Transcriptome-wide identification and quantification of Caffeoylquinic acid biosynthesis pathway and prediction of their putative BAHDs gene complex in *A. spathulifolius*

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**Abstract:** The phenylpropanoid pathway is a major secondary metabolite pathway that helps plants overcome biotic and abiotic stress and produces various by-products that promote human health. Its by-product, chloroquinic acid (CQA), is a soluble phenolic compound present in many angiosperms. Hydroxycinnamate-CoA shikimate/quinate transferase (BAHDs superfamily enzyme) is a significant enzyme that plays a role in accumulating CQA biosynthesis. This study analyzed transcriptome-wide identification of the phenylpropanoid to chloroquinic acid biosynthesis candidate genes in *A. spathulifolius* flowers and leaves. Transcriptomic analyses of the flowers and leaves showed a differential expression of the PPP and CQA biosynthesis regulated unigenes. An analysis of PPP captive unigenes revealed the following: the major duplication of the key enzyme, *PAL*, 120 unigenes in leaves and 76 in flowers; the gene encoding *C3'H*, 169 unigenes in leaves and 140 unigenes in flowers; duplicated unigenes of *4CL*, 41 in leaves and 27 in flowers. In addition, *C4H* unigenes had 12 unigenes in the leaves of *A. spathulifolius* and four in the flowers. The characterization of the BAHDs superfamily members identified 82 in leaves and 72 in flowers. Among them, phylogenetic analysis showed that five unigenes encoded HQT and three encoded HCT in *A. spathulifolius*. The three HQT are common to both leaves and flowers, whereas the two HQT were specialized for leaves. The pattern of HQT synthesis was upregulated in flowers, whereas HCT was expressed strongly in the leaves of *A. spathulifolius*. Overall, 4CL, C4H, and HQT are expressed strongly in flowers, and caffeic acid and HCT show more expression in leaves. Therefore, CQA biosynthesis occurs in the flowers of *A. spathulifolius* rather than leaves.

**Keywords:** Phenylpropanoid pathway, Caffeoylquinic acid, BAHDs, hydroxycinnamoyl-coenzyme A: quinate hydroxycinnamoyl transferase, hydroxycinnamoyl-coenzyme A: shikimate/quinate hydroxycinnamoyl transferase, *A. spathulifolius*, DEGs

### 1. Introduction

Plant secondary metabolites (PSM) are a group of organic compounds that assist in protecting plants against biotic and abiotic stress [1-4]. PSM metabolites are non-essential to normal life but impart competence to stressful environments [5, 6]. Secondary metabolites can be divided into three types: terpenoids, polyketides, and phenylpropanoid [7, 8]. The phenylpropanoid (PP) metabolism, the production of enormous compounds by the intermediate process of the shikimate pathway, is present in bacterial, fungi, and plants but absent in animals [9]. These phenolic compounds assist in the plant defense system against insects and fungi [10]. The main compounds of PP metabolites products of phenylalanine (Phe) are precursors that regulate many metabolites, such as flavonoids, tannins, lignins, and phenylpropanoid [11]. The shikimate pathway network connects the carbon metabolism and the AAA (Aromatic
Amino Acid) by converting phosphate phenol pyruvate and erthrose4-phosphate in glycolysis pentose phosphate pathway to chorismate [12]. Phe is the main compound that regulates the phenylpropanoid pathway [13]. PP metabolites are highly involved in many aspects of plant development and morphologically support and respond to both biotic and abiotic stress conditions [14]. In PPP, transamination of the phenylpyruvate and 4-hydroxyphenylpyruvate to form Phe via the trans-cinnamate 4-monoxygenase enzyme(C4H) occurs. Phe helps derive the byproducts of phenol compounds in stress environments and is involved in the production of iso-flavonoids, especially in diseased plants and flavonoids in UV irradiation during symbionts and salicylic acid in plant-pathogen interactions [15, 16]. Unlike caffeoyl-shikimate, CQA is rarely regulated in lignin biosynthesis but is involved in biotic and abiotic stresses, particularly UV radiation [16]. Hydroxycinnamic acids (HCs) are a major class of phenolic compounds in every plant. HCs include caffeic acid, which occurs mainly as an ester with quinic acid rather than shikimic acid, and is called 5-caffeoylquinic acid (CQA) [17]. HCs produce caffeic acid, coumaric acid, ferulic acid, and sinapic acid through PPP in plants. The effects of adding a combination of hydroxycinnamoyl-coenzyme A: shikimate hydroxycinnamoyl transferase (HCT) or hydroxycinnamoyl-coenzyme A: quinate hydroxycinnamoyl transferase (HQT) to C3'H help catalyze the H-unit of p-Coumaroyl CoA into Caffeoyl CoA. This process results in the production of chlorogenic acid or chloroquinic acid (CGA or CQAs) [18-21]. The HCT and HQT belong to BAHD acyltransferase superfamily. The HCT enzymes use coenzyme A-activated acyl donors and HQT uses quinate as a acceptor rather than shikimate acid [22] The HQT is involved in chloroquinic acid in some angiosperm [23]. HQT is estimated to be one of the key enzymes for the synthesis of CQA in plants [20]. These assist human diets. CQA has biological effects in blood circulation; its main function is to inhibit the oxidation of low-density lipoprotein in vitro [24, 25]. Various organic compounds of hydroxycinnamic acid are based on various types of BAHDs proteins abundant in apples, peaches, berry, carrot, and coriander. These compounds are chemically unstable, degradable and form other compounds [26, 27]. The derived byproduct of hydroxycinnamic acid protects against degenerative and age-related diseases in animals [28-31]. Most major soluble phenolic compounds can be found in Solanum species, such as eggplant, potato and tomato [32-34]. These compounds accumulate to substantial levels in coffee, apples, plums, and pears [35-38]. They are absorbed directly by the small intestine but most CQA is absorbed in the large intestine by the esterase of the gut microflora to release caffeic acid [39]. Previous studies evaluated many Asteraceae species for PP derived byproducts in different tissue of plants. In particular, the sunflower family has been studied widely for CQA production in sprouts, leaves, and roots [40-43]. In this study, transcriptome and quantitative PCR were used to analyze the CQA biosynthesis response in the leaves and flowers of A. spathulifolius. The differential expression of PPP involved unigenes throughout the leaf and flower transcriptome was analyzed. In addition, this study analyzed the BAHDs enzyme members to the putative HXXXD domain of HQT (quinic acid) and HCT (shikimic acid). Finally, the putative PPP and BAHDs unigenes were quantified in different plants parts of A. spathulifolius. The identification of these PPP and BAHDs provided valuable insights
for elucidating the response metabolites of chlorogenic acid or chloroquinic acid biosynthesis in *A. spathulifolius* and an importans source of ornamental plants to useful drug discovery.

2. Results

2.1. Assembly and gene annotation

The assembled flower reads produced 146,337 unigenes, with an average contig 811.58 bp in length. The assembled bases of transcripts had an N50 value of 1,279 bp in length, and overall alignment rates of 91.71% to the flower paired-end reads. The GC content was 38.09% on average. In contrast, the leaf transcriptome was reported previously [44]. The assembly completeness was measured by BUSCO analysis to be 91.4% (leaf) and 91.7% (flower), which are the complete transcripts to the eudicots database via tblastn aligns (Figure S1), indicating the good quality of unique transcripts. The flower of *A. spathulifolius* retrieved using the Nr database was 65,129 and 48,896 against the KEGG database, and 70,019 unigenes were identified in the Pfam database (Table 1). The PP pathway-involved unigenes in flowers showed a transcript of 1,128 and in the leaf had 1,287 unigenes. The Gene ontology terms indicated 40.9% to the phenylpropanoid metabolic process, 31.8% to the response to wounding, 22.7% to the lignin biosynthetic process, and 18.2% to the cinnamic acid biosynthetic process to the biological process function (Table 2).

### Table 1. The statistical comparisons of flower and leaf of *A. spathulifolius* to the annotation of unigenes.

| S.No | sample | Total base | unigenes | Uniprot | KEGG | PPP | BAHD |
|------|--------|------------|----------|---------|------|-----|------|
| 1    | Flower | 98467589   | 146,337  | 65,129  | 70,019 | 1,128 | 82   |
| 2    | leaf   | 71660029   | 98,860   | 48,896  | 39,890 | 1,287 | 72   |

2.2. Functional characterization and DEGs of PP biosynthesis

The phenylpropanoid biosynthesis-involved unique transcripts were annotated to the KEGG to explore the CQA synthesis pathway in *A. spathulifolius*. The perspective diagram of the CQA biosynthesis pathway in *A. spathulifolius* revealed three different possible routes through the leaf and flower transcriptome: i) coumaric acid to p-cinnamoyl quinic acid (p-CQA) to C’3H, ii) cinnamoyl-CoA with C4H followed by p-CQA, and iii) p-coumaric acid +4CL+Caffeoyl shikimic acid to Caffeoyl CoA with HQT to form CQA (Figure 1). The total identified PPP involved unigenes showed DEGs in the flower, and leaf reveals 464 different unigenes were upregulated in the flower. In the leaf transcriptome, there were 704 upregulated unigenes compared to the flower. Among the DEGs comparison, 482 unigenes were upregulated in both leaf and flower. One hundred and forty-two unigenes were downregulated in the leaf and flower of *A. spathulifolius* (Figure 2a). Regarding the DEGs unigenes, the *PAL* showed considerable duplication of the genes in both cases of leaves with 120 unigenes, and the flower had 76 copies of *PAL* genes. *PAL1* was highly regulated in the flower with a 793.68 FPKM value, whereas the leaf showed 765.03 FPKM. The last step to synthesis was the Cytochrome P450 (C3’H),
which catalyzes the 3’-hydroxylation of $p$-coumaric esters of shikimic/quinic acids to form CQA. C$3’$H

**Phenylalanine Biosynthesis to Chloroquinic acid (CQA) in A. spathulifolius**

![Diagram](image)

**Figure 1.** Schematic diagram of Phenylalanine towards to Chloroquinic acid (CQA) biosynthesis in *A. spathulifolius*. 1, 2 and 3 indicates the different route pathway of chlorogenic acid production. **PAL**, phenylalanine ammonia-lyase; **C4H**, Cinnamic acid 4-hydroxylase; **4CL**, 4-coumarate-CoA ligase; **C3’H**, $p$-coumarate 3’Hydroxylases; **CSE**, Caffeoylshikimate esterase. **HCT**, Hydroxycinnamoyl CoA shikimate Hydroxycinnamoyl-transferase; **HQT**, Hydroxycinnamoyl CoA quinate Hydroxycinnamoyl-transferase.

**Table 2.** Enriched GO term of Biological Process (BP) function in *A. spathulifolius* regarding PPP.

| GO ID     | BP TERM                                      | %    | p-value     |
|-----------|----------------------------------------------|------|-------------|
| GO:0009698| phenylpropanoid metabolic process            | 40.9 | 9.00E-21    |
| GO:0009800| cinnamic acid biosynthetic process           | 18.2 | 1.10E-08    |
| GO:2000762| regulation of phenylpropanoid metabolic process | 18.2 | 3.80E-08    |
| GO:0009611| response to wounding                         | 31.8 | 4.90E-08    |
| GO:0009699| phenylpropanoid biosynthetic process         | 18.2 | 6.90E-06    |
| GO:0006559| L-phenylalanine catabolic process            | 13.6 | 3.10E-05    |
| GO:0009809| lignin biosynthetic process                  | 22.7 | 2.80E-04    |
| GO:0008152| metabolic process                            | 13.6 | 3.80E-04    |
| GO:0009411| response to UV                               | 13.6 | 2.40E-03    |
| GO:0080167| response to karrikin                         | 13.6 | 8.30E-03    |
| GO:0009813| flavonoid biosynthetic process               | 13.6 | 1.10E-02    |
Figure 2. (a) The geom point plot shows the up-regulated differential expressed genes of PPP in leaf and flower of A. spathulifolius; (b) A Venn diagram shows up and down regulation of PPP in A. spathulifolius leaf and flower.

had 169 unigenes in the leaf and 140 unigenes in the flower of A. spathulifolius. There were 27 unigenes in the flower and 41 in the leaf with regards to the 4CL enzymes. The two 4CL genes in the flower were highly regulated with 435–253.48 than in the leaf. The two leaf 4CL with 332.62 and 165 FPKM has been observed. C4H protein was found to have 12 unigenes in the leaves and four unigenes in the flower. The four unigenes of C4H were highly upregulated in the leaves, while three unigenes of C4H were upregulated in the flowers with FPKM value 160–77 in range (Figure 2b).

2.3. Estimation of BAHDs superfamily

The leaves and flowers of A. spathulifolius were compared. The BAHD superfamily genes were vastly duplicated in the flowers with 82 unigenes in that 30 unigenes with ‘HXXXD’ and ‘DFGWG’ were observed. In the leaf, 72 unigenes were related to the BAHD transferase family. Only 33 unigenes had two domains. The HXXXD domain is related to HQT (“HTLAD/HTLSD”), which promoted the production of CQA byproducts. In leaves, there are 704 upregulated PPP-involved unigenes; 82 genes were BAHD member unigenes among the upregulated unigenes. In contrast, 457 unigenes were found in the flower; 72 unigenes belonged to the BAHD clades, which were upregulated. The HXXXD domain recognizes the distribution of different BAHD complex genes; the most available domain was ‘HTLSD’ with five copies in the leaf and three unigenes in the flower (Figure 3a). The duplication of these unigenes showed different
Figure 3. (a) Distribution of “HXXXD” domain among the leaf and flower of *A. spathulifolius*. The blank space indicates not presents.; (b) The DEGs expression of up and down regulated BAHD transferase unigenes among the different transcripts.

FPKM values with one another in both the leaf and flower. In addition, the distributed domain varied from the flower to leaf; the flower HXXXD domain maintained different amino acid codons than the leaf, even though the leaf has an equal amount of the HXXXD domain, but it contains a duplication of the distributed domain, and some ‘HYVVD, HVVAD, HVMCD, HRVVD, and HKIAD’ which specialized to flower, are not present. The domain of ‘HAVVD’ contains two copies in both the leaf and flower. The HRIGD, HKIID, HAVAD, HATFD, and HAILD domain presented one copy in flowers, whereas it had two copies each in the leaves. The HTLAD (HQT)-related unigenes tended to have three duplication genes in the leaf, whereas the flower showed only two copies (Figure 3a). In the flower, the ‘HTLSD’ (HQT) genes were upregulated according to DEGs analysis compared to the leaf HQT. The unknown function of the ‘HTMSD’ domain in both the leaf and flower was strongly expressed in the same value of FPKM (>50) in *A. spathulifolius*. The lowest expressed domain was the single duplication copy of the ‘HRXXD’ domain of the HCs unigenes (Figure 3b). Most of the BAHD transferase protein between the flower and leaf showed the highest FRKM value from the highest of 87.58 to the lowest of <2. The HQT with the ‘HTLAD’ domain contained unigenes in the flower expressed strongly with an FPKM value of 87.58. The unigenes leaf ‘HAMSD’ domain with 50.48 were detected, respectively. This indicates that CQA production in the flower of *A. spathulifolius* is higher than the leaf.
Figure 4. Phylogenetic tree shows the different type of up-regulated HXXXD among leaf and flower of A. spathulifolius.

2.4. BAHDs protein Phylogenies

The unrooted phylogenetic trees were constructed from a complete ORF and with the presence of binding site, ‘HXXXD’ and ‘DFGWG’ conserved domain of the BAHD family members (Figure S1). The HTLAD and HTLSD domain of unigenes were grouped into the previously reported HQT genes. The HHAAD domain of A. spathulifolius leaf and flower was grouped into the HCT unigenes previously reported in other known Asteraceae. amount of the HXXXD domain, but it contains a duplication of the distributed domain, and some ‘HYVVD, HVVAD’ rather than ‘HTLXD’ also clade within the HQT unigenes, which is highly diverged. In contrast, the domain of ‘NIIVD’ showed the lowest number of duplications than the other distributed BAHD. The HKXXD distributed genes were grouped into one clade, showing that the HKIID and HKVAD are highly diverged duplicated BAHD family proteins with seven copies, even though HKIAD was grouped into another clade after the HRTSD domain. In contrast, most of the HAXXD-based domains were grouped into a single clade with seven duplicated unigenes. Among them,
the HRAAD domains showed the least diverged group of proteins in *A. spathulifolius*: two copies in the flower and one in the leaf. Based on the phylogenetic tree, the HXXXD domain highly diverged in leaf and flower of *A. spathulifolius*.

2.5. Quantification of PPP unigenes

Previous studies of *A. spathulifolius* leaf transcriptome-identified PP candidate genes were designed for reverse transcription (RT) to confirm the quality of isoform produced by assembled transcripts (Figure S2). The total assembled unigenes in both leaf and flower produced 345,781 unigenes. The DEGs analysis of the leaf and flower revealed the up and downregulated unigenes shown in MA plot. The quantification of DEGs showed that 146,78 were upregulated and 9,789 were downregulated; the left unigenes showed no differential expression and were defined in 0 values in the smear plot (Figure 5a). The quantification flower hydroxycinnamoyl transferase unigenes showed the top nine upregulated genes, and 11 downregulated unigenes in the flower, which was also distributed in the leaf transcriptome (Figure 5b). The production of CQA-derived hydroxycinnamoyl transferase enzymes in both the leaf unigenes of PPP was identified in
Figure 6. Quantification of PPP involved unigenes in different plant parts of *A. spathulifolius*. 4CL; 4-coumarate-CoA ligase, CAA; Coumaroyl-CoA, CCR; Cinnamoyl-CoA reductase, CSE; Caffeoylshikimate esterase.

Figure 7. Overlay of quantified expression of HXXXD – Hydroxycinnamoyl transferase unigenes in flower and leaf.

the different parts of the leaf, such as young leaf (YL), extended leaf (EL), and mature leaf (ML) stages (Figure 6A). qRT-PCR revealed the high production of *PAL*, showing large Log2fold changes (above >345 FPKM in mature and flower samples). The 4CL protein shows the high fold changes in flower and the second-highest activity in the elongation leaf and young leaf of *A. spathulifolius*. CAA shows the largest fold change of
<9.5 in the mature leaf and >5.5 to 4 fold change in the flower compared to the elongation leaf and young leaf of *A. spathulifolius*. The CSE unigenes were equal to the 4CL genes, showing a high ΔCt value in the flower than the leaf of *A. spathulifolius*. Finally, the HCT showed the lowest ΔCt in the flower, which was highly activated in the leaf rather than the flower. The random BAHD protein family was also analyzed using real-time PCR. Quantification of the ‘HCVCD’ gene showed the lowest expression among the BADH unigenes chosen. The highest expression was observed in ‘HTLSD’ with ΔCt in flower and second most unigene ‘HCLCD’, followed by the ‘HTLAD, HTMSD, and HTLGD’ BAHD family in the leaf and flower of *A. spathulifolius* (Figure 6). Based on the random ‘HXXXD’ domain, the protein of *A. spathulifolius* showed an unknown function of ‘HTMSD’ in the pathway mechanism in Aster. Predominately, the HQT (HTLAD and HTLSD) showed the highest ΔCt value compared to the root. Hence, the synthesis of production and upregulation of HQT was higher in flowers of *A. spathulifolius* than in the leaf. In contrast, the leaf of *A. spathulifolius* showed the HCT (Cinnamic acid rather than quince acid) expressed in producing compounds like caffeic acid and other cinnamic acid.

### 3. Discussion

Phenylpropanoid biosynthesis produces various secondary metabolites, most of which have beneficial effects on human health [45]. These metabolisms are of concern regarding diabetes, obesity, cancer, and cardiovascular disease that are a significant burden on the world health care system. CQA is an abundant polyphenol compound in the human diet produced by almost all plants [19, 31, 39]. Polyphenols compounds are abundant in coffee, which is why coffee plants have attracted considerable attention for CQA production (12, 13 & 14). CQA affects the glucose and lipids metabolism, where intermediates regulate the breakdown of glucose and lipids [19, 25]. A range of mingled pathways was reported to produce CQA through the phenylpropanoid pathway. Based on the KEGG pathway, *A. spathulifolius* could produce CQA as a byproduct in a three-way route [46, 47]. DEGs analysis of the transcriptome-produced PPP involved 1128 unigenes in the flower transcriptome and 1287 in the leaf transcriptome (Table 1). Among the PPP-involved genes, the PAL 1 enzyme showed the up regulation in both leaf and flower, which is the key product of PPP in the shikimate pathway [48]. The phenylalanine ammonia-lyase (PAL)-dependent phenylpropanoid route produces the byproduct of CQA isomers that assist in protein adducts [39, 49, 50]. PAL catalyzes the conversion of Phe to trans-cinnamic acid, which is the gateway to the PPP that leads to various derivative-like flavonoids, isoflavonoids, coumarin, anthocyanins, and lignins. This ultimately results in the synthesis of CQA and caffeic acid in plants, which respond differentially to biotic or abiotic stresses [11, 51]. Duplicated PAL unigenes in leaves and flowers have 120 and 76 copies, respectively, which was higher than Cynara species (Asteraceae) (102 PAL genes) [52, 53]. Overall, with the improvement of PAL activity and stability, they can be used as an efficient biocatalyst for the enzymatic production of Phe. **PAL, C4H, 4CL, HCT, and HQT** are important enzymes in this pathway, facilitating the biosynthesis of flavonoids and other important secondary metabolites from...
phenylalanine. In addition, there are 27 and 41 4CL unigene copies in the flower and leaf of *A. spathulifolius*. The 4CL protein was found at its highest levels after the PAL enzymes, which play important roles in PP biosynthesis and belong to the cytochrome P-450 family. A higher level of chlorogenic acid production was observed based on the 4CL activity even towards coumaric acid, ferulic acid, and cinnamic acid [33, 54, 55]. 4CL might also participate in the biosynthesis of flavonoids and other soluble phenolic compounds [56]. This duplication of PAL and 4CL highlights the importance of PP biosynthesis in *A. spathulifolius*. The C3’H enzyme belongs to the p450 monooxygenases family of the CYP98 family. This enzyme does not use p-coumaric acid as a substrate; it uses the shikimate (HCT) and quinic (HQT) ester of p-coumaric acid instead. This process catalyzes the 3’-hydroxylation of p-coumaric esters of shikimic/quinic acids to form CQA [18, 46, 57]. The duplication of C3’H 169 copies in the leaf and 140 unigenes in the flower indicates the involvement of Caffeoyl CoA in the synthesis of CQAs and other flavonoids. The up-or-down regulation of phenylpropanoid biosynthesis through the leaf and flower transcriptome shows 704 unigenes upregulated in the leaf and 413 unigenes in the flower (Fig 2b). Duplication of the PPP-involved unigenes suggests that the complexity of the molecular mechanism of PP biosynthesis in *A. spathulifolius* to produce various byproducts. A comparison of the leaf and flower DEGs revealed 482 unigenes equally upregulated than downregulated (142) in *A. spathulifolius*. This duplication of the core components unigenes suggests that they may have been recruited for major plant phenylpropanoid metabolites for their specialized tissues in *A. spathulifolius*. Hence PP biosynthesis depends strongly in an enzyme-dependent manner and accumulates in the flower and leaf of *A. spathulifolius*. The DEGs of HCs (HXXXD) in the leaf and flower show the binding sites of shikimate and quinic acid, which have competition sites to synthesize CQA or caffeic acid in PPP. The superfamily of BADH is a large class of acyl CoA-dependent transferase, which is distinct between the conserved domains. The HXXXXDG and DFGWG consensus sequences are highly conserved among plants. There are two conserved domains in BAHDs: A C-terminal ‘DFGWG’, which may or may not be present in all BAHD, but it provides structural stability of enzymes; HXXXD, which has a solid role in catalyzing the acyltransferase group [58, 59]. *A. spathulifolius* BAHDs retain a functional copy of the HXXXX conserved site was predicted to have leaf (82) and flower (71). Two HCT proteins contain the same motif HHAAD in the middle part. In contrast, three HQT share the motif HTLS/AD. The two motifs of HXVVD also show a close relation to HQT (HTLXD) proteins, showing that although they do not share the same amino acid conserved motif, they share other conserved codons to the HQT protein with 78.7% of pairwise identity.

The HQT enzymes conserved the domain with HTLSD and HTLAD compared to Cynara and Helianthus of the Asteraceae family. Furthermore, two new domains, ‘HXVVD’, are the same as HQT with the phylogeny tree and alignment of codons. A *H. annuus* study revealed a large amount of CQAs in the sprout [43], where *C. cardunculus* whole-genome mapping showed that among 32 BAHDs proteins, only three of them showed similarity to the HCT and HQT enzymes [60]. In vivo studies of CQA synthesis in *C. intybus* revealed two HCT and three HQT genes involved in HCT or HQT with C3’H
to synthesize Caffeoyl CoA[61]. The HTLSD and HTLAD (HQT) sites were predicted in the leaf and flower of A. spathulifolius, which is the same as in C. cardunculus L, var. scolymus. In that study, 69 BAHDs proteins with the catalytic site of ‘HXXXD’ were identified genome-wide with three HQT and two HCT proteins. The new domain ‘H(Y/R) VVD’ may also be one of the HQT enzymes, but more analysis will be needed for confirmation. HCT and HQT are directly involved in CQA biosynthesis in tobacco [20].

Performing the quantitative PCR on HQT and HCT unigenes showed that HQT was expressed dramatically compared to the HCT unigenes. These duplication and new clade of BAHDs super family may indicates the PSM is essential to the endurance and successive reproductive fitness of a green plant species in its natural habitat. These results highlight the evolutionary relationships and conserved domain concurrence among the different HQT and HCT isoforms in the Asteraceae family plants, such as chicory and globe artichoke. Based on the transcriptome-wide identification and characterization of CQA in A. spathulifolius, the candidate genes involved in the core caffeoylquinic acid synthesis pathway (PAL, C4H, 4CL, HCT, C3’H, HQT) were highly distributed and duplicated throughout the genome. Therefore, the diverged domain of the BAHDs gene complex could affect CQA synthesis in A. spathulifolius leaves and flowers. In addition, the HQT is strongly expressed in flowers, and HCT is expressed in the leaves. These metabolites are a diverse set of compounds with numerous functions in plant-environment interactions. The results suggest that PSM increases the diversity of higher plants, including A. spathulifolius. Overall, these results revealed substantial CQA biosynthesis in A. spathulifolius that could benefit human health.

4. Materials and Methods

4.1. RNA Isolation, cDNA Library Construction, and Illumina Sequencing

The total RNA of the whole flower of Aster spathulifolius was extracted using the protocol reported by Bretial et al. [62]. cDNA Library construction and RNA-Sequencing were performed using the Genomics Macrogen Laboratory (South Korea). The TruSeq method was used to make short fragments of mRNA. The short fragments were then as used as templates for the cDNA library. All short fragments were linked to the sequencing adapter, and the fragments were sequenced for Paired-End (PE) reads using illumina sequencing. For leaf transcriptome, the material used in previous studies (SRR10724565) [44] and flower RNA-sequences submitted under NCBI-SRA database with accession no: SRR14001926 were applied.

4.2. Denovo assembly and functional annotation

The raw reads were checked for Fastq quality control [63]. Below-quality value ≤ 30% (Q20) reads were removed using the trimmomatic tool [64]. The clean reads were assembled to retrieve unique transcripts using the trinity program with kmer size 25 with the following pipeline: inchworm, chrysalis, and butterfly [65]. Finally, the unique reads transcripts were checked for coding function annotations using the ‘Trinotate and TrinotateWeb’ pipeline, as mentioned in a previous study [66]. The gene annotation for all assembled unigenes was aligned to the Swiss-Prot protein database (https://www.uniprot.org/), and the Kyoto Encyclopedia of Genes and Genomes (KEGG,
4.3 Identification of PPP unigenes

The peptide sequence of the Arabidopsis thaliana database of PPP protein was downloaded from the TAIR website [70, 71]. The identity of the PPP unigenes was retrieved [63] using local blastX at a value of 1 × 10⁵ using the NCBI-Blast tool [72] and against the KEGG database [73] and the BioCyc for pathway prediction (https://biocyc.org/ARA/organism-summary?object=ARA). In addition, the PPP involved unigenes of *A. spathulifolius* were set as a database (as a reference database) to blast again to the total transcripts to predict the unrecognized sequences for reverification.

4.4 Differentially expressed genes (DEGs) analysis

Transcripts of *Aster spathulifolius*, differential gene expressions (DEGs) of the abundance unigenes were determined by RSEM (RNA-Seq by Expectation-Maximization) [74, 75], which first generates and processes the total transcripts and then aligns them to raw reads of *A. spathulifolius* flower and leaf. RSEM is used to calculate the fragments per kilobase per million (FPKM) and the transcripts per million (TPM) values of the total unigenes to analyze the maximum number of genes expressed or abundant in *A. spathulifolius*. The genes were defined as DEGs with the options of FDR-corrected p < 0.01 and log2FC > 1. Both FPKM and TPM values were calculated to estimate the expression levels of the unigenes involved in the phenylpropanoid pathway (PPP) and caffeoylquinic acid biosynthesis. Complete DEGs analysis was performed under the Trinity pipeline [65] of RSEM with the option of Bowtie2 [75, 76] and EdgeR [77]. The thresholds for considering the significance of DEGs were FDR ≤ 0.01 and |log2 (fold change)| ≥ 1, which is the visualization of result under ggplot2 (https://ggplot2.tidyverse.org/reference/geom_point.html).

4.5 Structure of BAHD family member unigenes

The protein sequences of BAHD from *Cynara, Cichorium* from Asteraceae (same family), and *Solanum* [20, 61, 78, 79] plants were chosen to identify the HCT and HQT domain and predict the conserved domain of (HXXXD) of HCT and HQT under many BAHD family member proteins. The BAHD structural variance was differentiated by aligning the protein sequences by MAFFT [80] with the default parameters and the PHYML (LG) maximum likelihood tree with 100 bootstrap replications [81].

4.6 qRT-PCR expression studies:

Three stages of the leaf (young, extended, and mature leaf), a whole flower, and root were used to quantify the CQA biosynthesis-involved unigenes in *A. spathulifolius*. The PP pathway to CQA involving the unigenes from transcriptome leaf data was identified through KEGG annotation and BLAST-p analysis. The unigenes were then searched for the full open reading frame (ORF) to design the forward and reverse primers for further analysis. An amplicon size and primer structure of seven candidate unigenes and random
BAHDs unigenes were selected to quantify the HQT genes (Table S1). cDNA was synthesized by Reverse Transcription System (A3500, Promega, South Korea), the final 10 µl product was incubated at 70°C for 5 min to synthesize the c-DNA using Oligo(dT)15 Primer. Finally, a quantification assay was carried out using GoTaq® qPCR Master Mix (A6001, Promega) followed by standard cycling conditions as a guideline (Applied Biosystems 7500 step one plus).

6. Patents

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1.

**Author Contributions:** Jean Claude S. conceived and designed the experiments, analyzed the data and wrote the paper; Jean Claude S and SunMi Park performed the experiments.

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