

Relaxation of tyrosine pathway regulation underlies the evolution of betalain pigmentation in Caryophyllales

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Summary

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- Diverse natural products are synthesized in plants by specialized metabolic enzymes, which are often lineage-specific and derived from gene duplication followed by functional divergence. However, little is known about the contribution of primary metabolism to the evolution of specialized metabolic pathways.
- Betalain pigments, uniquely found in the plant order Caryophyllales, are synthesized from the aromatic amino acid L-tyrosine (Tyr) and replaced the otherwise ubiquitous phenylalanine-derived anthocyanins. This study combined biochemical, molecular and phylogenetic analyses, and uncovered coordinated evolution of Tyr and betalain biosynthetic pathways in Caryophyllales.
- We found that *Beta vulgaris*, which produces high concentrations of betalains, synthesizes Tyr via plastidic arogenate dehydrogenases (TyrA_a/ADH) encoded by two ADH genes (*BvADHα* and *BvADHβ*). Unlike *BvADHβ* and other plant ADHs that are strongly inhibited by Tyr, *BvADHα* exhibited relaxed sensitivity to Tyr. Also, Tyr-insensitive *BvADHα* orthologs arose during the evolution of betalain pigmentation in the core Caryophyllales and later experienced relaxed selection and gene loss in lineages that reverted from betalain to anthocyanin pigmentation, such as Caryophyllaceae.
- These results suggest that relaxation of Tyr pathway regulation increased Tyr production and contributed to the evolution of betalain pigmentation, highlighting the significance of upstream primary metabolic regulation for the diversification of specialized plant metabolism.

Introduction

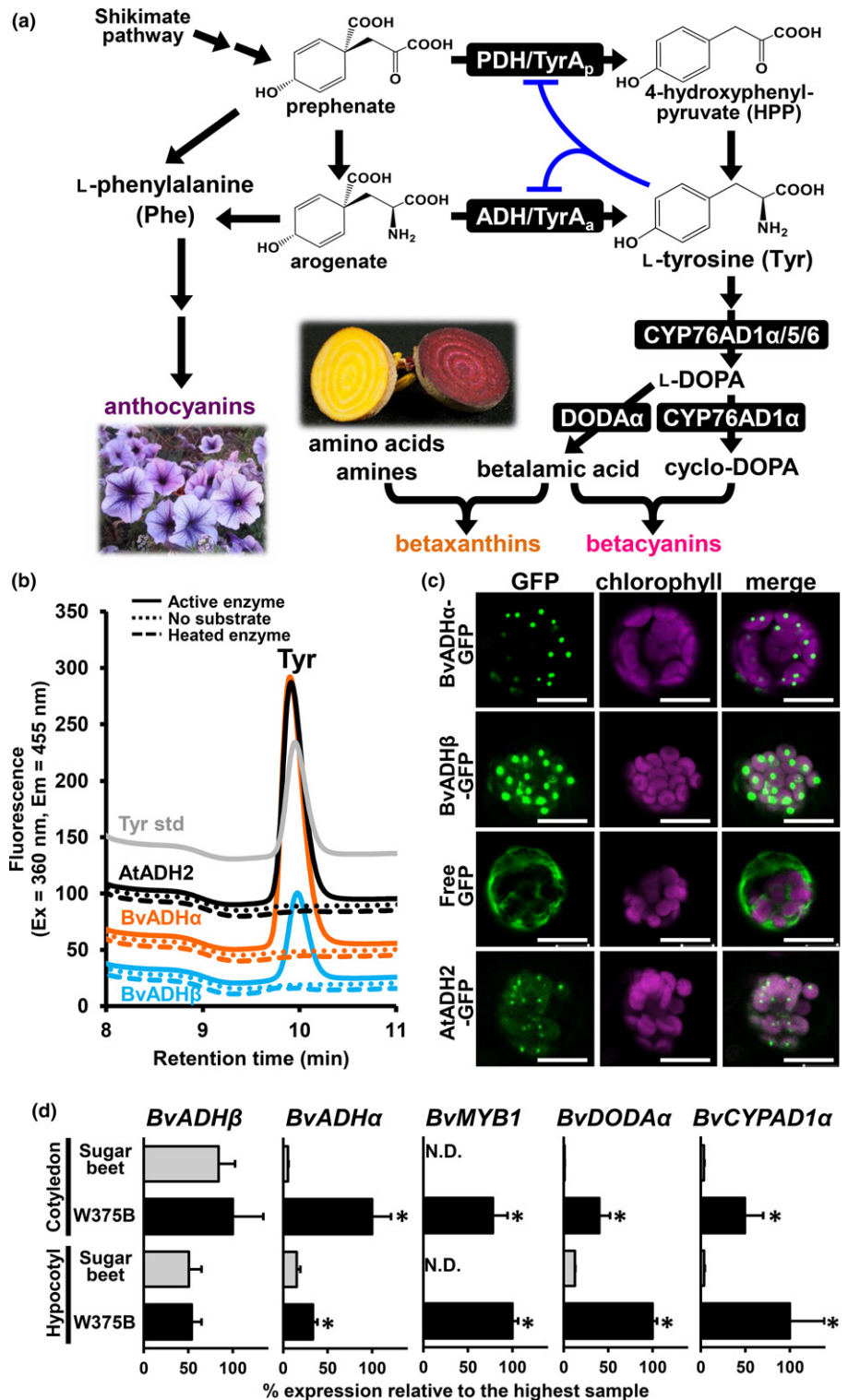
Plants synthesize numerous specialized metabolites (also known as secondary metabolites), which play crucial roles in plant adaptation. In contrast to well-documented diversification of plant enzymes directly involved in specialized metabolism (Mizutani & Ohta, 2010; Chen *et al.*, 2011; Pichersky & Lewinsohn, 2011; Weng, 2014; Moghe & Last, 2015), relatively little is known about the evolution of primary metabolic enzymes that provide precursors to the production of various specialized metabolites.

L-Tyrosine (Tyr) is an essential aromatic amino acid required for protein biosynthesis in all organisms; however, it is synthesized *de novo* only in bacteria, fungi and plants, but not in animals. Consequently, animals have to consume Tyr or L-phenylalanine (Phe) that can be hydroxylated to Tyr (Pencharz *et al.*, 2007). Besides protein biosynthesis, plants also use Tyr to produce a diverse array of specialized metabolites that are important for defense (e.g. dhurrin; Gleadow & Møller, 2014), stress tolerance (e.g. tocopherols; Mene-Saffrane *et al.*, 2010), and pollinator attraction (e.g. betalains; Tanaka *et al.*, 2008). Notably, humans have a long history of utilizing Tyr-derived specialized

metabolites, such as the psychedelic alkaloid mescaline derived from the cactus *Lophophora williamsii* (Ibarra-Laclette *et al.*, 2015) and the analgesic morphine derived from *Papaver somniferum* (opium poppy; Beaudoin & Facchini, 2014; Millgate *et al.*, 2004).

Tyr is synthesized from prephenate, which is converted from the final product of the shikimate pathway, chorismate (Siehl, 1999; Tzin & Galili, 2010; Maeda & Dudareva, 2012). In most bacteria and fungi, prephenate is oxidatively decarboxylated by prephenate dehydrogenase (TyrA_p/PDH, hereafter referred only as PDH; EC 1.3.1.12) to 4-hydroxyphenylpyruvate (HPP), which is transaminated to Tyr (Bentley, 1990; Fig. 1a). However, most plants first transaminate prephenate into arogenate and subsequently decarboxylate into Tyr by arogenate dehydrogenase (TyrA_a/ADH, hereafter referred only as ADH; EC 1.3.1.78; Rippert & Matringe, 2002a,b), both steps occurring in the plastids (Dal Cin *et al.*, 2011; Rippert *et al.*, 2009; Fig. 1a). The Tyr pathway is usually highly regulated at PDH and ADH. These homologous enzymes are strongly feedback inhibited by Tyr and control carbon flow between the two competing Tyr and Phe pathways (Gaines *et al.*, 1982; Bentley, 1990; Rippert &

Fig. 1 *Beta vulgaris* have two arogenate dehydrogenase (ADH/Tyr_a) enzymes localized in the plastids. (a) Tyrosine (Tyr) and betalain biosynthetic pathways in plants. Tyr can be synthesized from prephenate via ADH/Tyr_a or prephenate dehydrogenase (PDH/Tyr_p). Tyr is exported from the plastid to cytosol and then converted to L-dihydroxyphenylalanine (L-DOPA) by CYP76AD1 α , CYP76AD5, and CYP76AD6 (CYP76AD1 α /5/6). L-DOPA is then eventually converted to betalains, red betacyanins and yellow betaxanthins. Biosynthesis of Tyr competes for arogenate or prephenate substrate with that of L-phenylalanine (Phe), the precursor of anthocyanins. Blue lines denote feedback regulation by Tyr. DODA, L-DOPA dioxygenase. (b) Arogenate substrate was incubated with the purified recombinant enzymes of BvADH α or BvADH β together with NADP⁺ cofactor and the production of Tyr was analyzed. The HPLC traces were offset for presentation. *Arabidopsis thaliana* ADH2 (AtADH2) was used as a control for the ADH assay. (c) Green fluorescence protein (GFP) was fused at the C-terminal of BvADH α and BvADH β and transiently expressed in *Arabidopsis* protoplasts. Free GFP and GFP-fused *Arabidopsis* ADH2 (AtADH2) were used as controls for cytosolic and plastidic localization, respectively. Representative images show GFP fluorescence and chlorophyll autofluorescence in green and magenta, respectively. Bars, 10 μ m. (d) Expression levels of BvADH α and BvADH β were compared with those of betalain pathway genes in the cotyledon and hypocotyl of 7-d-old sugar beet and red beet (W357B). Significant differences between the two genotypes: *, $P < 0.05$ (Student's *t*-test). Bars represent percentage expression relative to the sample with the highest expression. Data are means of three biological replicates \pm SE. nd, not detectable.



Matringe, 2002a,b; Fig. 1b). A recent report showed that, in addition to plastidic ADH enzymes, some plants possess a PDH enzyme(s) that is not inhibited by Tyr and is localized to the cytosol (Rubin & Jensen, 1979; Siehl, 1999; Schenck *et al.*, 2015, 2017). Clearly, there is evolutionary variation in the Tyr pathway(s) in different plant lineages that warrants investigation.

Betalains are a class of Tyr-derived pigments that, within the flowering plants, occur exclusively in the order Caryophyllales where they replace the otherwise ubiquitous anthocyanins (Mabry, 1964; Tanaka *et al.*, 2008). Within Caryophyllales, the majority of families are betalain pigmented. In two families, Molluginaceae and Caryophyllaceae, however, evolutionary

reversions from betalain to anthocyanin pigmentation have occurred (Brockington *et al.*, 2015), highlighting the fact that these two classes of water-soluble pigments have never been found in the same organism (Bate-Smith, 1962; Mabry, 1964; Clement & Mabry, 1996; Brockington *et al.*, 2011). Betalains and anthocyanins are synthesized from Tyr and Phe, respectively, but have similar physiological functions in pollinator attraction and stress tolerance (Tanaka *et al.*, 2008). Betalains are also used as a natural food dye (E162) and have anticancer and antidiabetic properties (Neelwarne & Halagur, 2012; Lee *et al.*, 2014; Khan, 2015). Furthermore, intermediates in the betalain pathway are important pharmaceuticals (e.g. L-dihydroxyphenylalanine (L-DOPA) for the treatment of Parkinson's disease) or are substrates for other pharmaceutical agents (e.g. the production of dopamine and isoquinoline alkaloids such as morphine). Consequently, understanding the coordinated regulation of Tyr and betalain biosynthesis has the potential to enhance the production of Tyr, and the yield of Tyr-derived plant natural products important for human health.

Betalain biosynthesis starts with hydroxylation of Tyr to L-DOPA by at least three closely related cytochrome P450 enzymes (CYP76AD1, CYP76AD5 and CYP76AD6; Fig. 1a) (Polturak *et al.*, 2016; Sunnadaniya *et al.*, 2016). L-DOPA is further converted into betalamic acid or cyclo-DOPA by L-DOPA dioxygenases (DODA, Christinet *et al.*, 2004; Gandía-Herrero & García-Carmona, 2012) or CYP76AD1 (Hatlestad *et al.*, 2012), respectively (Fig. 1a). Betalamic acid then spontaneously reacts with cyclo-DOPA or amines to produce various forms of betacyanins or betaxanthins, respectively, which are usually further glycosylated. Recent studies found that the two key enzymes within the betalain pathway, DODA and CYP76AD1, duplicated just before the emergence of betalain pigmentation (Brockington *et al.*, 2015). Subsequently, one of the duplicated copies (DODA α and CYP76AD1 α) in both genes became specialized for betalain biosynthesis and were lost or downregulated in the anthocyanin-producing families such as Molluginaceae and Caryophyllaceae (Brockington *et al.*, 2015). Despite recent and rapid progress in understanding the betalain pathway enzymes and their evolution, little is known about the regulation of primary Tyr metabolism in relation to the evolution of this novel Tyr-dependent betalain pathway.

In the present study, we first investigated the Tyr biosynthetic pathway and its regulation in table beet (*Beta vulgaris*), which accumulates high concentrations of betalains (Goldman, 1996). Using comparative genomics, biochemical and cellular analyses, we found plastidic ADH enzymes from *B. vulgaris* that exhibit relaxed sensitivity to Tyr inhibition *in vitro* and *in vivo*. Phylogenetic analysis combined with recombinant enzyme characterization further demonstrated that de-regulated ADH enzymes emerged during the evolution of betalain pigmentations in the core Caryophyllales, and were lost or downregulated following disappearance of betalains. Furthermore, transient expression of the de-regulated beet ADH in *Nicotiana benthamiana* led to high accumulation of Tyr *in planta*. The results revealed the important contribution of primary Tyr pathway regulation to the unique

evolution of a plant specialized metabolic pathway, betalain biosynthesis.

Materials and Methods

Plant source and growth conditions

Beta vulgaris L. varieties, red beet (*W357B*), yellow beet (*Touch Stone*) and white beet (*Blankoma*), were provided by Dr Irwin Goldman from the University of Wisconsin-Madison, Department of Horticulture (Goldman, 1996), whereas sugar beet (Big Buck) and sea beet (PI 562585) were commercial sugar beets obtained from the Heirloom Seeds (West Finley, PA, USA) and the National Plant Germplasm System (NPGS), respectively. Spinach (*Spinacia oleracea*), Pigeonberry (*Rivina humilis*), four o'clock (*Mirabilis jalapa*) and common purslane (*Portulaca oleracea*) were grown from seed with a growing mix soil (Fafard[®], Agawam, MA, USA) in a growth chamber under 12 h light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$), 22°C and 60% humidity. After 1 month of growth, their leaves were harvested for RNA extraction.

Identification and cloning of ADH homologs from Caryophyllales

BLASTP searches were performed using the protein sequences of arogenate dehydrogenase (ADH) and prephenate dehydrogenase (PDH) enzymes from *A. thaliana* (AtADH1/At5g34930, NP_173023; AtADH2/At1g15710, NP_198343), *Glycine max* (GmPDH, KM507071), *Synechocystis* sp. PCC6803 (SyADH, WP_010872597), *Escherichia coli* (EcPDH, WP_052912694) and *Aquifex aeolicus* (AaPDH, WP_010881139) as queries against the sugar beet genome (*Beta vulgaris* <http://bvseq.molgen.mpg.de/>) (Supporting Information Fig. S1b). Potential ADH candidates were identified based on a broad phylogenetic analysis that included various plant ADH and PDH sequences (Fig. S1c,d).

Genomic DNA was extracted using Tris-sodium chloride-EDTA/sodium dodecyl sulfate buffer and precipitated with isopropanol and 200 mM ammonium acetate. For RNA isolation, the method described by Wang *et al.* (2011) was used with some modifications. The tissues were ground in a mortar with liquid nitrogen and powder polyvinylpyrrolidone (PVP). After addition of 700 μl fresh pre-warmed lysis buffer (2% CTAB, 2 M NaCl, 100 mM Tris-HCl pH 8, 25 mM EDTA and 5% β -mercaptoethanol), the samples were shaken vigorously for 2 min and incubated in a water bath at 65°C for 5 min. The RNA was converted into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and SuperScript IV Reverse Transcriptase with oligo dT₂₀ primer or random primers (Invitrogen).

Cloning primers were designed with the Invitrogen primer design (<http://tools.lifetechnologies.com/content.cfm?pageid=9716>) and the PCR IN-FUSION[®] primers designing program (<http://bioinfo.clontech.com/infusion/convertPcrPrimersInit.do>, Clontech, Mount View, CA, USA). All ADH candidate genes,

except for PoADH α , were PCR-amplified from cDNA using gene-specific primers (Table S1) and Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) with the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of amplification at 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, with a final extension at 72°C for 10 min. The PCR fragments were purified using QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and were inserted into the pGEX-2T vector (GE Healthcare, Piscataway, NJ, USA) at *EcoRI* and *BamHI* sites using the In-Fusion cloning method (Clontech, Madison, WI, USA). PoADH α was gene-synthesized (Biomatik, Cambridge, ON, Canada) and directly cloned into the same pGEX-2T vector. For generation of His-tagged proteins, the cloned PCR fragments were inserted into the pET28a vector (Novagen, Madison WI, USA) at *NdeI* and *EcoRI* sites.

Recombinant enzyme expression and purification

The His-tagged recombinant protein expression was carried out as we described previously (Dornfeld *et al.*, 2014). For glutathione S-transferase (GST)-tagged recombinant protein expression, the cloned pGEX-2T vectors were introduced into Rosetta-2 *E. coli* competent cells (Novagen, Madison, WI, USA) and cultured overnight at 37°C, 200 rpm in 10 ml LB medium containing Ampicillin (100 $\mu\text{g ml}^{-1}$). The 10 ml of the overnight culture were transferred to 1 l LB medium with Ampicillin (100 $\mu\text{g ml}^{-1}$ and further incubated at 37°C and 200 rpm until the OD₆₀₀ reached 0.3. The temperature was then changed to 18°C and, after 1 h, isopropyl β -D-1-thiogalactopyranoside (IPTG, 400 mM final concentration) was added to induce recombinant protein expression. After overnight incubation at 18°C under constant shaking at 200 rpm, cultures were harvested by centrifugation at 2000 *g* for 10 min at 4°C, and the pellet was washed with 0.9% NaCl solution. The samples were harvested and resuspended in 25 ml of lysis buffer (phosphate-buffered saline (PBS) pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and proteases inhibitor cocktail (Amresco, Solon, OH, USA). The resuspended cells were sonicated for periods of 20 s for 5 min. The cell lysate was centrifuged at 10 000 *g* for 30 min at 4°C, and the supernatant was applied to Fast Protein Liquid Chromatography (FPLC, AKTApure25 FPLC system; GE Healthcare) equipped with GSTrapTMFF (GE Healthcare). Prior and after injection, the column was washed with five times bed volume wash buffer A (PBS, pH 7.6) followed by five times bed volume of wash buffer B (10 mM reduced glutathione dissolved in 50 mM Tris-HCl, pH 8). The recombinant enzymes containing GST-tag were eluted with ten-bed volumes of the elution buffer B and collected into Eppendorf tubes containing 500 μl . Recombinant enzymes eluted in the fraction five and six, which were combined and desalted using a gel filtration column (Sephadex G50-80 resin; Sigma-Aldrich) in the reaction buffer (200 mM HEPES (pH 7.6), 50 mM KCl, 10% ethylene glycol). Enzyme concentrations were measured using Bradford assay (Bio-Rad) and the enzyme purity was estimated by running on SDS-PAGE gel and analyzing with ImageJ (<http://imagej.nih.gov/ij/>).

ADH and PDH activity assays *in vitro*

ADH and PDH activity from beet tissues (Fig. S2a,b) were analyzed by using the leaves and stem/root crude protein extract of red beet (*W357B*). The beets were grown in a glasshouse for 12 wk with a temperature of 22–25°C and 16 h of ambient and supplemented lights. Protein extraction was performed by grinding 1 g of tissues in liquid nitrogen and resuspending the powder in the extraction buffer (200 mM HEPES (pH 7.6), 50 mM KCl, 10% ethylene glycol, 1 mM PMSF, 1 mM DTT and plant proteases inhibitor cocktail (Ameresco)). The extracts were desalted using the gel filtration column (Sephadex G50-80 resin; Sigma-Aldrich) into the reaction buffer. The ADH or PDH assays were performed by mixing the desalted protein extract with 1 mM NADP⁺ and 1 mM L-arogenate or prephenate in a total volume of 10 μl or 25 μl , respectively. L-Arogenate was prepared by enzymatic conversion from prephenate (Sigma-Aldrich), as described previously (Schenck *et al.*, 2015). The reactions were started by adding the enzyme (crude extract or recombinant enzyme) and incubated at 37°C for 45 min. The reaction was stopped with two times volume of methanol. The same ADH and PDH assay protocols were used for initial characterization of purified recombinant BvADH enzymes.

For detection of Tyr product from the ADH assays, 10 μl of the reaction mixture was first derivatized with the equal volume of the 40 mM OPA (*o*-phthalaldehyde) solution (Sigma-Aldrich) mixed in 100 μl methanol, 5 μl 2-mercaptoethanol and 900 μl 0.4 M boric acid) for 3 min, injected to high pressure liquid chromatography (HPLC, Agilent 1260) equipped with the Eclipse XDB-C18 column (5 μm , 3.0 \times 150 mm; Agilent, Santa Clara, CA, USA), and separated by a 30 min linear gradient from 20% to 45% methanol in 0.1% ammonium acetate at a flow rate of 0.8 ml min⁻¹. The substrate and product of ADH assays (Tyr and arogenate, respectively) were detected by a fluorescence detector (Agilent) with excitation at 360 nm and emission of 455 nm. For PDH assays, the reactions were stopped by addition of NaBH₄, which converts the reaction product HPP into hydroxyphenyllactic acid (HPLA), followed by neutralization with 100 μl of 6 N HCl as described by Schenck *et al.*, 2015. The HPLC was equipped with Zorbax SB-C18 column (Agilent) using a 6 min isocratic elution at 25% methanol in 0.1% phosphoric acid, followed by a 20 min linear gradient of 25–60% methanol at a flow rate of 1.0 ml min⁻¹. The HPLA were monitored by absorption at 270 nm.

In order to test the electron donor and substrate preferences of purified recombinant enzymes, the ADH and PDH reactions were performed as described above, except for 12 min with 400 μM L-arogenate and 1 mM cofactor (NAD⁺ or NADP⁺). The reaction was stopped by placing the tubes on ice and immediately measured for the production of the reduced cofactor, NAD(P)H, at 340 nm by spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific). The quantification was based on the standard curve of authentic NADPH.

In order to examine Tyr sensitivity of the purified recombinant enzymes, ADH assay was performed as described previously (Schenck *et al.*, 2015), but in the presence or absence of different

concentrations of Tyr. Tyr was first dissolved in 0.025 N NaOH at 100 mM (as the water solubility of Tyr is very low, < 2 mM), which was diluted to 4 mM to 10 μ M final concentration in 0.0025 N NaOH. The reactions contained 500 mM HEPES (pH 7.6) to maintain the final pH at 7.6. The production of reduced cofactor (NADPH) was monitored at 340 nm using a spectrophotometer every 2 min for 10 min. In addition, other effectors (L-phenylalanine (Phe), L-tryptophan (Trp) and betanin) were used to test possible inhibition of the enzyme ADH activity at a final concentration of 1 mM. All of the reactions were performed under nonsaturated condition, where activity increased linearly depending on reaction times and enzyme concentrations.

Transient expression of *BvADH α* and *BvADH β* in *Nicotiana benthamiana*

ADH α and *ADH β* sequences used for *N. benthamiana* agroinfiltration were amplified from *Beta vulgaris* var. *vulgaris* variety 'Boltardy' (Chiltern Seeds, Wallingford, UK) swollen hypocotyl and leaf tissue cDNA libraries, respectively, which were prepared using BioScript Reverse Transcriptase (Bioline Reagents, London, UK). Transcripts were amplified by PCR using gene specific primers (Table S1) and Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). Vectors for transient transformation were constructed with Golden Gate cloning using the MoClo Tool Kit (Weber *et al.*, 2011; Addgene, Cambridge, MA, USA), with the *Bp*I and *Bsa*I restriction sites eliminated after cloning. The turboGFP sequence used in this assay was a variant codon-optimized for plants contained in the MoClo Plant Parts Kit (Engler *et al.*, 2014; Addgene). *BvADH α* , *BvADH β* and *turboGFP* sequences were ultimately cloned into the pICH86988 binary vector under control of the Cauliflower Mosaic Virus 35S promoter and the *Agrobacterium tumefaciens* octopine synthase (OCS) terminator.

Transient gene expression assays in *N. benthamiana* were performed according to the previously described agroinfiltration method with some modifications (Sparkes *et al.*, 2006). All constructs were transformed into the *Agrobacterium tumefaciens* GV3101 strain, and grown in LB media supplemented with kanamycin (50 mg l⁻¹), gentamycin (25 mg l⁻¹) and rifampicin (50 mg l⁻¹) until reaching an OD₆₀₀ of 1.5. Cultures were then brought to a final OD₆₀₀ of 0.5 in infiltration media (10 mM MgCl₂, 0.1 mM acetosyringone, 10 mM MES at pH 5.6) for 3 h before infiltration. Infiltration spots corresponding to 35S::*BvADH α* , 35S::*BvADH β* and 35S::*turboGFP* were performed in the same leaves of 6-wk-old *N. benthamiana* plants alternating the position of the spots between plants in a clockwise manner to account for intraleaf variation (Barshandy *et al.*, 2015). Infiltrated tissue was sampled 3 d post-infiltration from five biological replicates for Tyr quantification and quantitative real-time PCR (qRT-PCR) analysis.

For quantification of Tyr and other amino acids, c. 40 mg FW tissues were harvested, lyophilized, sent from the University of Cambridge (UK) to the University of Wisconsin-Madison (USA), and analyzed exactly as described. Tyr and other amino acids were extracted and measured as described previously (Wang

et al., 2017) and quantified based on standard curves of authentic amino acid standards (Sigma-Aldrich).

Phylogenetic analysis

Amino acid sequences from genomes (full open reading frame (ORF)) and transcriptomes (full or partial ORF) of Brockington *et al.* (2015) were used for phylogenetic analysis following methods described in Brockington *et al.* (2015) with minor modifications. In addition, we carried out analysis of dN/dS ratio in *ADH α* to test for relaxed selection in anthocyanic lineages (Table S2; see Methods S1 for a full description).

Subcellular localization of GFP-fused ADH enzymes

The subcellular localization experiments of GFP-fused ADH enzymes were conducted as described previously (Schenck *et al.*, 2015).

Accession numbers

The GenBank accession numbers for the sequences mentioned in this article are: *BvADH β* *W357B* red beet variety (KY207366), *BvADH β* *Boltardy* red beet variety (MF346292), *BvADH β* *Big Buck* sugar beet variety (KY207367), *BvADH β* *Touch Stone* yellow beet variety (KY207368), *BvADH β* *Blankoma* white beet variety (KY207369), *BvADH β* *Sea beet* PI562585 variety (KY207370), *BvADH α* *Big Buck* sugar beet variety (KY207371), *BvADH α* *W357B* red beet variety (KY207372), *BvADH α* *Boltardy* red beet variety (MF346291), *BvADH α* *Blankoma* white beet variety (KY207373), *BvADH α* *Touch Stone* yellow beet variety (KY207374), *BvADH α* *Sea beet* PI562585 variety (KY207375), *SoADH β* (KY207376), *SoADH α* (KY207378), *NaADH β* (KY207377), *MjADH α* (KU881770), *RhADH α* (KY207379), *PoADH α* (KY207380), *SmADH α* (KY274179), *PpADH α* (KY274180) and *HIADH α* (KY274181).

Results

Beta vulgaris has two ADH enzymes

In order to first investigate how *B. vulgaris* synthesizes Tyr, protein crude extracts of red beet leaf and root/stem tissues were analyzed for ADH and PDH activity, the production of Tyr or HPP from arogenate or prephenate, respectively. On the one hand, Tyr was produced from arogenate in the red beet extracts of both leaves and roots/stems (Fig. S2a) similar to soybean leaf extract, which was previously shown to have both ADH and PDH activity (Schenck *et al.*, 2015). On the other, unlike the soybean leaf extract, HPP production was not detected in the leaf and root/stem extracts of red beet (Fig. S2b). These results showed that red beet has ADH but not PDH activity.

In order to identify the gene(s) responsible for the ADH activity in *B. vulgaris*, previously reported plant and microbial *ADH* and *PDH* genes (Bonvin *et al.*, 2006; Hudson *et al.*, 1984; Legrand *et al.*, 2006; Rippert & Matringe, 2002a,b; Schenck

et al., 2015; Fig. S1b) were used to BLAST against the genome of sugar beet, another cultivar of *B. vulgaris* (Dohm *et al.*, 2014) (assembly v.1.2 <http://bvseq.molgen.mpg.de>). Two *B. vulgaris* sequences homologous to these *ADH* and *PDH* genes were found on chromosome 8 of the *B. vulgaris* genome 25.3 kbp apart (Fig. S1a). They were more similar to plant *ADHs* and *PDHs* (59–61% similarity at amino acid levels) than bacterial ones (24–40% similarity; Fig. S1b). Within plants, the two *ADH* candidate genes from *B. vulgaris* both belong to the canonical ADH clade containing Arabidopsis *ADHs* (Rippert & Matringe, 2002a,b), rather than the noncanonical clade containing legume *PDHs* (Schenck *et al.*, 2015, 2017), and appear to be derived from a recent duplication within the order Caryophyllales (Fig. S1c,d).

For biochemical characterization, these two putative BvADHs were expressed in *E. coli* as recombinant enzymes, which were further purified using affinity chromatography and subjected to ADH and PDH assays. On the one hand, both of the beet recombinant enzymes showed ADH activity (i.e. the production of Tyr from arogonate; Fig. 1b) and strongly preferred NADP⁺ over NAD⁺ (Fig. S3) similar to other plant ADH enzymes and activities (Byng *et al.*, 1981; Gaines *et al.*, 1982; Rippert & Matringe, 2002a,b). On the other, neither of the beet enzymes exhibited detectable PDH activity (Fig. S2c), which is consistent with the lack of PDH activity in beet tissues (Fig. S2b) and also confirmed the absence of *E. coli* PDH contamination (Hudson *et al.*, 1984). Therefore, these two genes were designated as *B. vulgaris* arogonate dehydrogenases (*BvADHα* and *BvADHβ*).

Both BvADHs are plastid-localized but only *BvADHα* expression is correlated with betalain pathway genes

Most plant enzymes involved in the aromatic amino acid pathways are localized within the plastids (Rippert *et al.*, 2009; Dal Cin *et al.*, 2011; Maeda & Dudareva, 2012), and both BvADH proteins also have a predicted N-terminal plastid transit peptide (Fig. S4). To experimentally determine the subcellular localization of BvADHs, a green fluorescent protein (GFP) was fused to the C-terminal of BvADHs, expressed in Arabidopsis protoplasts, and analyzed for their localization using confocal microscopy. The fluorescence signal of GFP fused with BvADHα or BvADHβ overlapped with chlorophyll autofluorescence, which was different from the free GFP control and similar to GFP fused with plastidic Arabidopsis ADH (Rippert *et al.*, 2009) (*AtADH2*, Fig. 1c). These results suggest that both BvADHs are targeted to the plastids and that Tyr is produced mainly by the plastidic arogonate pathway in *B. vulgaris*.

In order to examine expression patterns of *BvADHs*, especially in comparison to the betalain pathway genes, expression levels of *BvADHα* and *BvADHβ* were analyzed and compared with those of *DODAα*, *CYP76AD1α* and *BvMYB1* in cotyledon and hypocotyl tissues of sugar and red beets (Fig. 1d). Consistent with previous studies (Hatlestad *et al.*, 2012; 2015), *DODAα* and *CYP76AD1α*, as well as *BvMYB1* transcription factor, were much more highly expressed in red than sugar beet. Interestingly, *BvADHα* expression showed similar trends and was significantly higher in red than sugar beet in both cotyledon and hypocotyl

tissues. On the other hand, *BvADHβ* expression levels were very similar between genotypes in both tissue types (Fig. 1d). These results showed that expression of *BvADHα*, but not *BvADHβ*, is correlated with those of betalain pathway genes in *B. vulgaris*.

BvADHα but not BvADHβ exhibits relaxed sensitivity to Tyr

Both ADH and PDH enzymes are usually inhibited by Tyr in most organisms (Gaines *et al.*, 1982; Connelly & Conn, 1986; Bentley, 1990; Rippert & Matringe, 2002a,b; Sun *et al.*, 2009). To determine if the BvADHs are also feedback-regulated by Tyr, ADH activity of the recombinant BvADH enzymes was analyzed in the presence and absence of Tyr as an effector molecule. The ADH activity of GST-tagged BvADHβ was inhibited by 80% and 100% in the presence of 100 μM and 1 mM Tyr, respectively (Fig. 2), similar to the Tyr-sensitive Arabidopsis *AtADH2* (Rippert & Matringe, 2002a,b). By contrast, ADH activity of BvADHα was reduced only by half at 1 mM Tyr (Fig. 2). Similar results were obtained for histidine (His)-tagged ADH enzymes, where BvADHα showed much less sensitivity to Tyr than *AtADH2* (Fig. S5), although the expression of His-tagged BvADHβ was not successful. Other aromatic amino acids (Phe and Trp) as well as betanin, the major betacyanin accumulated in red beet, did not significantly reduce the ADH activity of BvADHα, BvADHβ or *AtADH2* at 1 mM (Fig. S6). These results revealed that BvADHα, but not BvADHβ, has relaxed sensitivity to Tyr inhibition.

Heterologous expression of *BvADHα* but not *BvADHβ* increases Tyr accumulation in plants

In order to test if BvADHα having relaxed sensitivity to Tyr can enhance the production of Tyr in *planta*, *BvADHα* and *BvADHβ* were transiently expressed in *N. benthamiana* through Agrobacteria infiltration (Fig. S7a; Sparkes *et al.*, 2006) and their impacts

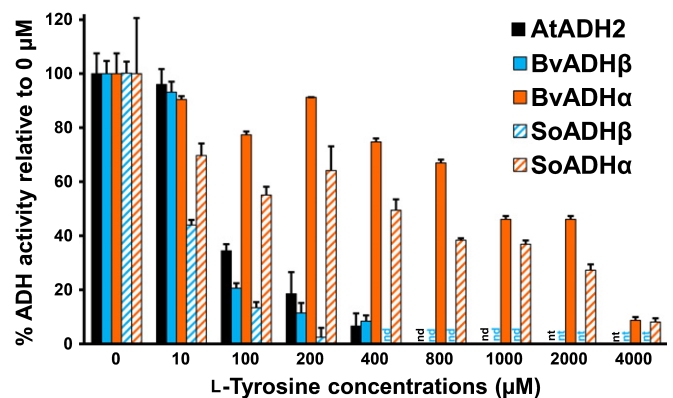


Fig. 2 Beet and spinach arogonate dehydrogenase (ADH)α but not ADHβ have reduced sensitivity to L-tyrosine (Tyr). ADH activity was measured at different Tyr concentrations using NADP⁺ cofactor and purified recombinant ADH enzymes of beet (*BvADHα*, *BvADHβ*), spinach (*SoADHα*, *SoADHβ*), and Arabidopsis (*AtADH2*). Data are expressed as the percentage of respective control activity without Tyr (0 μM) and means of three independent experiments ± SE. nd, not detectable; nt, not tested.

on Tyr production were analyzed. A control vector expressing GFP was also infiltrated as a negative control (Fig. S7a). *BvADH α* expression resulted in > 10-fold increase in Tyr concentrations relative to the GFP control, whereas the increase of Tyr due to *BvADH β* expression was not significantly different (Figs 3a; Table S3). Interestingly, Phe concentrations were decreased significantly under *BvADH α* , but not *BvADH β* expression (Fig. 3b). Other amino acid concentrations were largely unaffected by *BvADH α* or *BvADH β* expression (Table S3). These results demonstrate that *BvADH α* expression leads to elevated accumulation of Tyr *in planta*.

BvADH α orthologs emerged during the evolution of betalain pigmentation in Caryophyllales

Domestication has modified metabolic traits in various crops (Hanson *et al.*, 1996; Rapp *et al.*, 2010; Rong *et al.*, 2014). Thus,

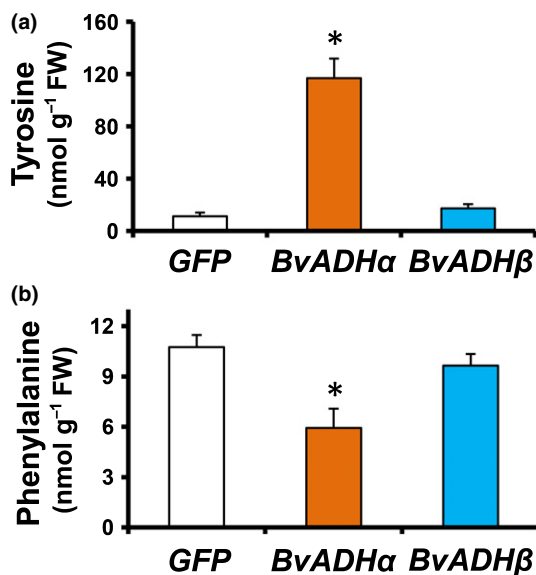


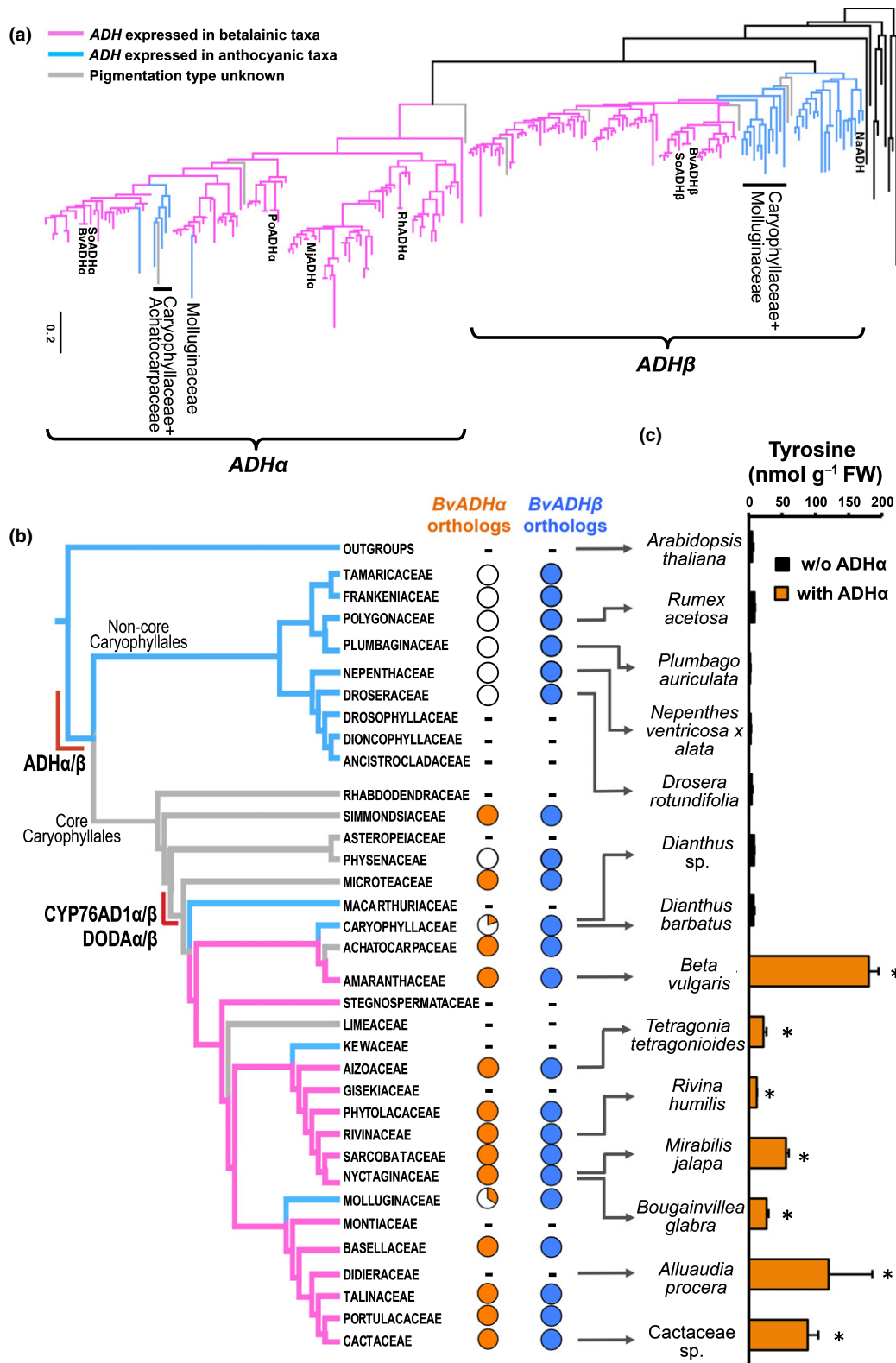
Fig. 3 Heterologous expression of *BvADH α* but not *BvADH β* increases tyrosine concentrations in *Nicotiana benthamiana*. *Agrobacterium tumefaciens* carrying the construct of 35S::GFP, 35S::*BvADH α* or 35S::*BvADH β* was infiltrated to *N. benthamiana* leaves, which were analyzed for amino acid contents using GC-MS. Concentrations of tyrosine (a) and phenylalanine (b). Significant differences from the 35S::GFP control: *, $P < 0.05$ (Student's *t*-test). Data are means \pm SE ($n = 5$). GFP, green fluorescent protein.

we hypothesized that the *BvADH α* enzyme with relaxed Tyr regulation was selected during domestication and intensification of color in table beets, that have been used at least since the Roman times (Biancardi *et al.*, 2012; Dohm *et al.*, 2014). To test this hypothesis, the nucleotide and protein sequences of *BvADH α* (and *BvADH β*) were compared among different domesticated beets, red beet (*W357B*), sugar beet (*Big Buck*), yellow beet (*Touch Stone*) and white beet (*Blankoma*), as well as their wild relative, sea beet (Biancardi *et al.*, 2012) (*Beta vulgaris* subsp. *maritima*). Several single nucleotide polymorphisms (SNPs) were detected among different lines in both *BvADH α* and *BvADH β* (Fig. S4a,b). However, only a few of them affected the amino acid sequences and were within and near the N-terminal signal peptide of *BvADH α* and *BvADH β* , respectively (Fig. S4c,d). Thus, the mature enzyme regions of *BvADH α* were unaltered during domestication.

In order to further test if the *ADH α* enzymes with reduced Tyr sensitivity are restricted to the species *B. vulgaris*, the corresponding genes for *BvADH α* and *BvADH β* were cloned from a closely related species within the same Amaranthaceae family, spinach (*Spinacia oleracea*), whose draft genome is available (<http://bvseq.molgen.mpg.de>). Spinach *ADH α* and *ADH β* orthologs (*SoADH α* and *SoADH β*) had 77 and 83% identity (respectively) at amino acid levels to the corresponding *BvADH α* s in the mature enzymatic regions. The recombinant enzymes of spinach *ADH α* s showed similar Tyr sensitivity to beet *ADH α* s: *SoADH α* , but not *SoADH β* , exhibited reduced Tyr sensitivity (Fig. 2). These results suggest that the reduced Tyr sensitivity of *BvADH α* at least at the enzyme level was not the result of selection during domestication of beet cultivars, but was already present in the common ancestor of the beet and spinach *ADH α* enzymes.

In order to determine the origin and molecular evolution of *BvADH α* , we mined genome and transcriptomic data across the Caryophyllales for *ADH* orthologs and performed a phylogenetic analysis (Figs 4a, S1e). The results indicate that a gene duplication event on the branch leading to stem Caryophyllales produced *ADH α* and *ADH β* lineages. Although *ADH β* orthologs were expressed across the entire Caryophyllales, expression of *ADH α* closely parallels betalain production in Caryophyllales (Fig. 4b). *ADH α* expression is undetectable from the anthocyanin clade that diverged before the earliest inferred origin of betalain synthesis (hereafter referred to as noncore Caryophyllales;

Fig. 4 Phylogenetic distribution of arogenate dehydrogenase (*ADH α*) in Caryophyllales. The blue and pink branches represent anthocyanin- and betalain-producing families, respectively, whereas families with unclear/unidentified pigmentation are shown in gray. (a) Maximum-likelihood phylogeny of *ADH* genes in Caryophyllales. Scale bar indicates inferred number of amino acid substitution per site. *ADH* enzymes characterized in this study are indicated at the end of each branch. (b) Presence and absence of *BvADH α* and *BvADH β* orthologs detected from genome or transcriptome data was mapped to the family-level phylogenetic tree of the Caryophyllales order. Filled circles denote that corresponding orthologs were detected in all species within the family, whereas partially filled circles indicate that the filled portion of the species within each family had corresponding orthologs. Open circles denote that no corresponding orthologs were detected. Red lines indicate estimated timings of duplication events of *ADH* and betalain pathway genes (*CYP76AD1* and *DODA*). Dashes (–) represent families with no available transcriptomic or genomic data. (c) L-Tyrosine (Tyr) contents were analyzed in various Caryophyllales species. *Arabidopsis thaliana* was used as outgroup. Orange bars indicate species having *ADH α* orthologs. Young leaf tissues were used for all samples except a Cactaceae species, in which flowers were used to avoid succulent tissues. Significant difference from *Arabidopsis* (*, $P < 0.05$) were based on the fixed effect model (see the Materials and Methods section). Also, a statistical analysis based on the mixed effect model showed significant differences between two groups, plants with and without *ADH α* ($P < 0.0001$). Bars represent means \pm SE ($n =$ four biological replicates).



Brockington *et al.*, 2009). Two families in the Caryophyllales, Molluginaceae and Caryophyllaceae have reverted from betalain to anthocyanin pigmentation (Brockington *et al.*, 2011, 2015). Presence of the *ADHα* orthologs in the transcriptomes of Molluginaceae and Caryophyllaceae was much less common than the

presence of *BvADHβ* (Fig. 4a,b). Thus, the presence of *ADHα*, but not *ADHβ*, closely mirrors the distribution of betalain pigmentation across Caryophyllales, similar to the pattern in two other genes of the betalain pathway, *CYP76AD1α* and *DODAα* (Brockington *et al.*, 2015).

Betalain-producing species have deregulated BvADH α enzyme and elevated Tyr concentrations

In order to further test experimentally if *ADH α* orthologs across Caryophyllales share the unique property of reduced Tyr inhibition, *ADH* genes from representative members of Caryophyllales (Brockington *et al.*, 2011) were cloned and the Tyr sensitivity of encoded enzymes was evaluated. On the one hand, an ADH β enzyme from the anthocyanin-producing noncore Caryophyllales, *Nepenthes ventricosa* \times *alata* (NaADH β , Nepenthaceae; Fig. 4b), was strongly inhibited by Tyr (Fig. 5) similar to beet and spinach ADH β (Fig. 2). On the other, ADH α orthologs from betalain-producing families – *Rivina humilis* (RhADH α , Rivinaceae), *Mirabilis jalapa* (MjADH α , Nyctaginaceae) and *Portulaca oleracea* (PoADH α , Portulacaceae) – all shared relaxed Tyr inhibition and retained 42% to 68% of ADH activity even at 1 mM Tyr (Fig. 5).

In order to test if Tyr-insensitivity of the recombinant ADH α enzyme is also detectable *in vivo*, Tyr sensitivity of leaf ADH

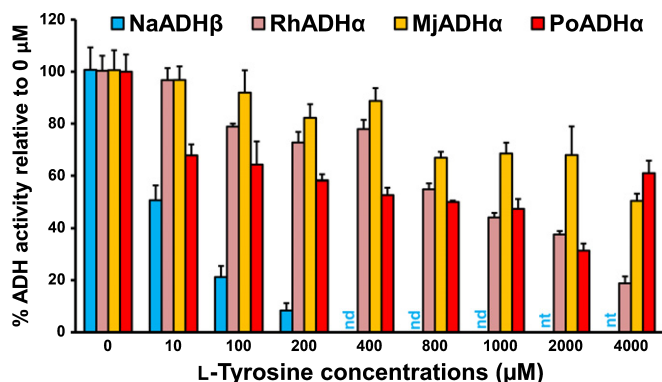


Fig. 5 Arogenate dehydrogenase (ADH) α from various species of core Caryophyllales also exhibit relaxed sensitivity. ADH activity was measured under different L-Tyrosine (Tyr) concentrations using purified recombinant ADH enzymes of *Nepenthes ventricosa* \times *alata* (NaADH β), *Rivina humilis* (RhADH α), *Mirabilis jalapa* (MjADH α) and *Portulaca oleracea* (PoADH α) ADH. Data are expressed as the percentage of respective control activity without Tyr (0 μ M) and the mean of three independent experiments \pm SE. nd, not detectable; nt, not tested.

Table 1 L-Tyrosine (Tyr) sensitivity of arogenate hydrogenase (ADH) activity from plant tissue extracts

Species	ADH activity (nmol mg ⁻¹ protein)		Inhibition (%)
	0 mM 3-fluoro-Tyr	0.5 mM 3-fluoro-Tyr	
<i>Spinach oleracea</i>	66.4 \pm 5.0	52.7 \pm 1.9	20.7
<i>Dianthus barbatus</i>	18.1 \pm 0.3	0.9 \pm 0.2	95.0
<i>Arabidopsis thaliana</i>	93.5 \pm 5.2	7.8 \pm 0.5	91.6

Total protein extracts of spinach, *Dianthus barbatus* and *Arabidopsis* leaf tissues were used to analyze ADH activity in the presence and absence of 0.5 mM Tyr analog (3-fluoro-Tyr), which were used to calculate percentage inhibition. ADH activity was measured with 1 mM arogenate substrate and 1 mM NADP⁺ cofactor during 2 h incubation (see Supporting Information Fig. S8). Data are means \pm SE ($n = 4$).

activity was analyzed from species containing *ADH α* (i.e. spinach) and ones lacking *ADH α* (i.e. *Arabidopsis thaliana*; *Dianthus barbatus*, Caryophyllaceae). Spinach rather than beet was used due to its cleaner background during HPLC-based enzyme assay. As shown in Table 1 and Fig. S8, ADH activity of *A. thaliana* and *Dianthus barbatus* tissues was strongly inhibited (92–95%) by 0.5 mM of 3-fluoro-DL-Tyr effector, whereas that of spinach was much more resistant to 3-fluoro-DL-Tyr inhibition (only *c.* 21% inhibited), consistent with the presence of SoADH α with relaxed sensitivity to Tyr (Fig. 2).

In order to further test if the presence of deregulated ADH α leads to increased Tyr accumulation in betalain-producing species, Tyr concentrations were quantified in young leaves of a variety of Caryophyllales species with or without ADH α and also in *A. thaliana* as a comparison. On the one hand, anthocyanin-producing species from noncore Caryophyllales (e.g. *Nepenthes ventricosa* \times *alata*) and Caryophyllaceae (e.g. *Dianthus barbatus*) had Tyr concentrations (2.1–8.8 nmol g⁻¹ FW) comparable to that of *Arabidopsis* (5.3 nmol g⁻¹ FW). On the other, although large variations were observed, betalain-producing ADH α -containing species had significantly higher Tyr concentrations (from 12 to 180 nmol g⁻¹ FW) than *Arabidopsis* (Fig. 4c). These results demonstrate that betalain-producing species have ADH α with relaxed sensitivity to Tyr inhibition and accumulate elevated concentrations of Tyr.

ADH α orthologs underwent relaxed selection and gene loss in lineages that have reverted from betalain to anthocyanin pigmentation

Interestingly, when *ADH α* orthologs were recovered from Caryophyllaceae or Molluginaceae transcriptomic data, they were often recovered in partial sequences, indicating general low abundance. Within the Caryophyllaceae, *ADH α* orthologs was only detected in the subfamily Paronychioideae (Greenberg & Donoghue, 2011), which forms a paraphyletic clade to the rest of the family. To test for relaxed selection in anthocyanic lineages we further examined a subset of *ADH α* orthologs with sequences either verified by Sanger sequencing or by transcriptome read mapping and manual inspection of read coverage (Methods S1; Notes S1–S4). Although no obvious acceleration of substitution was observed in Caryophyllaceae from nucleotide coding sequences (CDS; Fig. S9a), there was apparent acceleration in their amino acid sequences (Fig. S9b). Furthermore, the dN/dS ratio in Caryophyllaceae *ADH α* (0.166) was elevated compared to the rate among betalain-producing *ADH α* (0.0743) under the Partitioned MG94xREV Model, assuming homogenous synonymous and nonsynonymous rates across sites. In addition, we found evidence of relaxed selection (as opposed to intensification of positive selection) that contributes to the increase in nonsynonymous rate in Caryophyllaceae under the RELAX framework ($P = 5.6E-8$, Table S4) (Wertheim *et al.*, 2014). Moreover, the genome assembly of the anthocyanic carnation (*Dianthus caryophyllus*, Caryophyllaceae subfamily Caryophylloideae that nested within subfamily Paronychioideae; Greenberg & Donoghue, 2011; Yagi *et al.*, 2014) lacked *ADH α* ortholog and

only contained *ADH β* ortholog, suggesting complete gene loss of *ADH α* in the subfamily Caryophyllaceae. Species within the anthocyanic Caryophyllaceae, therefore, exhibit the transition from relaxed selection to gene loss of *ADH α* orthologs, which associates with the loss of betalain pigmentation in Caryophyllaceae.

Discussion

This study found that *Beta vulgaris* has ADH but no PDH enzymes or activity (Figs 1b, S2, S3). This is similar to most plants (Gaines *et al.*, 1982; Connelly & Conn, 1986; Rippert & Matringe, 2002a,b) but different from legumes that have both ADH and PDH (Rubin & Jensen, 1979; Siehl, 1999; Schenck *et al.*, 2015, 2017). Thus, *B. vulgaris* synthesizes Tyr via the ADH pathway that occurs within the plastids (Rippert *et al.*, 2009) (Fig. 1c). We also found that *B. vulgaris* possesses two paralogous genes encoding the ADH enzymes, namely *ADH α* and *ADH β* (Fig. S1c,d). Interestingly, *ADH α* but not *ADH β* exhibited relaxed sensitivity to Tyr inhibition (Fig. 2). Although recent studies reported that the legume PDH enzymes are also Tyr-insensitive (Schenck *et al.*, 2015, 2017), *BvADH α* and legume PDHs have two major differences. First, legume PDHs are localized in the cytosol (Schenck *et al.*, 2015), whereas *BvADH α* (and *BvADH β*) was targeted to the plastids (Fig. 1c). Second, legume PDHs completely lost Tyr sensitivity (Schenck *et al.*, 2015) but *BvADH α* was still inhibited by Tyr at higher concentrations (Figs 2, S5). The maintenance of inhibition at higher Tyr concentration is likely necessary because Phe biosynthesis is also localized within the plastids, and thus *BvADH α* is directly competing for the arogenate substrate with Phe biosynthesis (Fig. 1a). Complete loss of ADH regulation by Tyr would, therefore, deplete Phe and essential Phe-derived compounds (e.g. proteins, lignin).

Other insensitive ADH/PDH enzymes have been found previously in microorganisms (Legrand *et al.*, 2006) and the structural analyses of Tyr-sensitive and -insensitive enzymes identified histidine 217 as a possible residue responsible for its Tyr sensitivity (Legrand *et al.*, 2006; Sun *et al.*, 2009). Also, phylogeny-guided structure–function analysis revealed that converting a single active site aspartate 222 residue into a nonacidic residue played a key role in the evolution of the legume PDH enzymes and simultaneously introduced prephenate substrate specificity and Tyr insensitivity (Schenck *et al.*, 2017). However, the corresponding histidine and aspartate residues are still present in *BvADH α* (Fig. S10), suggesting that different mechanisms, and as yet unidentified residues are involved in the relaxed Tyr sensitivity of *BvADH α* .

Previous analyses of molecular evolution of *DODA α* and *CYP76AD1 α* , two enzymes which convert Tyr into betalains (Christinet *et al.*, 2004; Gandía-Herrero & García-Carmona, 2012; Hatlestad *et al.*, 2012), revealed that both of these genes arose through gene duplication, just before the origin of betalain pigmentation in Caryophyllales (Brockington *et al.*, 2015). Likewise, this study found that *ADH α* orthologs arose by gene duplication, before the emergence of *DODA α* and *CYP76AD1 α*

(Fig. 4a,b), intimately associated with the origin of betalain pigmentation. One of the duplicated copies, *ADH α* , underwent neofunctionalization and became much less sensitive to Tyr inhibition, which is the key regulatory mechanism of Tyr biosynthesis (Rippert & Matringe, 2002a,b; Maeda & Dudareva, 2012). *ADH α* enzymes with relaxed Tyr sensitivity are maintained in all betalain-producing species of Caryophyllales, at least the ones that we analyzed (Figs 2, 5). Furthermore, the expression pattern of *BvADH α* is distinct from that of *BvADH β* and similar to those of the betalain biosynthetic genes (*DODA α* and *CYP76AD1 α*) and *MYB1* transcription factor (Fig. 1d), suggesting that the alteration of *ADH α* enzyme property was accompanied by changes in its expression profile. Although similar examples of biochemical and transcriptional changes during the evolution of plant specialized metabolic enzymes/genes have been reported (Weng *et al.*, 2012; Moghe & Last, 2015; Panchy *et al.*, 2016; Kajikawa *et al.*, 2017; Xu *et al.*, 2017), here we revealed a unique example of coordinated evolution of primary amino acid pathway (i.e. Tyr biosynthesis) and its downstream specialized metabolism (i.e. betalain biosynthesis).

In the anthocyanic Caryophyllaceae, the transition of betalain pigmentation to anthocyanin pigmentation was associated with down-regulation, relaxed natural selection, and deletion of *ADH α* (Figs 4, S1c,d, S9; Table S4). Similar downregulation and deletion of genes were also observed during the loss of flower petals (Zhang *et al.*, 2013) and arbuscular mycorrhizal symbiosis (Delaux *et al.*, 2014) in various plant lineages. Together these lines of evidence suggest that maintenance of *ADH α* is superfluous, following loss of betalain pigmentation. The ultimate cause of reversion of betalain to anthocyanin pigmentation in multiple lineages within the core Caryophyllales is currently unknown. It may be due to a number of factors, including: (1) metabolic cost of nitrogen-containing alkaloid betalain pigments, (2) shift in pollinator populations that are attracted by unique spectra (e.g. blue) of some anthocyanins, (3) increased demand for other Phe-derived compounds (e.g. tannins, flavonoids), or (4) simple genetic drift enabled by the presence of still intact Phe, phenylpropanoid, core flavonoid pathways in betalain-producing plants (Shimada *et al.*, 2005; Brockington *et al.*, 2011; Xu *et al.*, 2016).

A mechanism underlying the mutually exclusive distribution of betalain and anthocyanin pigments has long fascinated evolutionary biologists (Brockington *et al.*, 2011; Des Marais, 2015). Our analyses now provide one possible explanation. The relaxation of the Tyr-mediated feedback inhibition may direct more carbon flow towards Tyr, and away from Phe biosynthesis (Fig. 1a), as demonstrated by increased Tyr and decreased Phe levels upon transient expression of *ADH α* (Fig. 3). This may create a surplus of Tyr at the expense of Phe-derived products such as anthocyanins. Furthermore, betalain-producing, *ADH α* -containing core Caryophyllales species accumulated more Tyr than plants not possessing *ADH α* (Fig. 4c). The involvement of other factors such as transcriptional regulation of betalain, anthocyanin and Tyr/Phe pathway genes remain to be examined (Hatlestad *et al.*, 2015; Ambawat *et al.*, 2013); however our data provide a fascinating insight into the contribution of Tyr biosynthesis regulation to the evolution of a novel betalain pigment biosynthesis.

Prior heterologous reconstructions of specialized metabolic pathways resulted in significant accumulations of Tyr-derived plant natural products, such as a cyanogenic glycoside, dhurin, in *Arabidopsis* (*c.* 4% per DW; Tattersall *et al.*, 2001; Kristensen *et al.*, 2005) and betalains in tobacco (330 mg kg⁻¹ approaching red beet extract of 760 mg kg⁻¹, Polturak *et al.*, 2016, 2017). In other cases, however, *DODA* and *CYP76AD1* expression in *Arabidopsis* still required feeding of Tyr for betalain production (Harris *et al.*, 2012; Sunnadeniya *et al.*, 2016). Therefore, 'pulling' a precursor (e.g. Tyr) may not always be enough to efficiently produce its downstream product, and 'pushing' the precursor supply may be also important. Indeed, in red beets, increased Tyr concentrations have a strong positive correlation with enhanced accumulation of betalains (Wang *et al.*, 2017), suggesting that elevated production of Tyr plays important role in overall production of betalains. Over 100-fold increase in Tyr accumulation observed in *N. benthamiana* leaves expressing *ADH α* (Fig. 3) further demonstrates an exciting opportunity to introduce Caryophyllales *ADH α* enzymes into other plants and microbes, deregulate Tyr biosynthesis, and boost the availability of Tyr and the production of Tyr-derived products (e.g. vitamin E, isoquinoline alkaloids including morphine).

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Author contributions

S.L.N. and H.A.M. designed the research; Y.Y. and S.A.S. performed phylogenetic analysis; T.F. and S.F.B. characterized the *P. polygonifolia*, *S. marina* and *H. latifolia ADH α* ; A.T. and S.F.B. performed transient and RT-PCR of *BvADH α* and *BvADH β* expression in *N. benthamiana*; M.W. quantified amino acid concentrations of the *N. benthamiana* tissues and analyzed Tyr sensitivity of ADH activity from plant tissues; S.L.N. performed the rest of the experiments; S.L.N., Y.Y., S.F.B., M.W. and H.A.M. analyzed data; and S.L.N., Y.Y., S.F.B. and H.A.M. wrote the paper.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Physical location, homology and phylogeny of BvADH α and BvADH β .

Fig. S2 ADH but not PDH activity was detected from *B. vulgaris* tissues or recombinant enzyme.

Fig. S3 BvADHs prefer NADP⁺ over NAD⁺ as cofactor.

Fig. S4 No amino acid changes were found in the mature protein coding region of BvADH α among different *B. vulgaris* varieties.

Fig. S5 Recombinant His-tagged BvADH α also exhibits reduced sensitivity to Tyr relative to AtADH2.

Fig. S6 BvADHs are not inhibited by phenylalanine, tryptophan, and betanin.

Fig. S7 Transgene expression and tyrosine concentrations of individual leaf samples of infiltrated *Nicotiana benthamiana*.

Fig. S8 Tyr sensitivity of ADH activity from plant tissues.

Fig. S9 ADH α sequences used for testing relax selection.

Fig. S10 Histidine 217 residue responsible for Tyr sensitivity of *Aquifex aeolicus* PDH (AaPDH) is still present in BvADH α .

Table S1 Primers used as indicated in the text and methods

Table S2 Sequences of Caryophyllales (ingroups) and non-Caryophyllales (outgroups) used in this study

Table S3 Amino acid concentrations of *Nicotiana benthamiana* leaves expressing *GFP*, *BvADH α* or *BvADH β*

Table S4 RELAX analysis support the acceleration in amino acid substitution in Caryophyllales is due to relaxed purifying selection, instead of intensified positive selection

Methods S1 Relaxation of tyrosine pathway regulation underlies the evolution of betalain pigmentation in Caryophyllales.

Notes S1 Alignment of ADH amino acid sequences inferred using MAFFT.

Notes S2 ADH α codon alignment used in RELAX analysis.

Notes S3 Trimmed ADH α codon alignment used in RELAX analysis.

Notes S4 Trimmed ADH α amino acid alignment used in RELAX analysis.

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See also the Commentary on this article by Moghe & Smith, 217: 471–473.