TPS genes ubiquitously present in terrestrial plants [7]. The enzymes encoded by these genes catalyze the synthesis of monoterpenes (C-10), sesquiterpenes (C-15), and diterpenes (C-20) using acyclic substrates geranyl diphosphate (GPP; C-10), farnesyl diphosphate (FPP; C-15), and geranylgeranyl diphosphate (GGPP; C-20), in that order. Varying numbers of TPS genes were observed in plants, ranging from a single gene in Physcomitrella patens to over a hundred genes in both Eucalyptus grandis and Eucalyptus grandis, which resulted in the vast volatile terpene product spectrum [7, 50–52]. In the representative A. coerules, a total of 42 TPSs were identified. Gene expression analysis indicated
that only some TPSs were highly expressed in flowers (Fig. 1), which seemed paradoxical to the diverse terpenes detected. This paradox could be partly interpreted by the versatile TPS proteins, as a single TPS could transform a specific kind of substrate into multiple products, and most TPS proteins had broad substrate spectra. For example, the highly expressed TPS7, TPS8 and TPS9 characterized in Aquilegia could catalyze either GPP, FPP or NPP into a series of volatile terpenes (Fig. 3). Moreover, amino acid alterations in the suggested TPS active sites could have significant effects on catalytic features, such as functional plasticity and specificities, which was validated by the site-directed mutations of TPS7 and TPS8 (Figs. 4, 5). Evolutionarily, the occurrence of single/multiple mutations within a gene might enable organisms to adapt to new or changing environments. For instance, when a gene is duplicated and then one copy of the duplicated gene can be functionally modified by mutation or even become a novel gene with a completely different function from the original gene. Theoretically, the large diversity of TPS genes might have evolved from a parental gene via three routes: gene silencing, neofunctionalization and subfunctionalization [53]. Consequently, sustained TPS gene duplication accompanied by functional divergence has been considered as a crucial factor responsible for the chemodiversity of floral volatile terpenes [7, 10]. However, the low expression of TPS genes detected in Aquilegia could not be arbitrarily considered to be silenced, as some of these genes might be induced by specific stimuli, such as biotic or abiotic stresses, or expressed in other tissues or developmental stages in addition to flowers [54, 55]. Furthermore, TPSs play important roles in the determination of immense variations in the terpenome among and within terrestrial plant taxa [1, 23, 56]. Notwithstanding these general conceptions, concrete illustrations of how duplicated TPS genes have evolved across multiple plant species populations are still scarce.

Generally, the functional characterization of Aquilegia TPSs and their key amino acids identified from this taxon exhibit broad plasticity, which indicates a crucial cornerstone toward rapid functional divergence within and among Aquilegia species. The TPS product specificity is prominently variable and elementarily depends on how easily the substrate carbocation can be stabilized in the active pocket or center of the enzymes [57, 58]. Most terpene synthases have active pockets with a propensity toward catalytically accepting more than one acyclic substrate ranging from IPP (C-5) to FPP (C-15) to synthesize multiterpene profiles [59]. The TPSs with verified potential for multisubstrate usage are extensively divergent, spreading across the seven terpene
synthase subfamilies (TPS-a, –b, –c -d, –e/f, –g and TPS-h), further accentuating the capability that the multisubstrate usage of TPSs could be widespread more than previously believed. For example, detailed analysis of the substrate specificities of TPS isolated from Vitis vinifera has shown the existence of at least nine multisubstrate TPSs: two are mono-/sesqui-/diterpenes, and the others are mono-/sesquiterpene synthases [12, 38]. Similarly, Santalum species have seven functionally confirmed C-10/C-15 multisubstrate terpene synthases. The versatile Aquilegia TPSs further indicated that the capacity to use multiple substrates may be widespread across TPS families in multiple species [59, 60]. However, it seems that the subcellular compartments confine the TPS in utilizing specific substrates due to the subcellular localizations of monoterpane-related TPSs in plastids (GPPs or NPPs) versus sesquiterpene-related TPSs in the cytosol (FPP) [7, 42]. For instance, although plastid-localized AoTPS7 and cytosol-localized AjTPS8 can catalyze GPP, NPP or FPP in vitro, not all the products can be detected in planta (Figs. 1-5).

In general, the observed expression levels of TPS genes, subcellular localizations of TPS proteins, and their catalytic properties accounted for the emission patterns of Aquilegia floral volatile terpenes. Simply, an illustrative hypothetical model was constructed to interpret the molecular mechanism underlying the floral volatile terpene emissions in A. oxysepala and A. japonica from Changbai Mountain and Zuojia District (Fig. 7). TPS genes are responsible for the floral volatile terpenes within Aquilegia populations endemic to Northeast Asia, and they will be subfunctionalized when their expression levels are changed. Generally, the slowly expressed orthologs appear to accumulate mutations easily, among which the nonsynonymous amino acid mutations will contribute to the functional divergence of the TPS isogenes.

Nonsynonymous amino acid mutations responsible for the catalytic activity or product specificity of TPS proteins have also been observed elsewhere. For instance, some amino acid motif variations in the closely related TPS4 and TPS5 genes from two maize cultivars drove the stereospecificity of multiproduct sesquiterpene synthases [61]. N338A in 1,8-cineole synthase of Salvia fruticosa, Y402L in β-farnesene synthase of Artemisia annua and TPS5 4/10 in maize demonstrated that the byproducts of TPSs were determined not only by residues forming the center active pocket but also by adjacent residues impacting the conformation [29, 62–64]. In a similar manner, our study revealed that 413A, 503I and 529D perhaps influenced the catalytic activity of AjTPS7, while 420G, 538F and 545 L affected the ratio of β-sesquiphellandrene to β-bisabolene. Consistent with the opinion that organisms are always evolving, floral volatiles may be employed by plants as a strategy to cope with perpetually changing environmental settings. Our results provide evidence for a possible mechanism whereby Aquilegia or other angiosperms, instead of modifying the committed genes in the earlier terpene biosynthesis pathway, may employ a simple but effective scaffold that can, with only very few substitutions, provide diverse terpene release. Furthermore, our results showed some correlations between amino acid mutations and gene expression. As a result, we suggest that the change in the gene expression level occurs prior to the amino acid mutation, as not all the less expressed AjTPS7 from Zuojia populations had VID or VIG mutations when compared with AID in Zuojia AoTPS7 (Fig. 6). However, more investigations are still necessary to fully investigate this issue. The combination of product specificity and plasticity observed in Aquilegia terpene synthases may be a snapshot of an evolutionary mechanism that maintains a dynamic state between desirable specific activities and a capacity for rapid change.

Materials and methods

Plant materials and growth conditions

Plant materials of the two columbine species, A. oxysepala and A. japonica, and their natural hybrid were collected from Zuojia (43.87°N, 126.57°E) and Changbai Mountain (42.4°N, 128.15°E) in Northeast China (Figs. S1, S13). After volatile compound sampling, the floral samples obtained were promptly frozen in liquid nitrogen and stored at −80°C until further use.

To determine the correlation between the TPS expression levels and floral volatile terpene emissions, Aquilegia plants were cultivated in a greenhouse under a photoperiod of 16 h of light followed by 8 h in the dark, and flowers were separated into four developmental stages (Fig. S1). Wild-type plants of Arabidopsis thaliana ecotype Columbia (Col-0) used to generate protoplasts for subcellular localization were grown in a greenhouse at 22°C with an alternating photoperiod of 16/8 h light/dark. Rosette leaves of Arabidopsis plants grown for 3 to 4 weeks were used for protoplast isolation. For the in vivo activity assay of TPS proteins, tobacco plants were grown in a greenhouse under natural light settings. The expanded leaves of 4-week-old tobacco plants were used for Agrobacterium GV3101 infiltration experiments.

Analysis of floral volatile compounds in A. oxysepala, A. japonica and their hybrid

The volatile terpenes released from Aquilegia flowers were analyzed following a previously published method [18, 20]. Briefly, fresh flowers from A. oxysepala, A. japonica and their hybrid were sealed in a transparent and odor-free plastic device. A 100-mm fused silica fiber coated with DVB/CAR/PDMS (Sigma-Aldrich) was used to trap floral volatile compounds for 2 h at room temperature [18, 65] and then directly used for gas chromatography–mass spectrometry (GC/MS). The fibers were thermally desorbed at 260°C for 20 seconds in an Agilent 5975-6890 N GC/MS apparatus (Agilent Technologies) equipped with an HP-1 MS Fused-Silica Capillary Column (0.25 mm diameter, 30 m length, and
Figure 7. Proposed model for volatile terpene biosynthesis in flowers of Aquilegia populations endemic to Northeastern Asia. The parent TPS7 was highly expressed in A. oxysepala (Zuojia and the Changbai mountain population), the hybrid and A. japonica (the Changbai mountain population). The active parent TPS7 generated a high amount of (+)-limonene, whereas the mTPS7 mutated protein was hardly expressed in A. japonica (the Zuojia population), although it produced a low amount of (+)-limonene. The parent TPS8 was highly expressed in A. japonica (the Zuojia population) when compared with the less expressed mTPS8 in the other populations. Moreover, the parent TPS8 yielded a high amount of β-sesquiphellandrenes, while the mutant mTPS8 produced more β-bisabolenes. The arrows represent high expression. The squares and circles under TPSs indicate A. oxysepala and A. japonica, respectively. The different background colors of the same shape indicate the same species from different populations. The hexagon indicates the natural hybrid from the Zuojia population. The three-dimensional models of the TPSs were created according to the crystal structure of 5-epi-aristolochene synthase (PDB ID: 5IL3) from tobacco61. The active cavity is illustrated in dotted circles. Polymorphic amino acids in or adjacent to the active cavity are marked. The different background colors of (+)-limonene, β-sesquiphellandrenes and β-bisabolenes represent different contents produced by the enzymes.

0.25 μm film thickness). The detection parameter was held at 60°C for 3 min, subsequently raised at a rate of 5°C min⁻¹ to 100°C for 1 min, and then increased at a rate of 10°C min⁻¹ to 250°C for 10 min. Prospective floral volatile constituents were identified by equating mass spectra from GC/MS analysis with the mass spectra archived in NIST 2008 and a reference standard sample when applicable. The absolute content of volatile terpenoids was quantitatively analyzed according to the standard curve of (+)-limonene or β-caryophyllene.

TPS gene identification, sequencing analysis and cloning

Using FhTPS1 (GenBank NO. AFP23421.1) as a bait sequence, the predicted TPS genes were first mined from the assembled genome database of A. coerulea (Phytozome v12.1, https://phytozome-next.jgi.doe.gov/info/Acoerulea_v3_1) [32] by using the TBLASTN algorithm and subsequently subjected to a manual NCBI-BLASTX search. The best hits were taken as TPS candidate genes. In addition, RNA-seq analysis of flowers at different developmental stages was also carried out to screen the potential TPS genes in the selected Aquilegia species. Briefly, total RNA was first isolated from samples by employing the OminiPlant RNA Kit (DNase I) (CWBio, Beijing, PRC) following the manufacturer’s specified procedure. The RNA samples were either sent to BioMarker, Beijing, PRC for RNA-seq following the company’s standard process or subjected to cDNA synthesis. The cDNA sample was made in a final 25 μl reaction system including total RNA (1 μg), Oligo d(T)15 primers, and M-MLV Reverse Transcriptase (Promega, Madison, US) according to the manufacturer’s manual. Specific primers (Table S7) were designed to amplify the full-length cDNA sequences of TPSs. The sequences were ligated to the pESI-Blunt vector (Yeasen, Shanghai, PRC) and then subjected to sequencing confirmation.

Multiple sequence alignment of TPSs was performed by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) with default parameters. For phylogenetic evaluation, the aligned sequences were subjected to MEGA-X to generate a neighbor-joining tree with bootstrap analysis (1000 replicates) and gap handling by pairwise deletion [66]. The following GenBank accession numbers were used: AtTPS10 (ACF41947), AtTPS14 (NP001185286), AtTPS21 (NP001190374), AtGA1 (Q38802.1), and AtGA2
(Q9SAK2.1). Transcript names of the 42 A. coerulea TPSs used in the phylogenetic analysis and deposited in Phytozome v12.1 are listed in Table S7.

Quantitative real-time PCR analysis

To evaluate the expression profiles of the TPS genes in A. oxysepala, A. japonica and their hybrid, a qPCR assay was conducted in a 10 μl reaction volume containing 5 μl of TB Green® Premix Ex Taq (TaKaRa, Kusatsu, Japan), 0.5 μM of each oligonucleotide and 1 μl of cDNA template. The specific qPCR primers used in this study are listed in Table S7. The β-actin and IPP2 genes were utilized as endogenous controls [67, 68]. Relative gene expression was calculated using the 2-ΔΔCt formula [69]. All measurements presented in this study were prepared in triplicate.

Subcellular localization of Aquilegia TPS proteins

The previously used 35S:FhPAP1-GFP in the backbone of pUC19 was digested by NdeI and ClaI [70]. The whole open reading frames (ORFs) of AoTPS7, AjTPS8, AoTPS9, and AoTPS27 without termination codons were subcloned into the aforementioned digested pUC19 by a Minerva Super Fusion Cloning Kit (US Everbright® Inc., Suzhou, PRC) to generate 35S:AoTPS7-GFP, 35S:AjTPS8-GFP, 35S:AoTPS9-GFP, and 35S:AoTPS27-GFP, respectively. The plasmids were extracted using the GoldHi EndoFreePlasmid Maxi Kit (CWBio, Beijing, PRC) according to the instructions provided and then transfected into Arabidopsis protoplasts as described previously [71]. The GFP signals were visualized by fluorescence microscopy.

Heterologous expression of TPS proteins in E. coli and in vitro enzymatic assay

To express recombinant proteins in E. coli, the ORFs of Aquilegia TPSs were amplified with gene-specific primers (Table S7) and then subcloned into the pET32a vector. Afterward, an empty vector and vectors appropriately ligated with different TPS genes were used for transformation of E. coli strain BL21 (DE3). Recombinant TPS proteins were induced by 0.25 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 16°C for 16 h in a constant shaking incubator. Subsequently, the cells were harvested by centrifugation, resuspended in phosphate-buffered saline (PBS) and broken down by sonication. The crude proteins were then purified on a Ni-TED Sefinose™ column (Sangon Biotech, Shanghai, PRC) and analyzed by SDS-PAGE. Moreover, the recombinant proteins were confirmed by western blotting analysis using Anti His-Tag Mouse Monoclonal Antibody (CWBio, Beijing, PRC) following the standard protocol. The following enzymatic assays were performed as previously described with slight revisions [18, 72]. Briefly, the 300 μl reaction mixture consisted of 50 mM HEPES buffer (pH 7.4), 2 mM GPP (Sigma Aldrich), (E,E)-FPP (Sigma Aldrich), NPP (Echelon Biosciences) or (Z,Z)-FPP (Echelon Biosciences), 75 mM MgCl₂, 5 mM dithiothreitol, 5% glycerol and 60 μg protein. The mixture was incubated at 30°C for 2 h. The volatile products were captured by silica fiber and then analyzed by GC-MS as previously mentioned. Based on an in vitro enzymatic assay system, the kinetic parameters of AoTPS7 were calculated.

Chimera construction and site-directed mutagenesis

To create both chimeric cDNAs and site-directed mutants, PCR was used together with a revised version of the overlap extension strategy detailed in other studies [27, 28]. The PCR-based mutagenesis protocol (Fast Mutagenesis System, TransGen Biotech, PRC) was executed on pET32a-AoTPS7 and pET32a-AoTPS8 by employing primers containing the desired mutations (Table S7). The TPS7 Chimera N(Ao)C(Aj) and N(Aj)C(Ao) were created by overlapping PCR fragments of the targeted region (split at residue 308), which were employed to evaluate the role of C-terminal regions in the determination of enzyme activity and product specificity in Aquilegia TPS27. Briefly, complementary primers Chimera-F (Table S7) were designed to anneal to specific regions of the parent AoTPS7 and AjTPS7 to construct overlapping fragments. Subsequently, they were fused and amplified to generate the final chimeric construct. All chimeric constructs were subcloned back into the pET32a vector, and their sequences were verified as described elsewhere [27, 28].

In vivo characterization of terpene synthase

The obtained TPS7 genes were subcloned into the pBI121 binary vector and then transformed into Agrobacterium competent cells (strain GV3101). Agrobacterium harboring TPS7 or TPS8 was infiltrated into the juvenile leaves of 4-week-old N. tabacum (cv. K326) [20]. Then, the leaves infiltrated by Agrobacterium were cut into small pieces and cultured on MS medium to obtain transgenic seedlings [70]. The volatile terpenes of transgenic seedlings in the tissue culture vessels were captured by silica fiber and then analyzed by GC–MS as previously mentioned. Moreover, AoTPS8, AjTPS8 and AtFPS2 (A. thaliana farnesyl diphosphate synthase 2, At4g17190) were subcloned into the pEAQ-HT vector, which was reported to dramatically enhance target protein expression [73]. Agrobacterium harboring AoTPS8 or AjTPS8 was mixed with that carrying AtFPS2 (1:1) and infiltrated into the juvenile leaves of Nicotiana benthamiana. Four days later, the transiently transformed leaves were cut into small pieces and collected into odor-free bottles to detect volatile compounds.

Acknowledgments

We thank Professor Hongzhi Kong (Institute of Botany Chinese Academy of Sciences: Beijing, China) and Feng Chen (Department of Plant Sciences, University of Tennessee) for their discussion and comments on the manuscript. The pEAQ-HT vector was kindly shared by Professor Wei Li (Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences,
Shenzhen, China). This work was supported by the National Key R & D Program of China (2019YFD1000400; 2016YFD0101900), the National Natural Science Foundation of China [31972445, 31670382], and the Department of Science and Technology of Jilin Province [20190201299]C). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions
SY, NW, SK, YL and TB performed the experiments and analyzed the data with the help of GN, and LL helped to confirm and collect the Aquilegia species. The manuscript was drafted by SK, SY, and XG and revised by XG and BL. XG designed the experiments and supervised the research with LW. All authors have participated in this research and approved the final manuscript.

Data availability
RNA-seq data have been deposited in the NCBI repository (SRR15204285- SRR15204292). The sequences were uploaded to GenBank with the following numbers: AoTPS7, MZ666959; AJTPS7, MZ666960; HTPS7, MZ666963; AoTPS8, MZ666961; AJTPS8, MZ670776; and AoTPS9, MZ666964. Other data supporting the findings of this study are available within the paper and within its supplementary materials published online.

Conflicts 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.

Supplementary data
Supplementary data is available at Horticulture Research Journal online.

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