A Root-Expressed L-Phenylalanine:4-Hydroxyphenylpyruvate Aminotransferase Is Required for Tropane Alkaloid Biosynthesis in *Atropa belladonna*

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The tropane alkaloids, hyoscyamine and scopolamine, are medicinal compounds that are the active components of several therapeutics. Hyoscyamine and scopolamine are synthesized in the roots of specific genera of the Solanaceae in a multistep pathway that is only partially elucidated. To facilitate greater understanding of tropane alkaloid biosynthesis, a de novo transcriptome assembly was developed for Deadly Nightshade (*Atropa belladonna*). Littorine is a key intermediate in hyoscyamine and scopolamine biosynthesis that is produced by the condensation of tropine and phenyllactic acid. Phenyllactic acid is derived from phenylalanine via its transamination to phenylpyruvate, and mining of the transcriptome identified a phylogenetically distinct aromatic amino acid aminotransferase (ArAT), designated Ab-ArAT4, that is coexpressed with known tropane alkaloid biosynthesis genes in the roots of *A. belladonna*. Silencing of Ab-ArAT4 disrupted synthesis of hyoscyamine and scopolamine through reduction of phenyllactic acid levels. Recombinant Ab-ArAT4 preferentially catalyzes the first step in phenyllactic acid synthesis, the transamination of phenyllactic to phenylpyruvate. However, rather than utilizing the typical keto-acid cosubstrates, 2-oxoglutarate, pyruvate, and oxaloacetate, Ab-ArAT4 possesses strong substrate preference and highest activity with the aromatic keto-acid, 4-hydroxyphenylpyruvate. Thus, Ab-ArAT4 operates at the interface between primary and specialized metabolism, contributing to both tropane alkaloid biosynthesis and the direct conversion of phenylalanine to tyrosine.

INTRODUCTION

Plants synthesize a variety of chemically diverse specialized metabolites, many of which have defined roles in defense against abiotic or biotic stresses and herbivory or are involved in chemical attraction to facilitate predation, pollination, or seed dispersal (Tewksbury and Nabhan, 2001; Klee, 2010; Klahre et al., 2011; Weinhold and Baldwin, 2011; De Luca et al., 2012; Mithöfer and Boland, 2012; Dudareva et al., 2013). The bioactive properties of plant specialized metabolites have led to their exploitation by humans as flavors, fragrances, pigments, and medicines. One group of compounds, plant alkaloids, are widely used as stimulants, narcotics, and therapeutic agents and includes caffeine, nicotine, cocaine, morphine, scopolamine, vinblastine, and vincristine (Aniszewski, 2007; Gryniewicz and Gadzikowska, 2008; Kingston, 2009). Alkaloids are a structurally heterogeneous family of $\sim12,000$ compounds that are synthesized through diverse pathways and defined by the presence of a heterocyclic nitrogen atom (Ziegler and Fachini, 2008; O’Connor, 2010). The broad utility of alkaloids for humankind has led to considerable interest in understanding the pathways and regulatory mechanisms underlying their synthesis. The development of functional genomics technologies, particularly deep and quantitative transcriptome profiling, has greatly facilitated these efforts (Murata et al., 2008; Liscombe et al., 2009; Desgagné-Penix et al., 2010; Giddings et al., 2011; Farrow et al., 2012; Góngora-Castillo et al., 2012; Wizen et al., 2012). Tropane alkaloids are a class of alkaloids defined by the presence of a bicyclic nitrogen bridge across a seven-carbon ring. Tropane alkaloids are synthesized by several plant families, including the Erythroxylaceae, Proteaceae, and the Euphorbiaceae, but are particularly prevalent in the *Atropa*, *Hyoscyamus*, *Datura*, *Duboisia*, and *Brugmansia* genera of the Solanaceae (Griffin and Lin, 2000; Jirischitzka et al., 2013). Extracts of these Solanaceous species have been used for centuries as medicinals, and the bioactive component, atropine, which is a racemic mixture of D- and L-hyoscyamine, together with scopolamine, are the active ingredients in modern therapeutics used to treat motion sickness, postoperative nausea, arrhythmia, and tremors associated with Parkinson’s disease (Gryniewicz and Gadzikowska, 2008).
The biosynthesis of hyoscyamine and scopolamine (Figure 1) is not completely understood but is initiated by decarboxylation of the nonproteinogenic amino acid ornithine by ornithine decarboxylase to yield the polyamine putrescine (Michael et al., 1996). Putrescine is methylated by putrescine methyltransferase (PMT) to yield N-methylputrescine, which is subsequently converted into 4-methylaminobutanal by N-methylputrescine oxidase (Heim et al., 2007; Katoh et al., 2007; Biastoff et al., 2009). 4-Methylaminobutanal is postulated to undergo spontaneous cyclization to form an N-methyl-Δ5-pyrroline cation, which is ultimately converted to tropinone through an unknown mechanism (O’Connor, 2010; Jirschitzka et al., 2013). Tropinone represents a key branch point in the pathway (Hashimoto et al., 1991; Nakajima et al., 1993). Tropinone reductase II reduces tropinone to pseudotropine, which directly leads to the synthesis of callystegines, a class of polyhydroxylated alkaloids that resemble monosaccharides in structure and act as glycosidase inhibitors (Dräger, 2004). In contrast, tropinone reductase I reduces tropinone to tropine, which leads to the biosynthesis of hyoscyamine and scopolamine. Tropine is then condensed with a phenylactic acid moiety to form littorine, which undergoes P450-mediated rearrangement to hyoscyamine aldehyde (Robins et al., 1994; Li et al., 2006; O’Connor, 2010). An uncharacterized alcohol dehydrogenase is proposed to convert hyoscyamine aldehyde to hyoscyamine, which is subsequently converted to scopolamine in a two-step epoxidation reaction catalyzed by hyoscyamine 6-hydroxylase (H6H) (Matsuda et al., 1991; Hashimoto et al., 1993).

Several of the enzymes and genes involved in tropine alkaloid biosynthesis in the Solanaceae have been identified and characterized, and in all cases, both transcripts and proteins accumulate preferentially or exclusively in roots, the primary site of tropine alkaloid synthesis (Hashimoto et al., 1991; Kanegae et al., 1994; Nakajima and Hashimoto, 1999; Suzuki et al., 1999a). Several steps in tropine alkaloid biosynthesis remain to be identified, including the conversion of 4-methylaminobutanal to tropinone, the formation of phenylactic acid and its condensation with tropine to form littorine, and the identification of the putative alcohol dehydrogenase that converts hyoscyamine aldehyde to hyoscyamine. In addition, the identity of transcriptional regulators and tropine alkaloid transporters remains unknown, although several such proteins involved in the synthesis of the structurally related compound nicotine are now characterized (Shoji et al., 2009, 2010; Todd et al., 2010; Hildreth et al., 2011; Zhang et al., 2012).

As part of a project to develop genomics-based resources for medicinal plants (http://medicinalplantgenomics.msu.edu/), a de novo assembly of the Deadly Nightshade (Atropa belladonna) transcriptome representing 44,198 unigenes, covering a total 33 Mb of the transcriptome, was generated from diverse tissues by RNA sequencing (RNA-seq). In addition, a rapid, targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based method for the simultaneous detection of hyoscyamine, scopolamine, and selected precursors in a single extract was developed, together with a tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) system, to facilitate functional annotation of candidate genes (Ratcliiff et al., 2001; Liu et al., 2002; Dong et al., 2007). These resources were used to identify a phylogenetically distinct, root-expressed aromatic amino acid aminotransferase (ArAT), designated AbArAT4, which preferentially catalyzes the transamination of phenylalanine (l-Phe) to phenylpyruvate, the initial step leading to formation of littorine, a key intermediate in hyoscyamine and scopolamine biosynthesis.

RESULTS

De Novo Assembly of the A. belladonna Reference Transcriptome

Compared with the model crop species of the Solanaceae family, tomato (Solanum lycopersicum), potato (Solanum tuberosum), pepper (Capsicum annuum), and petunia (Petunia hybrida), there is a paucity of genomics-based resources for species of the Hyoscyameae and Datureae tribes that synthesize the tropine alkaloids hyoscyamine and scopolamine. To facilitate gene discovery efforts related to tropine alkaloid biosynthesis and generate nucleotide resources for comparative genomic analysis across the Solanaceae family, a de novo transcriptome assembly of A. belladonna was generated using a combination of paired-end (PE) and single-end (SE) read RNA-seq generated on the Illumina platform (Table 1). RNA from leaves, stems, flowers, roots, and whole fruits of mixed developmental stages from a single reference plant were pooled and used to construct a normalized cDNA library. To maximize recovery of the complete A. belladonna transcriptome, 11 tissues were selected to augment the RNA-seq data. SE RNA-seq reads (Table 1). RNA samples were derived from three pooled biological replicates. Notably, both the normalized cDNA pool for PE sequencing and the individual tissue samples for SE sequencing contained samples extracted from roots, the primary site of tropine alkaloid biosynthesis (Hashimoto et al., 1991; Suzuki et al., 1999a, 1999b).

Using the Oases transcriptome assembler (Schulz et al., 2012), a de novo assembly of the A. belladonna transcriptome was generated. The final assembly compiled from ~110 million reads contained 81,183 assembled sequences with an N50 larger than 1 kb and an average length of 900 bp (Table 2). The Oases algorithm generates isoforms of a locus, which represent true alternative splice forms, alleles, close paralogs, and close homologs. An analysis of the assembled transcriptome revealed 44,198 unigenes (loci), of which, 31,258 loci have a single isoform and 12,940 loci have more than one isoform (Table 2). Low-complexity sequences and possible contaminants were removed, resulting in a total of 80,624 high-quality transcripts, representing 43,951 unigenes (loci). Out of 80,624 high-quality transcripts, a peptide could be predicted from 67,848 using ESTscan (Iseli et al., 1999). Contaminants were defined as sequences derived from a kingdom other than Viridiplantae, such as bacteria, fungi, virus, or arthropods; only a small number of the sequences (539) were removed from the data set, suggesting the high quality of our tissue samples and resulting assembly.

Functional Annotation and Quality Assessment of the Assembly

Gene Ontology Slim (GOSlim) terms (Ashburner et al., 2000) were assigned to 25,614 out of 67,848 predicted peptides. Of these, ~67% (17,336) were categorized into 32 Biological Process categories, ~85% (21,944) into 25 Molecular Function categories (Table 3).
categories, and ~31% (7918) into 23 Cellular Components categories (Supplemental Figure 1). The most represented Biological Processes were cellular process (17.5%), metabolic process (11.7%), and biosynthetic process (11.1%). Nucleotide binding (15.8%), hydrolase activity (14.4%), and catalytic activity (14.1%) were the most prevalent Molecular Functions. For the Cellular Component categories, we observed that over 40% of the sequences were localized to membranes, 15% to the nucleus, and around 12% to the intracellular compartment (Supplemental Figure 1). Overall, the relative composition of the A. belladonna transcriptome over the various functional categories was similar to transcriptomes generated with other closely related Solanaceae species (Rensink et al., 2005).

Functional annotation was assigned using a combination of sequence similarity search results, including UniRef100 (Suzek et al., 2007), the Arabidopsis thaliana proteome (Lamesch et al., 2012), and Pfam domains (Punta et al., 2012). As shown in Figure 2, a total of 66,250 (82.2%) assembled transcripts had a significant match to UniRef100 and 63,522 (78.8%) aligned to the Arabidopsis proteome, whereas Pfam domains were assigned to 42,225 (52%) assembled transcripts. The assembly was further characterized by searching sequence similarity against the 236 known A. belladonna peptide sequences available in GenBank, of which 217 (91.9%) were detected at full length or near full length (>80% coverage) within the transcriptome assembly. Together, these data infer the high quality nature of the A. belladonna transcriptome assembly.

**Orthologous and Paralogous Gene Families**

Clustering of the predicted A. belladonna proteome with the predicted proteomes of potato, tomato, and Arabidopsis (79,565 sequences total) generated a total of 18,519 orthologous and close paralogous clusters of two or more members (Supplemental Data Set 1). Of these, 15,999 clusters contained a total of 19,036 predicted A. belladonna peptides, while 14,271 transcripts were not assigned to any cluster. The majority of the A. belladonna predicted peptides (10,851) were identified in clusters including the four species (Supplemental Figure 2) and the gene families in this group include four to 87 members (Supplemental Data Set 1). A total of 2800 clusters were restricted to Solanaceae species (A. belladonna, tomato, and potato) and are thus termed Solanaceae lineage-specific (Figure 3; Supplemental Data Set 1). The Solanaceae lineage-specific families contained a total of 9702 proteins in gene families of three to 53 members. A sizeable proportion of A. belladonna transcripts (3004) was grouped within the Solanaceae lineage-specific families, including those encoding the known tropane alkaloid biosynthesis enzymes putrescine methyltransferase, ornithine decarboxylase, and tropinone reductase I and II. This grouping is consistent with prior findings that report
a high degree of sequence conservation within the Solanaceae family (Rensink et al., 2005). The OrthoMCL algorithm identifies close paralogs, and 1205 clusters were A. belladonna specific, containing 2681 predicted proteins in gene families of two to 10 members. It is important to note that some of these clusters may represent alternative splice forms from alleles and close paralogs as the Oases assembler may not distinguish between these transcript variants.

**Representation and In Silico Expression Analysis of A. belladonna Transcripts Involved in Tropane Alkaloid Biosynthesis**

Several genes encoding enzymes involved in the synthesis of hyoscyamine and scopolamine have been isolated from Datura, Hyoscyamus, and Atropa spp (Figure 1, Table 3). tBLASTn searches of the A. belladonna transcriptome assembly using each of the known tropane alkaloid biosynthetic enzymes as the query sequence revealed the presence of highly homologous unigenes within the transcriptome (Table 3). For several of these enzymes, predicted full-length transcripts were identified in the A. belladonna transcriptome, but genes whose predicted full-length sequence exceed 1.5 kb (e.g., N-methylputrescine oxidase and littorine mutase) were represented by multiple transcripts, suggesting their incomplete assembly (Table 3).

Tropane alkaloid biosynthesis occurs in the roots of Solanaceae species with the transcripts of known biosynthetic genes preferentially or specifically accumulating in root tissues (Hashimoto et al., 1991; Suzuki et al., 1999a, 1999b). The A. belladonna transcriptome contains three cDNA libraries derived from root tissues: secondary root, primary tap root, and sterile seedlings (Table 2). An analysis of transcript abundance measured by fragments per kilobase of transcript per million fragments mapped indicates that the known genes involved in tropane alkaloid biosynthesis are preferentially expressed in root tissues, with the majority of transcripts displaying highest abundance in secondary roots (Table 3). These data indicate that the de novo assembled A. belladonna transcriptome contains the known transcripts involved in tropane alkaloid biosynthesis and provides an accurate quantitative view of their abundance, indicating that this resource should have utility in identifying novel genes involved in tropane alkaloid biosynthesis and transport.

**Identification of an Aromatic Amino Acid Aminotransferase as a Candidate Tropane Alkaloid Biosynthetic Enzyme**

The condensation of phenyllactic acid with tropine forms littorine, a precursor of hyoscyamine and scopolamine (Figure 1) (Humphrey and O’Hagan, 2001). The phenyllactic acid moiety is derived from L-Phe in a two-step conversion in which L-Phe is transaminated to...
Ab-ArATs that likely perform similar roles in these three species (Figure 4). Ab-ArAT4 is notably more divergent (Supplemental Table 2) and is the lone ArAT in A. belladonna that lacks clear tomato or potato orthologs, as one might expect for an enzyme involved in the synthesis of a species-specific compound class like tropane alkaloids.

The known genes involved in tropane alkaloid biosynthesis possess distinct expression patterns across the A. belladonna tissue panel, with the highest transcript abundances observed in root tissues and little to no expression in non-root tissues (Table 3). Thus, we hypothesized that other pathway genes would follow a similar expression pattern. Examination of ArAT family member expression levels across eleven diverse A. belladonna tissues (Table 4) indicates that only ArAT4 closely matches the expression pattern of known tropane alkaloid biosynthesis genes (Table 3), with moderately high expression in secondary root, primary root, and sterile seedlings and low expression in non-root tissue samples. In contrast, the five other ArAT family members are either expressed constitutively in all tissues (e.g., ArAT2 and 3) or predominantly in above ground tissues (e.g., ArAT1, 5, and 6), and all are expressed at lower levels than ArAT4 in roots. The distinct phylogenetic relationship of Ab-ArAT4 and the similarity of its expression pattern to other tropane alkaloid biosynthetic genes make it a likely candidate for the transaminase leading to phenyllactate, a precursor of littorine (Figure 1).

Ab-ArAT4 Is Required for Tropane Alkaloid Biosynthesis

VIGS is a powerful approach for moderately high-throughput characterization of gene function in plants and is particularly effective in members of the Solanaceae family, including silencing of root expressed genes involved in alkaloid biosynthesis (Liu et al., 2002; Li et al., 2006; Todd et al., 2010). Here, the utility of

The majority of putative plant ArATs remain uncharacterized but the closely related Arabidopsis proteins encoded by At5g36160 and At5g3970, Ps-TyrAT from Papaver somniferum, Ph-PPY-AT from petunia, and Cm-ArAT1 from melon (Cucumis melo) all utilize L-Phe and L-Tyr as amino donors (Prabhu and Hudson, 2010; Lee and Facchini, 2011; Riewe et al., 2012; Yoo et al., 2013). An amino acid alignment of the predicted protein sequence of the Ab-ArAT proteins and selected homologs indicates that each contains a conserved catalytic lysine that corresponds to residue 270 in Ab-ArAT1, together with conserved residues required for the binding of the pyridoxal-5′-phosphate cofactor (Supplemental Figure 3). Ph-PPY-AT was previously shown to be localized in the cytosol rather than the chloroplast (Yoo et al., 2013); similarly, the Ab-ArATs identified in this study are not predicted to be localized to any organelles and lack the typical transit peptides that are characteristic of chloroplast-localized proteins. Phylogenetic analysis of ArATs from Arabidopsis, Atropa, and other Solanaceae, including the fully sequenced tomato and potato genomes, indicated an expansion of the ArAT family in Solanaceae compared with Arabidopsis (Figure 4; Supplemental Table 1) and suggested potential orthologous relationships. For example, subclades A, B, D, and E contain putative orthologs of tomato, potato, and A. belladonna ArATs that likely perform similar roles in these three species (Figure 4). Ab-ArAT4 is notably more divergent (Supplemental Table 2) and is the lone ArAT in A. belladonna that lacks clear tomato or potato orthologs, as one might expect for an enzyme involved in the synthesis of a species-specific compound class like tropane alkaloids.

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Transcript abundance was determined by RNA-seq analysis and data are presented as fragments per kilobase of transcript per million mapped reads.

VIGS for functional genomics in *A. belladonna* was investigated using the TRV two-component system, with constructs assembled in the TRV2 vector by ligation-independent cloning (Dong et al., 2007). Initially, the effectiveness of VIGS in *A. belladonna* was assessed using constructs targeting *PHYTOENE DESATURASE* (PDS) and *ACTIN*. The characteristic photochemical phenotype of PDS-silenced plants (Liu et al., 2002) was consistently observed 10 d after infiltration, with phenotypes increasing in severity in plants 3 weeks postinfiltration (Supplemental Figure 4). Similarly, *ACTIN*-silenced plants displayed a stunted phenotype compared with the TRV2 empty vector controls similar to that previously described in *Nicotiana benthamiana* (Ryu et al., 2004) (Supplemental Figure 4).

In addition, VIGS was performed to silence *PMT* following by *PHYTOENE DESATURASE* (*PDS*), the two most highly expressed family members, *ArAT2* and *ArAT6*, which are the two most highly expressed family members involved in catalyzing early and late steps in the biosynthesis of scopolamine, respectively (Hashimoto et al., 1991; Matsuda et al., 1991; Blastoff et al., 2009; Figure 1). Silencing of *PMT* resulted in a 77 and 66% reduction in hyoscyamine and scopolamine levels, respectively (Figure 5A). Congruent with the reduction in hyoscyamine and scopolamine, phenylacetic acid was reduced by ~95% in the *ArAT4*-silenced plants, whereas tropine, which condenses with phenyllactate to form litorine (Figure 1), was increased ~4-fold (Figure 5A). However, the levels of L-Phe and phenylpyruvate were not substantially altered by *ArAT4* silencing. In addition, tropine was reduced in the *ArAT4*-silenced plants by ~50%. *ArAT4* transcripts were monitored in root tissues of the silenced plants by quantitative RT-PCR analysis, revealing a 52% reduction in *TRV2:AbArAT4* plants compared with the TRV2 empty vector controls (Figure 5B).

To assess the possibility that off-target cosilencing might affect tropane alkaloid biosynthesis, expression of the other *ArAT* family members was assessed in the *TRV2:ArAT4*-silenced plants relative to TRV2 empty vector controls. Cross-silencing within the *Ab-ArAT* gene family was found to be minimal to nonexistent in the *TRV2:ArAT4*-silenced plants. In particular, expression of *ArAT2* and *ArAT3*, which are the two most highly expressed family members after *ArAT4* in the roots of *A. belladonna* (Table 4), was not altered by *ArAT4* silencing (Figure 5B). Of the remaining three weakly expressed family members, *ArAT1* expression was unaltered, while *ArAT5* and *ArAT6* expression was slightly but significantly reduced.

### Table 3. Identification and Expression Analysis of *A. belladonna* Unigenes Involved in Tropane Alkaloid Biosynthesis

<table>
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<tr>
<th><em>A. belladonna</em> Unigene Identifier</th>
<th>Annotation</th>
<th>Callus</th>
<th>Leaf</th>
<th>Stem</th>
<th>Seedling</th>
<th>Primary Root</th>
<th>Secondary Root</th>
<th>Flower Buds</th>
<th>Flowers</th>
<th>Green Fruit</th>
<th>Ripe Fruit</th>
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<td>886.4</td>
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<td>178.1</td>
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<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Transcript abundance was determined by RNA-seq analysis and data are presented as fragments per kilobase of transcript per million mapped reads.
Ab-ArAT4 and Tropane Alkaloid Biosynthesis

Ab-ArAT4 Possesses Phenylalanine:4-Hydroxyphenylpyruvate Aminotransferase Activity

The reported activity of several ArATs indicates they can use L-Tyr, L-Phe, and L-Trp to varying degrees as amino donors, with 2-oxoglutarate generally being the preferential amino acceptor (Prabhu and Hudson, 2010; Lee and Facchini, 2011; Riewe et al., 2013). The kinetic parameters of purified recombinant Ab-ArAT4 were assessed using the three aromatic amino acids as amino donors together with several potential oxoacid acceptors as substrates (Table 5). The potential substrates and products of these reactions are presented in Supplemental Figure 6.

A. belladonna Ab-ArAT4 displayed typical Michaelis-Menten kinetics with $K_m$ for the potential amino donors L-Phe, L-Tyr, and L-Trp of 2.1, 4.1, and 19.5 mM, respectively (Table 5; Supplemental Figure 7). Of the oxoacid acceptors tested 4-hydroxyphenylpyruvate (4-HPP), oxaloacetate, and phenylpyruvate were similar at ~0.5 mM, while 2-oxoglutarate and pyruvate were 5.2 and 18.7 mM, respectively. It was surprising that 2-oxoglutarate, which is typically the preferred cosubstrate of many characterized ArATs, was such a poor cosubstrate for Ab-ArAT4. When catalytic efficiency ($k_{cat}/K_m$) was measured, that for L-Phe and 4-HPP was at least an order of magnitude higher than any other oxoacid and amino donor pair, indicating these are the preferred enzyme cosubstrates. Consistent with the primary role of ArAT4 being to channel L-Phe toward scopolamine biosynthesis, the relative maximum activity of the forward reaction using L-Phe as the amino donor and 4-HPP as the amino acceptor to generate phenyllactic acid and L-Tyr was ~250-fold higher than the reverse reaction. When utilizing L-Phe and 4-HPP as substrates, the activity of ArAT4 was largely insensitive to changes in pH, with a broad optimum between 6.0 and 9.0 and a $t_{1/2}$ (temperature at which 50% of activity is lost) of ~59°C (Supplemental Figure 8).

Overall, and congruent with its role in tropane alkaloid biosynthesis, these data indicate that Ab-ArAT4 preferentially catalyzes the transamination of L-Phe to form phenylpyruvate, which is diverted toward scopolamine biosynthesis. However, unexpectedly, Ab-ArAT4 has a strong preference for 4-HPP as the preferred amino acceptor rather than 2-oxoglutarate.

**Table 4. ArAT Transcript Abundance (Fragments per Kilobase of Transcript per Million Mapped Reads) in Select A. belladonna Tissues as Determined by RNA-seq Analysis**

<table>
<thead>
<tr>
<th>A. belladonna Unigene Identifier</th>
<th>Gene Name</th>
<th>Tissue Type</th>
<th>Primary Root</th>
<th>Secondary Root</th>
<th>Flower Buds</th>
<th>Flowers</th>
<th>Green Fruit</th>
<th>Ripe Fruit</th>
<th>Seed</th>
</tr>
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<tbody>
<tr>
<td>aba_locus_11780_iso_1_len_1427_ver_2</td>
<td>ArAT1</td>
<td>Callus</td>
<td>2.7</td>
<td>15.2</td>
<td>1.3</td>
<td>7.1</td>
<td>0.7</td>
<td>0.7</td>
<td>3.8</td>
</tr>
<tr>
<td>aba_locus_3474_iso_1_len_1708_ver_2</td>
<td>ArAT2</td>
<td>Leaf</td>
<td>28.3</td>
<td>38.4</td>
<td>47.1</td>
<td>43.1</td>
<td>50.8</td>
<td>85.2</td>
<td>41.1</td>
</tr>
<tr>
<td>aba_locus_4922_iso_1_len_1502_ver_2</td>
<td>ArAT3</td>
<td>Stem</td>
<td>10.4</td>
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<td>15.8</td>
<td>18.7</td>
<td>11.1</td>
<td>33.6</td>
<td>16.2</td>
</tr>
<tr>
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<td>ArAT4</td>
<td>Seedling</td>
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<td>0.0</td>
<td>0.0</td>
<td>30.6</td>
<td>12.2</td>
<td>168.1</td>
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<td>0.0</td>
<td>16.9</td>
<td>6.5</td>
<td>64.4</td>
<td>2.0</td>
</tr>
<tr>
<td>aba_locus_16374_iso_1_len_467_ver_2</td>
<td>ArAT6</td>
<td>Stem</td>
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<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<td>4.2</td>
<td>29.1</td>
</tr>
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<td>Leaf</td>
<td>2.4</td>
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<td>0.0</td>
<td>1.9</td>
<td>0.0</td>
<td>1.8</td>
<td>8.1</td>
</tr>
</tbody>
</table>

(Figure 5B; Table 4). To directly test whether these other ArAT family members are involved in tropane alkaloid biosynthesis, the impact of silencing each gene on scopolamine levels was investigated (Supplemental Figure 5). Whereas silencing of ArAT4 reduced scopolamine levels by 66% (Figure 5A), silencing of the other five ArATs did not affect scopolamine levels in A. belladonna roots (Supplemental Figure 5). Together, these data allow us to conclude that ArAT4 is the predominate ArAT involved in tropane alkaloid biosynthesis in A. belladonna.
acid homeostasis, particularly the levels of the aromatic amino acids. To test this hypothesis, the abundance of the proteinogenic amino acids was determined in TRV2 and the TRV2:ArAT4 VIGS lines (Supplemental Table 3). Although statistically significant increases in arginine and lysine levels were observed in the ArAT4-silenced lines compared with TRV2 empty vector controls, overall there was little change in amino acid abundance and no statistically significant change in the abundance of the aromatic amino acids. Together, these data indicate that although ArAT4 channels L-Phe toward tropane alkaloid biosynthesis and simultaneously synthesizes L-Tyr from 4-HPP, flux through this pathway is insufficient to perturb aromatic amino acid homeostasis.

DISCUSSION

The Solanaceae is a diverse plant family of ~90 genera and 3000 species that occupy a broad geographic range and includes plants of economic importance as food, ornamental, and medicinal crops (Knapp et al., 2004). In addition, members of the Solanaceae serve as model systems for investigating fundamental processes involved in plant growth and development, including the ripening of fleshy fruit (tomato), tuber formation (potato), pollination and petal senescence (petunia), plant pathogen and pest interactions (tomato, potato, and Nicotiana spp), and the biosynthesis of specialized metabolites (tomato, pepper, Nicotiana, Hyoscyamus, Datura, and Atropa spp). The development of genomics tools, including genome sequences and transcriptome data sets for several of these model species, is facilitating characterization of these biological processes (Xu et al., 2011; Ashrafi et al., 2012; Bombarely et al., 2012; Tomato Genome Consortium, 2012; Kim et al., 2014; Qin et al., 2014). In this study, a deep transcriptome of A. belladonna, a member of the Solanaceae family often used for investigating tropane alkaloid biosynthesis (Suzuki et al., 1999a, 1999b; Rothe et al., 2003; Richter et al., 2005), was generated from diverse tissues, facilitating in silico quantitative analyses of gene expression (Tables 1 to 3). The A. belladonna transcriptome contains sequences of all known genes involved in tropane alkaloid biosynthesis and confirms that they are preferentially expressed in roots, with the highest transcript abundance observed in secondary roots (Suzuki et al., 1999a, 1999b; Li et al., 2006). The utility of the A. belladonna transcriptome for identifying enzymes involved in tropane alkaloid biosynthesis was demonstrated through the identification and characterization of Ab-ArAT4, an ArAT that possesses a novel L-Phe:4-HPP transaminase activity and channels L-Phe toward the synthesis of scopolamine (Figure 7).

In general, primary metabolism is largely conserved in plants, whereas specialized metabolism, which evolved from primary metabolism, is highly variable and gives rise to the myriad of chemical compounds synthesized within the plant kingdom (Weng et al., 2012). In addition to their role in protein synthesis, in plants, the aromatic amino acids L-Phe and L-Tyr are incorporated into the structural polymers lignin, cutin, and suberin and a wide range of specialized metabolites, including flavonoids, volatiles, alkaloids, and tocopherols (Maeda and Dudarova, 2012). In plants, L-Phe and L-Tyr are synthesized in plastids from prephenate, which is derived from chorismate formed through the shikimate pathway. Prephenate is aminated to form arogenate, which is subsequently converted to L-Tyr through the action of arogenate dehydrogenase.
and L-Phe by arogenate dehydratase (Dal Cin et al., 2011; Maeda et al., 2011; Maeda and Dudareva, 2012). However, plant genomes have retained multiple ArATs that likely utilize L-Phe and L-Tyr as substrates but, like Ab-ArAT4, are not predicted to be plastid localized. Recently, a petunia ArAT, Ph-PPY-AT, was shown to be localized to the cytoplasm and able to directly catalyze the formation of L-Phe from phenylpyruvate using L-Tyr as the amino donor, yielding 4-HPP as a by-product (Yoo et al., 2013). This reaction is analogous to the formation of L-Phe from phenylpyruvate in bacteria and demonstrates the existence of a second, direct, extraplastidic route to L-Phe biosynthesis in plants and led to a proposed role for Ph-PPY-AT in modulating aromatic amino acid homeostasis (Whitaker et al., 1981; Fischer et al., 1993; Yoo et al., 2013). In contrast, our data support a direct role for Ab-ArAT4 only in specialized metabolism, as silencing of this gene reduces tropane alkaloid accumulation but does not affect the abundance of the aromatic amino acids (Figure 5; Supplemental Table 3).

The lack of impact on primary metabolism as a result of silencing AbArAT4, and particularly the finding that L-Phe, L-Tyr, and phenylpyruvate levels are unaltered, despite a 95% reduction in phenyllactate abundance (Figure 5; Supplemental Table 3), may be due to several factors. For example, at least 30% of photosynthetically fixed carbon is devoted to the production of L-Phe, which is subsequently channeled toward the synthesis of structural polymers and specialized metabolites (Razal et al., 1996; Maeda and Dudareva, 2012). In contrast, tropane alkaloids typically accumulate to <1% of dry weight in Solanaceous species (Griffin and Lin, 2000). Therefore, disruption of the relatively small amount of L-Phe flux that is typically channeled toward tropane alkaloid biosynthesis, by silencing of Ab-ArAT4, may be insufficient to produce a difference in the steady state levels of L-Phe or phenylpyruvate pools. Furthermore, aromatic amino acid biosynthesis is highly regulated and compensatory mechanisms may be enacted in the ArAT4 VIGS lines that subsequently limit...
changes in the steady state pools of L-Phe and L-Tyr. Indeed, the proposed role of Ph-PPY-AT in aromatic amino acid homeostasis was only uncovered in a genetic background in which the typical chloroplast localized L-Phe biosynthesis pathway was silenced (Yoo et al., 2013). Feedback inhibition of L-Phe biosynthesis and/or diversion of L-Phe to other metabolites also represent potential mechanisms that could suppress free L-Phe accumulation in Arabidopsis silenced lines. For example, L-Phe biosynthesis is subject to feedback inhibition of chorismate mutase and arogenate dehydratase (Jung et al., 1986; Eberhard et al., 1996). In addition, attempts to overproduce L-Phe in transgenic lines and the characterization of mutations in regulatory domains of L-Phe biosynthesis genes result in diversion of L-Phe toward additional aromatic acid-derived specialized metabolites (Tzin et al., 2009; Huang et al., 2010). These data are consistent with the high degree of regulation of aromatic amino acid synthesis in primary metabolism operating to minimize changes to the free pools of L-Phe and L-Tyr in Arabidopsis silenced lines.

Although silencing of Arabidopsis did not affect the abundance of the aromatic amino acids in A. belladonna roots, a modest but significant increase in both arginine and lysine was observed (Supplemental Table 3). The biological significance of these changes remains to be elucidated, but elevated arginine levels may reflect altered flux through to this metabolite pool due to the decreased utilization of ornithine, which is produced from arginine, in tropane alkaloid synthesis as a result of Arabidopsis silencing (Slocum, 2005). Similarly, while reduced levels of phenylpyruvate, hyoscyamine, and scopolinamide and increased tropane content were expected in Arabidopsis silenced lines, an unexpected reduction in tropane levels was also observed (Figure 5A). This suggests the presence of a previously unrecognized feedback loop in tropane biosynthesis. The mechanism through which this may occur is unknown but may involve tropane-mediated inhibition of enzymes involved in tropane biosynthesis, the identity of which are still unknown.

The mechanisms that underlie the evolution of specialized metabolism are diverse but frequently involve gene duplication or gene family expansion, coupled with subfunctionalization or neofunctionalization that may influence the expression pattern of a given gene or the activity of a protein or enzyme (Pichersky and Lewinsohn, 2011; Shoji and Hashimoto, 2011; Niemüller et al., 2012; Weng et al., 2012; Kaltenegger et al., 2013; Matsuba et al., 2013; Kang et al., 2014). Relative to Arabidopsis, there is an expansion of the ArAT gene family in A. belladonna, tomato, and potato, with two or three ArAT genes present in these species for each Arabidopsis gene (Figure 4). Phylogenetic analysis indicates that Ph-PPY-AT is likely orthologous to Arabidopsis. These two proteins share ~90% amino acid identity and reside within clade B, together with proteins from other members of the Solanaceae (Figure 4). In contrast, Arabidopsis is phylogenetically distinct, lacks obvious orthologs in tomato and potato, and on average shares ~67% identity to the proteins in clade B (Figure 4; Supplemental Table 2). Furthermore, Arabidopsis possesses a distinct root preferential expression pattern that closely matches other tropane alkaloid biosynthesis genes (Tables 3 and 4). The other five A. belladonna ArATs do not display the characteristic root-preferential expression pattern of Arabidopsis (Table 4) and either exhibit very low expression in roots (ArAT1, ArAT5, and ArAT6) or are constitutively expressed in all the tissues examined (ArAT2 and ArAT3). Whereas Arabidopsis is required for tropane alkaloid biosynthesis (Figure 5), silencing of the five additional Arabidopsis did not affect scopolamine levels (Supplemental Figure 5).

Despite the phylogenetic and sequence divergence of Arabidopsis and Ph-PPY-AT, both enzymes utilize the same compounds, but whereas Ph-PPY-AT preferentially utilizes L-Tyr and PPy as cosubstrates to catalyze the formation of L-Phe and 4-HPP (Yoo et al., 2013), Arabidopsis preferentially catalyzes the reverse reaction, performing transamination of L-Phe to form phenylpyruvate with 4-HPP as the cosubstrate to yield L-Tyr (Table 5). Diversity in the catalytic properties of Ph-PPY-AT and Arabidopsis also support their proposed roles in primary and specialized metabolism, respectively. For example, in addition to their preferred substrates, Ph-PPY-AT and Arabidopsis are also active with additional aromatic amino acids as amino donors and various keto-acids and amino acceptors (Tables 5; Yoo et al., 2013). However, while Ph-PPY-AT exhibits $V_{max}$ and $K_{cat}/K_m$ values for different substrate pairings that are similar to one another (Yoo et al., 2013), Arabidopsis has a clear preference for L-Phe and 4-HPP and possesses $V_{max}$ and $K_{cat}/K_m$ values with these substrates that are one to more than two orders of magnitude greater than those observed with other substrate pairings (Table 5). In addition, the $V_{max}$ of the forward and reverse reactions catalyzed by Ph-PPY-AT exhibit only a 2-fold difference, whereas Arabidopsis has ~250-fold greater preference for the forward over the reverse reaction (Yoo et al., 2013; Table 5). This increased potential for reversibility supports
the proposed role of Ph-PPY-AT in maintaining aromatic amino acid homeostasis, while the more specialized, predominantly unidirectional activity of Ab-ArAT4 channels L-Phe, and subsequently phenylpyruvate, toward tropane alkaloid biosynthesis (Figure 5).

Overall, the kinetic properties of Ab-ArAT4, together with its distinct transcript accumulation, divergent phylogenetic separation, and absence of a clear ortholog in members of the Solanaceae that do not produce scopolamine, suggest that this enzyme likely evolved from an ancestral enzyme involved in primary metabolism to a highly specialized enzyme with a dedicated role in tropane alkaloid biosynthesis. The activity and in vivo role of the majority of the ArATs phylogenetically related to Ab-ArAT4 and Ph-PPY-AT (Figure 4) remain unknown, but in light of the characterization of these two enzymes, it is possible that their homologs also utilize aromatic keto-acids as substrates rather than traditional keto-acids, such as 2-oxoglutarate. Therefore, it may be prudent to reexamine

Ab-ArAT4 Acts at the Interface of Tropane Alkaloid Biosynthesis and Aromatic Amino Acid Metabolism.

Ab-ArAT4 participates in the catabolism of L-Phe to direct phenylpyruvate (PPY) and phenyllactate toward tropane alkaloid biosynthesis and simultaneously catalyzes the anabolism of L-Tyr. The characterized enzymes of aromatic amino acid metabolism are shown in bold. Ab-ArAT4, *A. belladonna* Aromatic Aminotransferase 4; CM, chorismate mutase; ADH, arogenate dehydrogenase; ADT, arogenate dehydratase; PPA-AT, prephenate aminotransferase.

**Figure 6.** L-Phenylalanine:4-Hydroxyphenylpyruvate Aminotransferase Activity in ArAT4-Silenced Lines.

**(A)** Summary of the preferred reaction of ArAT4.

**(B)** ArAT activity was determined through monitoring the production of L-Tyr using L-Phe and 4-HPP as substrates in reaction mixes containing crude protein extracted from TRV2 and TRV2:ArAT4 VIGS lines. Data are presented as the mean of three technical replicates derived from a pooled set of three biological replicates for each genotype, ± se. Asterisks denote significant differences (**P < 0.01) as determined by Student’s t test.

**Figure 7.** Ab-ArAT4 Acts at the Interface of Tropane Alkaloid Biosynthesis and Aromatic Amino Acid Metabolism.

Ab-ArAT4 participates in the catabolism of L-Phe to direct phenylpyruvate (PPY) and phenyllactate toward tropane alkaloid biosynthesis and simultaneously catalyzes the anabolism of L-Tyr. The characterized enzymes of aromatic amino acid metabolism are shown in bold. Ab-ArAT4, *A. belladonna* Aromatic Aminotransferase 4; CM, chorismate mutase; ADH, arogenate dehydrogenase; ADT, arogenate dehydratase; PPA-AT, prephenate aminotransferase.

[See online article for color version of this figure.]
the kinetic properties of ArATs previously determined to exhibit maximal activity with 2-oxoglutarate as the cosubstrate to assess their activities with aromatic keto-acids (Prabhu and Hudson, 2010; Lee and Facchin, 2011; Riewe et al., 2012).

Tropane alkaloids are synthesized by several plant families and the structure of the tropane ring is conserved (Griffin and Lin, 2000). However, recent evidence suggests independent evolution of pathways leading to the reduction of tropinone in the Solanaceae and Erythroxylaceae (Jirschitzka et al., 2012). Decoration of the tropine skeleton by a phenyllactate molecule to form littorine, and subsequently hyoscyamine and scopolamine, is a reaction that predominantly occurs in specific genera of the Solanaceae, and the identity of the enzymes involved in these conversions are largely unresolved (Griffin and Lin, 2000; Humphrey and O’Hagan, 2001). Transcriptomics, coupled with silencing, metabolite, and biochemical analysis, identified a specialized role for ArAT4 in diverting L-Phe, via phenyllactate, into hyoscyamine and scopolamine in A. belladonna, establishing the identity of the first enzyme in this branch of the pathway. There have been many attempts to engineer increased production of hyoscyamine and scopolamine, particularly through overexpression of PMT and H6H (Moyano et al., 2003; Rothe et al., 2003; Zhang et al., 2004), which participate in early and late steps of the pathway, respectively (Figure 1). The identification of Ab-ArAT4 as the first step in the pathway that provides the phenyllactate moiety for the formation of littorine (Figure 1) provides a tool to potentially manipulate tropane alkaloid levels in the Solanaceae. For example, combining ArAT4 overexpression with previous transgenic strategies may increase the flux of metabolites through this branch of the pathway leading to increased availability of phenyllactate for subsequent condensation with tropine to form littorine (Figure 1). The tremendous flux that exists through L-Phe (Razal et al., 1996) suggests that it may be possible to divert more of this primary metabolite toward the production of phenyllactate to increase tropane alkaloid levels. In addition, the transcriptome resources generated through this research will facilitate additional characterization of tropane alkaloid biosynthesis in the Solanaceae.

METHODS

Plant Material and Growth Treatments

Seeds of Deadly Nightshade (Atropa belladonna) were purchased from Horizon Herbs. Seeds were surface sterilized with 50% bleach solution for 5 min followed by extensive washing with sterile water and were pre-treated with gibberellic acid (60% gibberellin A$_3$ at 0.1% w/v) for 24 hr prior to germination on moistened filter paper. Following radicle emergence, seedlings were transplanted into Jiffy Peat Pellets and grown at 22°C under 16-h-day/8-h-night cycle at 120 molm$^{-2}$s$^{-1}$ until the emergence of the sixth true leaf, after which they were transplanted into C900 pots (Nursery Supplies) and grown in Bacclo High Porosity Professional Planting Mix (Michigan Peat Company) supplemented with 125 ppm 14-3-14-7Ca-1Mg fertilizer (Greencare Fertilizer) at 20°C in greenhouses at Michigan State University, East Lansing, MI. Sterile seedlings were grown for 14 d on half-strength Murashige and Skoog modified basal medium with Gamborg vitamins, pH 5.7, containing 1.5% w/v sucrose and 0.6% w/v agar at 23°C under an 18-h-day/6-h-night cycle using a 50 μmol fluorescent light source. Callus was induced from hypocotyls of sterile seedlings on TCM2 media (Murashige and Skoog modified basal medium with Gamborg vitamins, pH 5.7, 1.5% w/v sucrose, 1.0 mg/L 6-benzylaminopurine, 1.0 mg/L naphthaleneacetic acid, and 0.6% w/v agar). Plates were incubated as described for growth of sterile seedlings and callus was harvested after 4 weeks.

RNA Isolation, cDNA Synthesis, and Normalization

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen), and RNA quality and quantity was determined using the 2100 Bioanalyzer (Agilent Technologies); 1 μg of total RNA isolated from leaves, stems, roots, flowers of mixed developmental stages, and whole fruits of mixed developmental stages containing seeds was pooled and used for cDNA synthesis using the SMART cDNA Library Construction Kit (Clontech Laboratories). cDNA was normalized to reduce high abundance transcripts using the duplex-specific nuclease-based normalization technology (Evrogen) (Zhuklev et al., 2004).

Library Construction, Transcriptome Sequencing, and Sequence Processing

Normalized cDNA was digested with SfiI to remove the adaptor sequences used in the normalization process, sheared, and size selected (average ~ 408 bp); 2.5 μg was utilized for library construction using the Paired-End DNA Sample Prep Kit (Illumina). In order to capture quantitative information of the A. belladonna transcriptome, RNA samples (10 μg) pooled from three biological replicates extracted from 11 diverse tissue samples were converted to cDNA using the mRNA-Seq kit (Illumina). Cluster generation was performed using the Illumina Cluster Station with size selected (range 235 to 410 bp) cDNA fragments and sequenced using the Illumina Genome Analyzer Ix platform at the Michigan State University Research Technology Support Facility Genomics Core. Sequence reads were cleaned and filtered using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html); *fastx_adapter_filter,* *“fastx_artifacts_filter,”* and *“fastx_quality_trimmer”* programs were used to trim and remove low-quality sequences using a minimum quality score of 20. After cleaning and filtering paired-end sequences, custom Perl scripts were used to filter single reads for use as single-end sequences.

De Novo Assembly Strategy

A de novo assembly using the quality filtered reads was conducted using the Oases transcriptome assembler v0.1.21 (Zerbino and Birney, 2008; Schultz et al., 2012). To optimize the assembly, Oases was run using k-mer lengths of 27, 29, 31, and 33. Metrics such as number of transcripts, number of transcripts longer than 250 bp, N50 contig length, maximum transcript length, and average transcript length were used to determine the optimal assembly.

To avoid increasing the number of redundant sequences in the final assembly, an iterative approach to de novo assembly was used. In the initial de novo assembly, two lanes of 55 nucleotide PE reads derived from the normalized PE library were assembled (k-mer length = 31, coverage cutoff = 4, and minimum contig length = 250 bp). This assembly was then used as a reference sequence for the A. belladonna core transcriptome by constructing an artificial genome in which contigs were concatenated into a single pseudomolecule. Redundant reads in the single tissue libraries were identified by mapping the 36 nucleotide SE reads to the first assembly using TopHat (v 1.2) (Trapnell et al., 2009) with default parameters. Using the unmapped reads from the single tissue libraries, a second de novo assembly was then performed using the PE reads together with the unmapped SE reads (k-mer length = 27, coverage cutoff = 4, and minimum contig length = 250 bp).

To leverage the PE read information and join fragmented contigs, a final assembly was then made using the “-long” Velvet option (long,
$k$-mer length = 31, coverage cutoff = 4, and minimum contig length = 250 bp) with the normalized PE library reads and the assembled contigs. Contigs in the final assembly were filtered to remove low-complexity sequences. Putative contaminants in the assembly were identified using BLAST searches against UniRef100 (Suzek et al., 2007) and removing contigs that were similar to non-plant sequences. A. belladonna protein sequences (339 peptides) from GenBank were used to quantify the quality and coverage of the assembly. Transcripts were concatenated into a single pseudomolecule, and expression abundances (fragments per kilobase of transcript per million fragments mapped) were determined in the transcriptome assembly for the 11 single tissue libraries in Table 1 as described by Gongora-Castillo et al. (2012).

Functional Annotation
Functional annotation of assembled transcripts was assigned using WU-BLAST (Chao et al., 1992) by in previously described except that silencing experiments were performed 1998; Quevillon et al., 2005) with an E-value HmmPfam tool from the HMMER package (v 2) (Krogh et al., 1994; Eddy, 1998; 2009) with an E-value cutoff of 1e-10. Pfam domains (Finn et al., 2010) were identified using HMMPfam from the HMMER package (v 3) (Krogh et al., 1994; Eddy, 1998; 2009) with an E-value cutoff of 1e-10. Gene Ontology (GO) terms (Harris et al., 2004) were assigned to the 80,636 high-quality assembled transcripts using the HmmPfam tool from the HMMER package (v 2) (Krogh et al., 1994; Eddy, 1998; Quevillon et al., 2005) with an E-value ≤ 1e-10. GO terms were mapped to GO slim terms using custom Perl scripts and the Map2Slim script from the go-perl (v 0.13) package (http://www.geneontology.org/GO.slims.shtml).

Orthologous and Paralogous Gene Family Analysis
OrthoMCL was used to identify orthologous and close paralogous genes using the default parameters (Li et al., 2003; Chen et al., 2007) and an E-value cutoff of 1e-10. To eliminate false grouping of paralogs due to alternative isoforms, a representative transcript defined as the model that produces the longest peptide sequence was used in the orthologous clustering. Transposable elements for each species were filtered out to avoid clusters composed solely of transposable elements.

Multiple Sequence Alignments and Phylogenetic Analyses
Sequence analysis was performed using MEGA version 5 (Tamura et al., 2011). Amino acid alignments were performed using MUSCLE (Edgar, 2004) and non-rooted phylogenetic trees constructed using the Maximum Likelihood method and the Jones-Taylor-Thornton model using default parameters. A bootstrap test of 2000 replicates was used to assess the reliability of the phylogeny. Estimates of evolutionary distance, p-distance, were calculated from an alignment constructed in MUSCLE. The amino acid alignment used to construct the phylogenetic tree in Figure 4 is available as Supplemental Data Set 2.

VIGS
VIGS experiments were performed using the two-component TRV system with target gene constructs assembled by ligation independent cloning in the TRV2-LIC vector (Ratcliff et al., 2001; Dong et al., 2007). PCR fragments of A. belladonna PDS, ACTIN, PMT, H6H, and ArATs were amplified from cDNA synthesized from RNA extracted from either the secondary roots or sterile seedlings of A. belladonna using primer pairs described in Supplemental Table 4. Recombinant clones were confirmed using a combination of PCR verification and DNA sequence analysis. Constructs were transferred into Agrobacterium tumefaciens strain GV3101 and bacterial growth, processing, and infiltration conditions were identical to those previously described except that silencing experiments were performed by infiltrating Agrobacterium cultures into the cotyledons of 3-week-old A. belladonna seedlings (Velasquez et al., 2009). Following infiltration, plants were grown in a growth room at 22°C with a 16-h photoperiod at a light intensity of 120 μmol in Sunshine Redi-earth Plug and Seeding Mix (Sun Gro Horticulture). Tissue samples for alkaloid extractions were harvested 4 weeks after infiltration, frozen in liquid N₂, and stored at −80°C.

Quantitative RT-PCR
RNA was extracted from root tissues as described above with inclusion of an on-column DNase treatment. Synthesis of cDNA was accomplished using the SuperScript III first-strand synthesis kit (Invitrogen) and 1 μg of RNA template. Gene-specific primers for Ab-ArATs, and the endogenous control elongation factor 1 (EF1; Supplemental Table 4), were designed using Primer Express 3.0 software (Applied Biosystems). PCR efficiency was determined for each set of primers using standard curves derived from serial dilutions of cDNA. Quantitative PCR was performed in 10-μL reactions using FAST SYBR master mix (Applied Biosystems) with 300 nM of each primer, 20 ng of cDNA template for ArAT4, and 80 ng of cDNA template for all other ArATs tested. Reactions were assembled using a Biomek 3000 liquid handler and DNAs amplified using an Applied Biosystems ABI Prism 7900HT real-time PCR system using the following program: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were analyzed using StepOne software, calculating average threshold cycle (CT) values and ΔCT mean for all ArATs-silenced and TRV2 controls using six biological and three technical replicates of each line. The EF1 gene was used as an internal standard for normalization. Values were normalized and 2^(-ΔΔCT) calculated to determine relative transcript levels of the genes.

Metabolite Extractions
Metabolites were extracted from frozen powdered root tissue using 0.1 g of tissue per 1 mL of 20% methanol, containing 0.1% formic acid and 10 μM propyl 4-hydroxybenzoate as an internal standard. Samples were vortexed and placed horizontally on an orbital shaker for 3 h at room temperature. Extracts were centrifuged for 30 min at room temperature and the supernatant transferred to vials for analysis by LC-MS/MS. The tissue pellets were dried at 37°C for 24 h, and the dry weight of the plant material calculated through retrieving the difference between the weights of the extraction tubes before and after the extraction.

Identification and Quantification of Tropane Alkaloids and Intermediate Compounds
The identification and quantification of tropane alkaloids and select precursors was accomplished by LC-MS/MS using a modified version of a previously described protocol for amino acid analysis (Gu et al., 2007). A Waters Quattro Micro API mass spectrometer coupled to a Shimadzu LC-20AD HPLC and SIL-5000 autosampler was utilized along with a Waters Symmetry C18 column (2.1 × 100 mm, 3.5-μm particle size) at a 50°C oven temperature. HPLC was performed using a 10-μL injection volume with a flow rate of 0.4 mL/min and a gradient ranging from 99:1 to 5:95 1 mM aqueous perfluorohexanoic acid (solvent A) and acetoneitrile (solvent B; Supplemental Table 5). Quantitative analyses were performed using electrospray ionization in positive ion mode with multiple reaction monitoring (Supplemental Table 6). Capillary voltage, extractor voltage, and radio frequency lens settings were 3.17 kV, 2 V, and 0.1, respectively. Flow rates of cone gas and desolvation gas were 30 and 600 L/h, respectively with the source temperature at 100°C and desolvation temperature at 350°C. Argon was used as the collision gas for collision-induced dissociation at a manifold pressure of 2 × 10^-3 mbar, with collision energies and source cone potentials optimized for each metabolite using Waters QuanOptimize software. A series of calibration standards and blanks were analyzed with each sample set.
Phenylpyruvic acid and ω-3-phenylactic acid were detected in extracts of *A. belladonna* by LC-MS/MS in negative ionization mode using an Applied Biosystems MDS SCIEX 3200 QTRAP mass spectrometer equipped with a Shimadzu LC-20AD HPLC pumps. Compounds were separated by HPLC using an Ascentis Express C18 column (2.1 × 100 mm, 2.7-μm particles) at a 40°C oven temperature with a 10-μL injection volume and a flow rate of 0.4 mL/min on a gradient ranging from 98:2 to 0:100 of 0.15% aqueous formic acid (solvent A) to methanol (solvent B; Supplemental Table 7). Measurements were performed using electrospray ionization in negative ion mode and optimized MRM transitions (Supplemental Table 8). Instrument parameters were as follows: ion spray voltage, 3750 V; source temperature, 550°C; ion source gas 1, 13; and ion source gas 2, 10. Data were processed and quantified using Applied Biosystems Analyst v. 1.4.2 software. The final concentration of metabolites was adjusted for the internal standard and calculated using standard curves of authentic compounds and normalized to dry weight.

Phenylpyruvate produced in enzyme assays using purified recombinant ArAT4 was detected by LC-MS/MS in negative ionization mode using a Waters Quattro Premier XE mass spectrometer equipped with an Acquity UPLC pump and autosampler. Phenylpyruvate was separated by HPLC using an Acquity BEH C18 column (2.1 × 100 mm, 1.7-μm particles) at a 50°C oven temperature with a 10-μL injection volume and a flow rate of 0.4 mL/min on a gradient ranging from 98.2 to 0:100 of 0.1% aqueous formic acid (solvent A) to methanol (solvent B; Supplemental Table 9). Measurements were performed using electrospray ionization in negative ion mode using a MRM transition of precursor ion > product ion m/z of 163.05 > 91. Capillary, cone, and collision voltages were at −3 kV, 22 V, and 10 V, respectively. Data were acquired using Waters MassLynx software and analytes processed for calibration and quantification using QuanLynx software. The final concentration of phenylpyruvate was calculated using a standard curve and the data presented as nmol per minute per μg recombinant enzyme.

**Amino Acid Analysis**

Amino acids were detected by LC-MS/MS as previously described (Gu et al., 2007). Leucine and isoleucine were combined as a single analyte due to poor resolution of these isomeric compounds in plant extracts. The final concentration of metabolites was calculated using standard curves of authentic compounds and calculated as either μmol per minute per mg recombinant enzyme, nmol formed per μg crude *A. belladonna* root protein, or as pmol per mg dry weight.

**Heterologous Protein Expression and Purification**

The predicted full-length open reading frame of ArAT4 was amplified from root cDNA with *Kod* DNA polymerase using the primers AbArAT4 ENT-F and AbArAT4 ENT STOP-R (Supplemental Table 4) and cloned into the pET100/D-TOPO vector as an N-terminal His-TAG fusion. Following sequence verification, the construct was transformed into *Escherichia coli* strain *Nico21* (DE3) (New England Biolabs). A fresh transformant was selected and inoculated into selective Terrific Broth medium and grown overnight at 37°C. The overnight culture was diluted 1:100 into 8 liters of fresh selective Terrific Broth and shaken to an OD₆₀₀ of 0.6, prior to induction by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM. The expression cultures were grown at 16°C for 20 h, and cells were harvested by centrifugation for 15 min at 4750g at 4°C. Cells were resuspended in a volume equal to 20% of the original culture volume of lysis buffer (50 mM potassium maleate, 50 mM sodium phosphate, pH 6.5, 100 mM potassium chloride, and 10% glycerol, with 0.1 mM pyridoxal 5'-phosphate (PLP), 1 mM DTT, and 10 μg/mL lysozyme added just prior to use) containing one tablet of Complete Protease Inhibitor Cocktail (Roche) per 50 mL of buffer. Resuspended cells were incubated at 4°C for 4 h and lysed by sonication. After adding 2-oxoglutarate to a final concentration of 5 mM, the crude lysate was incubated in a 70°C water bath until the lysate temperature reached 60°C. This temperature was maintained for 15 min, followed by cooling in an ice water bath until the lysate temperature was below 10°C. The lysate was centrifuged for 10 min at 20,000g at 4°C to remove cell debris, insoluble protein, and denatured protein. The supernatant was decanted and chitin bead slurry (New England Biolabs) was added at a rate of 0.5 mL of slurry to each 50 mL of supernatant and incubated at room temperature for 30 min. The chitin beads were pelleted by centrifugation at 4750g for 10 min, and the supernatant was passed over a Poly-Prep Chromatography Column to remove any remaining chitin beads. Imidazole was added to this supernatant to a concentration of 10 mM, MgCl₂ was added to 5 mM to chelate the EDTA contained in the protease inhibitor cocktail, and the pH was adjusted to 8.0 with potassium hydroxide. The supernatant was broken into four 450-mL batches. His-tagged ArAT4 was then purified using a 0.1 mL bed volume of Ni-NTA beads (Qiagen) according to the manufacturer’s instructions. The tagged protein was washed with 1× PBS, pH 7.4, containing 20 mM imidazole and eluted with 1× PBS, pH 7.4, containing 250 mM imidazole. The eluate was exchanged for a solution of 1× PBS, pH 7.4, 1× Complete Protease Inhibitor Cocktail, 1 mM DTT, and 0.1 mM PLP using an Amicon Ultra-30 module (EMD Millipore). Protein fractions were quantified by the Bradford assay (Bio-Rad Laboratories), separated by SDS-PAGE using 10% polyacrylamide gels, and visualized with Coomassie Brilliant Blue R250 stain. The enzyme was diluted to 0.1 mg/mL in a final buffer of 40% glycerol, 0.5× PBS, pH 7.4, 0.5× Complete Protease Inhibitor Cocktail, 0.5 mM DTT, and 0.05 mM PLP. Enzyme solution was stored at −20°C for up to 10 d. This enzyme stock solution was diluted as needed into cold 1× PBS immediately prior to use.

**Enzyme Activity Assays and Kinetics**

For determination of the optimum pH of ArAT4, enzyme assays were performed at 30°C for 15 min in 50-μL reaction mixes of 25 mM buffer at the reported pH, containing 0.1 mM PLP, 0.1 mM EDTA, 10 mM L-Phe, 7 mM 4-HPP, and 10 μg of purified enzyme. Reactions were stopped by the addition of 10 μL of 5% formic acid. For determination of the k₅₀ of ArAT4, purified recombinant enzyme was diluted 1:100 into 1× PBS and incubated at the temperatures indicated for 15 min and returned to ice prior to utilizing in standard assays containing 10 ng of purified enzyme, 10 mM L-Phe, 7 mM 4-HPP, 25 mM HEPES, pH 7.5, and the reagents noted above at 30°C. The kinetic parameters of ArAT4 were determined using a standard assay containing 25 mM HEPES buffer, pH 7.5, 0.1 mM EDTA, 0.1 mM PLP, and specified amounts of aromatic amino acid or 2-oxoadipate in a total volume of 100 μL. Reactions were terminated by addition of 20 μL 5% formic acid. Amounts of recombinant ArAT4 used in kinetic assays and the length of time the assays were incubated are indicated in parentheses. Kinetic assays contained 0.78 to 100 mM 2-oxoglutarate (100 ng enzyme for 1 h), 0.156 to 20 mM oxaloacetate (100 ng enzyme for 1 h), 1.953 to 250 mM pyruvate (500 ng enzyme for 2 h), 0.09 to 1 mM phenylpyruvate (500 ng enzyme for 1 h), 0.055 to 7 mM 4-hydroxyphenylpyruvate (10 ng enzyme for 15 min), 0.078 to 10 mM L-Phe (10 ng enzyme for 15 min with 4-HPP or 100 ng for 1 h with 2-oxoglutarate), 0.031 to 4 mM L-Tyr (500 ng for 1 h with phenylpyruvate), or 0.094 to 12 mM L-Trp (100 ng for 15 min). For all assays, reaction products were monitored and quantified by LC-MS/MS by methods previously indicated. Apparent V₅₀ and K₅₀ values were determined using nonlinear regression of the Michaelis-Menten equation using the Solver add-on of Microsoft Excel 2010. A calculated molecular mass of 51.09 kD was used for determination of K₅₀.

**Crude Protein Extraction and Analysis of ArAT Activity**

Extraction of crude *A. belladonna* root proteins was performed using previously described methods (Maeda et al., 2011), with the exception...
that filtration was performed using an Amicon Ultra-30 module (EMD Millipore). Powdered root tissue from three biological replicates was pooled for the extraction. In vivo assays of ArAT activity were performed using 50 μg of protein at 30°C for 1 h in 100-μL reaction mixes containing 25 mM HEPES, pH 7.5, 0.1 mM PLP, and 0.1 mM EDTA together with 10 mM L-Phe and 3.5 mM 4-HPP. Reactions were terminated by the addition of 25 mM HEPES, pH 7.5, 0.1 mM PLP, and 0.1 mM EDTA together with 10 mM L-5% formic acid and production of l-Tyr was determined by LC-MS/MS as described above.

**Accession Numbers**

The Illumina sequencing reads are available in the National Center for Biotechnology Information Sequence Read Archive under accession numbers SRX046282 to SRX046294, SRX060267, and SRX060269. Transcriptome assemblies and quantitative analysis of transcript abundance for the A. belladonna transcriptome are available at http://medicinalplantgenomics.msu.edu/. Full-length sequences corresponding to Ab-ArAT1 to 5 are deposited in GenBank under accession numbers KC954703 to KC954707.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Assignment of Gene Ontology Terms to A. belladonna Transcripts.

**Supplemental Figure 2.** Number of A. belladonna Unigenes in the Different Clusters as Identified by OrthoMCL.

**Supplemental Figure 3.** Amino Acid Alignment of Ab-ArATs and Selected Homologs.

**Supplemental Figure 4.** The Utility of Virus-Induced Gene Silencing for Functional Analysis of Alkaloid Biosynthesis in A. belladonna.

**Supplemental Figure 5.** The Impact of Silencing Additional ArATs on Tropane Alkaloid Biosynthesis in A. belladonna.

**Supplemental Figure 6.** Potential Forward and Reverse Reactions Catalyzed by ArATs.

**Supplemental Figure 7.** Kinetic Analysis of ArAT4.

**Supplemental Figure 8.** Physical Properties of the ArAT4 Enzyme.

**Supplemental Table 1.** Proteins Utilized for Phylogenetic Tree Construction.

**Supplemental Table 2.** Estimates of the Average Evolutionary Divergence of Solanaceae ArATs.

**Supplemental Table 3.** Amino Acid Levels in ArAT4-Silenced Lines.

**Supplemental Table 4.** Oligonucleotide Primers Used in This Study.

**Supplemental Table 5.** HPLC Mobile Phase Gradients Utilized for LC-MS/MS Analyses of Alkaloids and Select Precursors in Positive Mode.

**Supplemental Table 6.** Multiple Reaction Monitoring Parameters Utilized for LC-MS/MS Analyses of Tropane Alkaloids and Select Precursors in Positive Mode.

**Supplemental Table 7.** HPLC Mobile Phase Gradients Utilized for LC/MS/MS Analyses of Phenylpyruvic Acid and DL-3-Phenyllactic Acid Using an Applied Biosystems MDS SCIEX 3200 QTRAP Mass Spectrometer.

**Supplemental Table 8.** Multiple Reaction Monitoring Parameters Utilized for LC/MS/MS Measurements of Phenylpyruvic Acid and DL-3-Phenyllactic Acid Using an Applied Biosystems MDS SCIEX 3200 QTRAP Mass Spectrometer.

**Supplemental Table 9.** HPLC Mobile Phase Gradients Utilized for LC-MS/MS Analyses of Phenylpyruvic Acid Using a Waters Quattro Premier XE Mass Spectrometer.

**Supplemental Data Set 1.** OrthoMCL Clusters within the A. belladonna Transcriptome.

**Supplemental Data Set 2.** Multiple Sequence Alignment Used to Construct the Phylogeny Presented in Figure 4.

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**AUTHOR CONTRIBUTIONS**

C.S.B., C.R.B., A.D.J., D.D.-P., and J.C. conceived and designed the research. M.A.B., E.G.-C., and C.S.B. designed and performed research. M.A.B., E.G.-C., and C.S.B. analyzed the data. E.G.-C., K.L.C., and J.P.H. developed the bioinformatics pipeline. A.D.J. established mass spectrometry methods. M.A.B., E.G.-C., C.R.B., and C.S.B. wrote the article. All authors read and approved the article.

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A Root-Expressed L-Phenylalanine:4-Hydroxyphenylpyruvate Aminotransferase Is Required for Tropane Alkaloid Biosynthesis in *Atropa belladonna*

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