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Gene Note

An acyltransferase-like gene obtained by differential gene expression profiles of quinolizidine alkaloid-producing and nonproducing cultivars of *Lupinus angustifolius*

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Abstract Quinolizidine alkaloids (QAs) are one of the representative groups of plant alkaloids. To isolate genes involved in QA biosynthesis, we performed a differential gene expression analysis by PCR-select subtraction between a QA-producing bitter cultivar and a nonproducing sweet cultivar of *Lupinus angustifolius*. We obtained 71 and 43 clones specific to the bitter and sweet cultivars, respectively. Among the genes specifically expressed in the bitter cultivar, an acyltransferase-like gene (*LaAT*: *L*upinus *a*ngustifolius *a*cyl*t*ransferase) showing homology to the BAHD protein family was isolated. LaAT showed the strongest homology to the *Arabidopsis thaliana* BAHD acyltransferases involved in the formation of conjugated polyamines. Semi-quantitative RT-PCR revealed that *LaAT* expression was highest in the young leaves but barely detectable in the other organs of the bitter cultivar plant, whereas *LaAT* expression was undetectable in the sweet cultivar.

Key words: Acyltransferase, *Lupinus angustifolius*, quinolizidine alkaloid.

Quinolizidine alkaloids (QAs) are plant secondary metabolites that occur mainly in the genus *Lupinus* (Ohmiya et al. 1995; Saito et al. 1989). Several hundred structurally related compounds belonging to this group have been reported (Joseph 2008). QAs show a broad range of pharmacological properties (e.g., cytotoxic, oxytocic, antipyretic, antiviral, antibacterial, and antifungal activities) (Saito and Murakoshi 1995). Some QA-containing plants, such as *Sophora flavescens,* have been used as a source of crude drugs in Chinese-Japanese traditional medicine (KAMPO), and QAs have been shown to be the principal constituents responsible for the pharmacological activities of these medicinal plants (Tang and Eisenbrand 1992). In addition, QAs are assumed to play dispensable, but important roles in the survival of plants that produce them as defense compounds against pathogenic organisms or predators (Wink 2003). Many QA-producing species are cultivated as crops in Europe, the Americas, Australia, and the Mediterranean countries (Oram 1983). The alkaloid-free sweet cultivars have been bred in several lupin species. The sweet cultivar 'Uniharvest' and the bitter cultivar 'Fest' were derived from a cultivar of narrow-leaf lupin (*Lupinus angustifolius* cv. New Zealand Blue) (Oram 1983). The bitter and sweet cultivars have a similar genetic background, but the sweet cultivar is homozygous for the mutant *iuc* (*iucundus*) allele, which results in a low alkaloid phenotype. The bitter and sweet cultivars are similar in the case of traits such as flowering time and pod shattering but differ for alkaloid contents and the colors of the flower and seed coat (Oram 1983).

QAs are synthesized from L-lysine via decarboxylation and cyclization of cadaverine. The cyclic alkaloids are further modified by hydroxylation, esterification, and glycosidation to yield a variety of alkaloids (Figure 1) (Ohmiya et al. 1995). However, the biochemical information on the enzymes involved in the QA biosynthesis remains scanty (Facchini 2001). To further the understanding of the enzymes yielding these compounds, we have investigated several enzymes involved in the pathway at a biochemical and molecular biological level (Hirai et al. 2000; Okada et al. 2005; Saito et al. 1993; Suzuki et al. 1994). In this study, to identify additional enzymes involved in this biochemical pathway, we performed differential gene expression profiling of bitter and sweet cultivars by PCR-select subtraction. This method has been previously applied to the characterization of chemo-varieties of *Perilla frutescens* to identify anthocyanin biosynthetic genes (Yamazaki et al. 2008).

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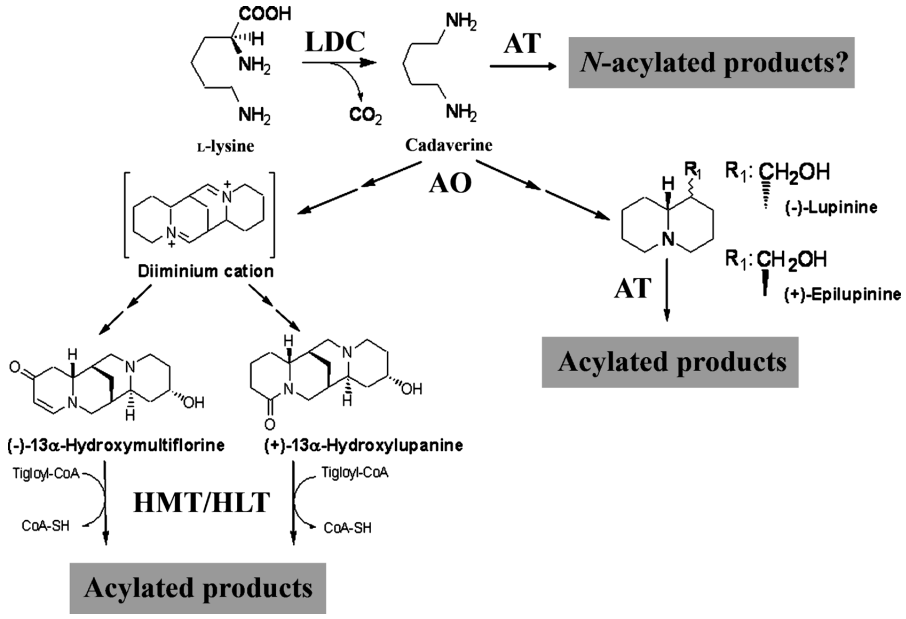

Figure 1. Proposed biosynthetic pathway of the ester-type quinolizidine alkaloids. LDC, Lysine decarboxylase; AO, amine oxidase; AT, acyltransferase; HMT/HLT, tigloyl-CoA:(–)-13α-hydroxymultiflorine/(+)-13α-hydroxylupanine-*O*-tigloyltransferase.

PCR-select subtraction analysis was conducted between cDNAs from the leaves of the bitter cultivar and sweet cultivar. From the first screening, 288 clones each were selected as specific candidates for the bitter and sweet cultivars. Using dot-blot hybridization, these clones were further delimited to 71 and 43 clones specific to the bitter and sweet cultivars, respectively. The (partial) sequences of these clones were determined, and the sequence homologies were analyzed by BLAST-X (Figure 2, Supplementary Table S1, S2). Among the bitter cultivar-specific clones, 30 clones represented genes that encoded metabolic enzymes. Of these, 3 genes exhibited homologies to *ornithine decarboxylase* (*ODC*), *copper amine oxidase* (*CuAO*), and *acyltransferase* (*AT*) and represented potential candidates for structural genes encoding QA biosynthetic enzymes. The gene that showed AT homology was designated *LaAT* for *Lupinus angustifolius acyltransferase*. Because it was potentially involved in an esterification step of QA biosynthetic pathway, it was subjected to further analysis. In contrast, of the 43 sweet cultivar-specific clones, no clone represented a gene apparently related to QA biosynthesis.

The sequence encoding the C-terminal end of LaAT was obtained by 3′-RACE (3′-Full RACE Core Set, TaKaRa), and the full-length *LaAT* was amplified by PCR using cDNA from the young leaves of the bitter cultivar. The full-length *LaAT* cDNA was 1359 bp and encoded an open reading frame (ORF) of 453 amino acids. The predicted protein was calculated to have a molecular mass of 50.65 kDa and an isoelectric point of 5.73. The *LaAT* cDNA contained a putative ATG start codon and encoded the N-terminal part of an AT-like

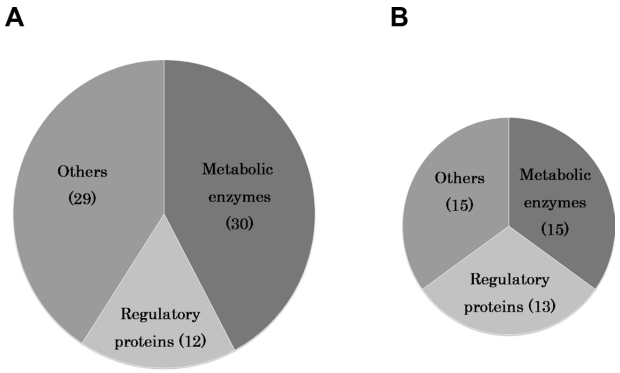

Figure 2. Profiling of sequences from the PCR-select cDNA subtraction method for bitter and sweet cultivars of *L. angustifolius*. (A) Bitter cultivar specific. (B) Sweet cultivar specific.

protein homologous to the BAHD superfamily. The BAHD family enzymes have been characterized as transferring a range of different acyl groups from their CoA thioesters to various plants metabolites, mostly secondary products. The addition of the acyl group to a hydroxyl moiety or nitrogen atom in these compounds results in an ester or amide (D'Auria 2006).

Based on the BLAST search, LaAT showed a high degree of homology to the BAHD acyltransferases, which are involved in the formation of conjugated polyamines in *Arabidopsis thaliana*. This homology included a 41% identity to spermidine disynapoyl transferase (SDT) and with 40% identity to spermidine dicoumaroyl transferase (SCT) (Luo et al. 2009). LaAT also showed 32% identity to 3′-*N*-debenzoyltaxol-*N*-benzoyltransferase (DBNTBT), which is involved in taxol biosynthesis in *Taxus canadensis* (Walker et al.

2002) and only 28% identity to tigloyl-CoA:(−)-13α-hydroxymultiflorine/(+)-13α-hydroxylupanine *O*-tigloytransferase (HMT/HLT) from *L. albus* (Okada et al. 2005). The overall sequence identity to other plant acyltransferases was ~30% in deduced amino acid sequences. The BAHD acyltransferases are recognized by several common motifs, including the HXXXD motif important for general base catalysis in the active site and the structural motif DFGWG located near the C terminus (Ma et al. 2004). Multiple alignments of amino acid sequences of LaAT revealed that LaAT contained the highly conserved sequences of the BAHD family including HXXXD and DFGWK, in which the last amino acid is mutated from glycine (G) to lysine (K) as showed in Figure 3.

A phylogenetic tree was constructed by the neighbor-joining method with the MEGA 4 program (Tamura et al. 2007) and used plant BAHD acyltransferases that

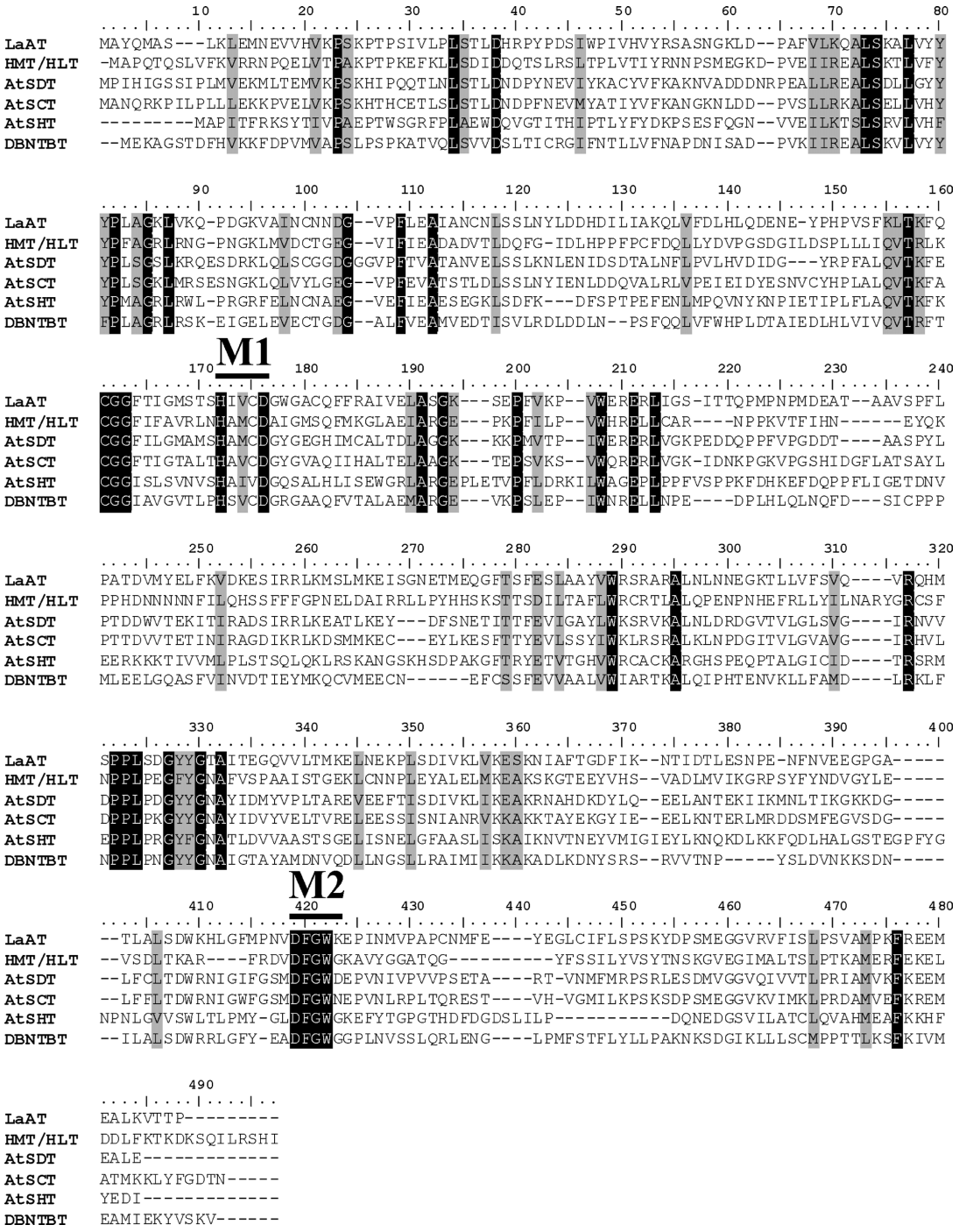

Figure 3. Multiple alignments of LaAT with several plant BAHD-family acyltransferases. Motif1 (M1) and 2 (M2) are conserved amino acid sequences in the BAHD acyltransferase family and are indicated with black lines: motif 1, HXXXD; motif 2, DFGWK.

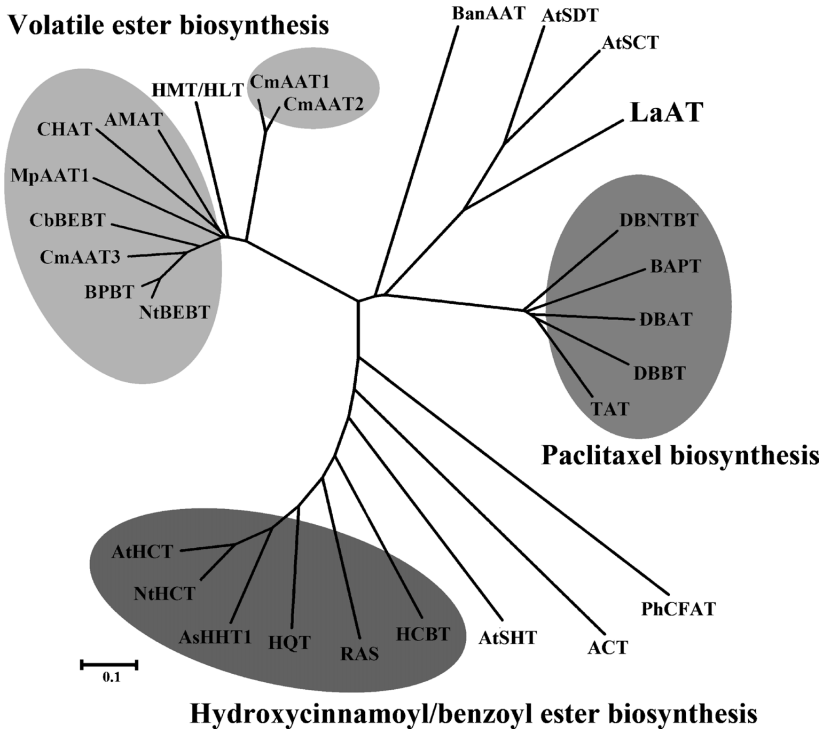

Figure 4. A molecular phylogenetic tree of the deduced amino acid sequences of LaAT with other plant acyltransferases belonging to the BAHD family. The tree was constructed by the neighbor-joining method by using the MEGA4 program. The lengths of the lines indicate the relative distances between nodes. Enzymes used for the alignment were as follows: LaAT (this study, AB581532), *L. angustifolius* acyltransferase; AtSCT (AAP81804), *A. thaliana* spermidine dicoumaroyl transferase; AtSDT (NP_179932), *A. thaliana* spermidine disynapoyl transferase; BanAAT (CAC09063), banana alcohol acyltransferase; CmAAT1-3 (CAA94432, AAL77060, and AAW51125, respectively), *Cucumis melo* alcohol acyltransferase; HMT/HLT (BAD89275), *L. albus* tigloyl-CoA:(-)-13a-hydroxymultiflorine/(+)-13a-hydroxylupanine *O*-tigloytransferase; AMAT (AAW22989), *Vitis labrusca* anthraniloyl-CoA:methanol acyltransferase; CHAT (AAN09797), *A. thaliana* (*Z*)-3-hexen-1-ol *O*-acetyltransferase; MpAAT1 (AAU14879), *Malus pumila* alcohol acyltransferase; CbBEBT (AAN09796), *Clarkia breweri* benzoyl-CoA:benzylalcohol *O*-benzoyltransferase; BPBT (AAU06226), *Petunia x hybrida* benzoyl-CoA:benzylalcohol/phenylethanol benzoyltransferase; NtBEBT (AAN09798), *Nicotiana tabacum* benzoyl-CoA:benzylalcohol *O*-benzoyltransferase; AtHCT (NP_199704), *A. thaliana* hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase; NtHCT (CAD47830), *N. tabacum* hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase; AsHHT1 (BAC78633), *Avena sativa* hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyltransferase; HQT (CAE46932), *N. tabacum* hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase; RAS (CAK55166), *Coleus blumei* rosmarinic acid synthase; HCBT (CAB06430), *Dianthus caryophyllus* anthranilate *N*-hydroxycinnamoyl/benzoyltransferase; AtSHT (NP_179497), *A. thaliana* spermidine hydroxycinnamoyl transferase; ACT (AAO73071), *Hordeum vulgare* agmatine coumaroyltransferase; PhCFAT (ABG75942), *Petunia x hybrida* coniferyl alcohol acyltransferase; TAT (AAF34254), *Taxus cuspidata* taxa-4(20),11(12)-dien-5a-ol-*O*-acetyltransferase; DBBT (Q9FPW3), *T. cuspidata* 2-debenzoyl-7,13-diacetylbaccatin III *O*-benzoyltransferase; DBAT (AAF27621), *T. cuspidata* 10-deacetylbaccatin III-10-*O*-acetyl transferase; BAPT (AAL92459), *T. cuspidata* baccatin III *O*-phenylpropanoyltransferase; DBNTBT (AAM75818) *T. canadensis* 3′-*N*-debenzoyltaxol-*N*-benzoyltransferase.

have been characterized at a biochemical level. As shown in Figure 4, LaAT, SDT, and SCT form the subfamily that is distinct from one to which HMT/HLT of *L. albus* belongs (Okada et al. 2005). Furthermore, based on its calculated molecular mass of 50.65 kDa, LaAT fits in well as a member of the BAHD family, with all the members of this family possessing molecular masses of ca 50±5 kDa (D'Auria 2006). These results clearly indicate that LaAT is one of the BAHD superfamily. Based on its close homology to SDT and SCT, LaAT might be either involved in the formation of QAs esters or in the formation of *N*-acylated polyamine conjugates.

The LaAT expression pattern was investigated by semi-quantitative RT-PCR. Total RNA was isolated from both young and mature leaves, cotyledons, hypocotyls, and roots of bitter and sweet cultivars of *L. angustifolius* by using the RNeasy Plant Mini Kit (Qiagen), and cDNAs were synthesized using a Superscript II RT kit (Invitrogen). A 1391 bp of *LaAT* was amplified by PCR using Ex *Taq* DNA polymerase (TaKaRa) and specific primers (LaAT-F: 5′-CTCACATTTCAACCCACTAA-3′, LaAT-R: 5′-ATTCTAGGGTGTAGT AACCTTCA-3′). The PCR was performed with an initial denaturation at 95°C for 5 min, then 26 cycles each at 95°C for 30 s, at 55°C for 30 s, and at 72°C for 1.5 min. For normalization of the different RNA preparations, a 700 bp fragment of *L. angustifolius β-tubulin* was amplified with the following primers: Tul-F (5′-CAGGGAGGAATACCC-AGACA-3′) and Tul-R (5′-GGCAGTGAATTGCTC-ACTCA-3′). The PCR products were separated by electrophoresis using a 1% gel at 100V, and the gel was further stained by SYBR® Green (TOYOBO). In the

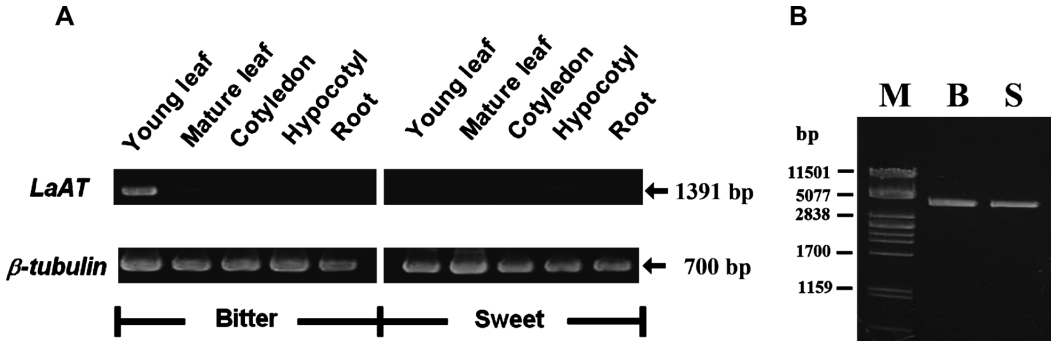

Figure 5. Tissue-specific and cultivar-specific expression of *LaAT*. (A) Total RNAs were isolated from various tissues and organs, as indicated, and then subjected to semi-quantitative RT-PCR analysis using *L. angustifolius β-tubulin* as an internal control. (B) PCR analysis of genomic DNA isolated from bitter and sweet cultivars of *L. angustifolius*. DNA was extracted from young leaves of both cultivars and subjected to genomic PCR analysis by using the same primer pair that used for semi-quantitative RT-PCR analysis. Lane M, λPstI marker; B, bitter cultivar; S, sweet cultivar.

bitter cultivar, the *LaAT* transcript level was the highest in the young leaves and barely detectable in the other organs. No detectable level of expression was observed in the tissues of the sweet cultivar plant (Figure 5A). These expression profiles confirmed the specific expression of LaAT in the bitter cultivar. It was also consistent with a report that QAs are synthesized in the shoot tissues of *L. angustifolius* (Lee et al. 2007). A genomic PCR was performed by using the genomic DNA extracted from young leaves of both bitter and sweet cultivars of *L. angustifolius* (Qiagen DNeasy Plant Mini Kit). Genomic PCR was carried out using Ex *Taq* DNA polymerase (TaKaRa) and the same specific primers used for semi-quantitative RT-PCR. The PCR was performed with an initial denaturation at 95°C for 5 min, then 25 cycles each at 95°C for 30 s, at 55°C for 30 s, and at 72°C for 1.5 min. The fragment obtained from genomic PCR, about 4 kb, indicates that there is *LaAT* in both bitter and sweet cultivars; therefore, the expression of *LaAT* may be regulated at a transcriptional level (Figure 5B).

In summary, differential gene expression profiling between the QA-producing bitter cultivar and nonproducing sweet cultivar of *L. angustifolius* was conducted. Based on its possible role in QA synthesis, an acyltransferase-like gene, *LaAT,* was cloned and found to be specifically expressed in young leaves, where QAs appear to be synthesized. Although the enzymatic function of *LaAT* has not been confirmed, this work represents a valuable first step toward a better understanding of the enzymes involved in the QA biosynthetic pathway.

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