Structural Evolution of Differential Amino Acid Effector Regulation in Plant Chorismate Mutases

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**Background:** Chorismate mutase is essential for aromatic amino acid biosynthesis.

**Results:** Structural and biochemical studies of three chorismate mutases from Arabidopsis reveal distinct sets of effector molecules.

**Conclusion:** Key residues in the effector site modulate the regulatory effects of ligands.

**Significance:** Evolution of effector control may lead to specialized regulation of this enzyme in plants.

Chorismate mutase converts chorismate into prephenate for aromatic amino acid biosynthesis. To understand the molecular basis of allosteric regulation in the plant chorismate mutases, we analyzed the three Arabidopsis thaliana chorismate mutase isoforms (AtCM1–3) and determined the x-ray crystal structures of AtCM1 in complex with phenylalanine and tyrosine. Functional analyses show a wider range of effector control in the Arabidopsis chorismate mutases than previously reported. AtCM1 is activated by tryptophan with phenylalanine and tyrosine acting as negative effectors; however, tryptophan, cysteine, and histidine activate AtCM3. AtCM2 is a nonallosteric form. The crystal structure of AtCM1 in complex with tyrosine and phenylalanine identifies differences in the effector sites of the allosterically regulated yeast enzyme and the other two Arabidopsis isofoms. Site-directed mutagenesis of residues in the effector site reveals key features leading to differential effector regulation in these enzymes. In AtCM1, mutations of Gly-213 abolish allosteric regulation, as observed in AtCM2. A second effector site position, Gly-149 in AtCM1 and Asp-132 in AtCM3, controls amino acid effector specificity in AtCM1 and AtCM3. Comparisons of chorismate mutases from multiple plants suggest that subtle differences in the effector site are conserved in different lineages and may lead to specialized regulation of this branch point enzyme.

Chorismate is a shikimate pathway-derived metabolite that exists at the branch point of aromatic metabolite synthesis in plants and microbes (1, 2). Chorismate can be converted into the aromatic amino acids phenylalanine, tyrosine, and tryptophan, as well as specialized metabolites like salicylic acid, anthocyanins, and lignin (2–5). In the biosynthesis of phenylalanine to tyrosine, chorismate mutase catalyzes the pericyclic Claisen rearrangement of chorismate to prephenate as the committed step in this pathway (Fig. 1) (6).

Although chorismate mutase activity is found in bacteria, fungi, and plants, the proteins that catalyze this reaction vary in both sequence and overall structure. The chorismate mutases from eukaryotes and most bacteria, also known as the AroQ, are typically dimeric α-helical proteins with each monomer consisting of ~250 amino acids (7). In some bacteria, such as Bacillus subtilis, the smaller dimeric AroH chorismate mutase consists of ~110 amino acid monomers (6, 8). The size difference between the two types of enzyme appears to be due to the presence of a regulatory region in the larger AroQ enzymes (9).

Within this regulatory region, an effector site binds aromatic amino acids to modulate enzymatic activity. For example, the effector site of Saccharomyces cerevisiae chorismate mutase (ScCM) (9) can bind either tryptophan or tyrosine (10). Tryptophan binding activates ScCM, and tyrosine leads to attenuation of prephenate synthesis. Thus, downstream metabolites provide reciprocal regulation of flux into either pathway leading from chorismate. This allows ScCM to divert chorismate flow from tryptophan synthesis to phenylalanine/tyrosine synthesis in high tryptophan conditions or to reduce phenylalanine/tyrosine synthesis through inhibition by downstream metabolites. Structural and biochemical studies of the yeast enzyme show that it contains an effector site in each monomer at the dimer interface and that binding of tyrosine and tryptophan alter the conformation of the enzyme between less active (T-state) and more active (R-state) forms, respectively (9–13).

In Arabidopsis thaliana (thale cress), three different chorismate mutase isofoms have been reported as follows: AtCM1, AtCM2, and AtCM3 (14–16). AtCM1 and AtCM3 both contain putative N-terminal plastid localization peptides, and AtCM2 is cytosolic (Fig. 2). In various plant species, chorismate mutase activity is found in both the plastid and cytosol (17–22). In petunia, a chorismate mutase homolog may be involved in the synthesis of phenylalanine-derived volatile phenylpropanoids and benzenoids (23). Biochemical studies using heterologous protein extracts of AtCM1 and AtCM3 suggest that both

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3 The abbreviations used are: ScCM, S. cerevisiae chorismate mutase; TSA, 8-hydroxy-2-oxa-biocyclo[3.3.1]non-6-ene-3,5-dicarboxylic acid; AtCM, A. thaliana chorismate mutase.
enzymes are regulated by aromatic amino acids (14–16); however, an analysis of the purified proteins was not performed. Although AtCM2 contains the putative regulatory effector binding domain, phenylalanine, tyrosine, and tryptophan do not affect its activity (15).

To understand the molecular basis of allosteric regulation in the plant chorismate mutases, here we examine the steady-state kinetic properties of the three Arabidopsis chorismate mutase isoforms and determine the x-ray crystal structures of AtCM1 in complex with phenylalanine and tyrosine. These analyses reveal a wider range of effector control in chorismate mutases of Arabidopsis than previously described for other plants and microbes. Specifically, AtCM1 is activated by tryptophan with phenylalanine and tyrosine acting as negative effectors; however, AtCM3 is activated by tryptophan, cysteine, and histidine. Site-directed mutagenesis of residues in the effector site that differ between AtCM1, AtCM2, and AtCM3 reveals key features leading to differential effector regulation in these enzymes. Moreover, sequence analysis of chorismate mutases from multiple plant species suggests that subtle differences in the effector site are conserved in different lineages.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were purchased from Sigma. Clones of the AtCM1 (U83587), AtCM2 (U18739), and AtCM3 (U60550) were obtained from the Arabidopsis Biological Resource Center.

**Generation of Bacterial Expression Constructs and Site-directed Mutagenesis**—The coding regions of AtCM1, AtCM2, and AtCM3 were PCR-amplified from respective TAIR cDNA clones. For AtCM2, an expression construct for N-terminally directed mutants of AtCM1 and AtCM3 were expressed and purified using the QuikChange PCR method (Stratagene).

**Protein Expression and Purification**—Expression constructs were transformed into *Escherichia coli* Rosetta II (DE3) cells (EMD Millipore). Cells were cultured in terrific broth until 

\[ A_{\text{opt}} = 0.6 - 0.8 \]

was obtained. Induction of protein expression used a final concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside overnight at 18 °C. Cells were pelleted by centrifugation and resuspended in 50 mM Tris, pH 8.0, 500 mM NaCl, 20 mM imidazole, 10% glycerol, and 1% Tween. Following sonication, cell debris was removed by centrifugation, and the resulting lysate was passed over a Ni²⁺-nitrilotriacetic acid column (Qiagen) column equilibrated in the lysis buffer. The column was then washed with 50 mM Tris, pH 8.0, 500 mM NaCl, 20 mM imidazole, and 10% glycerol. Bound His-tagged protein was eluted with 50 mM Tris, pH 8.0, 500 mM NaCl, 250 mM imidazole, and 10% glycerol. For protein crystallization, incubation with thrombin (1:2000 total protein) during overnight dialysis at 4 °C against wash buffer removed the His tag. Diazed protein was reloaded on a mixed benzanide-Sepharose/Ni²⁺-nitrilotriacetic acid column. The flow-through was loaded onto a Superdex-200 26/60 HiLoad FPLC size-exclusion column equilibrated with 25 mM Hepes, pH 7.5, and 100 mM NaCl. Protein concentration was determined by the Bradford method (Protein Assay, Bio-Rad) with bovine serum albumin as standard. Site-directed mutants of AtCM1 and AtCM3 were expressed and purified using the same methods as wild-type protein.

**Protein Crystallography**—Purified AtCM1 was concentrated to 9 mg mL⁻¹ and crystallized using the hanging drop vapor-diffusion method with a 2-μl drop (1:1 concentrated protein and crystallization buffer). Diffraction quality crystals were obtained at 4 °C with a crystallization buffer of 30% PEG-400, 0.1 M Hepes, pH 7.5, 0.2 M MgCl₂, and 1 mM of either phenylalanine or tyrosine. Crystals were flash-frozen in liquid nitrogen with mother liquor supplemented with 25% glycerol as a cryoprotectant. Diffraction data (100 K) was collected at the Argonne National Laboratory Advanced Photon Source 19-ID beamline. The data were indexed, scaled, and integrated with HKL3000 (24). Molecular replacement implemented in Phaser (25) using the yeast chorismate mutase (Protein Data Bank code 4CSM) as a search model was used to determine the structures of each AtCM1 complex. Iterative rounds of manual model building and refinement, which included translation-libration-screen models, used COOT (26) and PHENIX (27). Data collection and refinement statistics are summarized in Table 1. The final model of the AtCM1-phenylalanine complex included residues Arg-79–Val-290 and Val-307–Asp-340, the
phenylalanine ligand, and 83 waters. The final model of the AtCM1-tyrosine complex included residues Arg-79–Lys-289 and Val-307–Asp-340, the tyrosine ligand, and 130 waters. Coordinates and structure factors for AtCM1 complexed with phenylalanine (Protein Data Bank code 4PPV) and tyrosine (Protein Data Bank code 4PPV) and tyrosine complex included residues Arg-79–Lys-289 and 83 waters. The final model of the AtCM1 crystal structure about the sequence alignment.

**RESULTS**

**Functional Comparison of Arabidopsis Chorismate Mutases—**

Previous work on the three chorismate mutase isoforms from Arabidopsis (AtCM1–3) determined the $K_m$ value of chorismate and the general effects of the aromatic amino acids on activity for each enzyme; however, these studies were performed using yeast cell extracts (15, 16). To quantify the steady-state kinetic parameters of the AtCM isoforms, each enzyme was expressed in E. coli as N-terminal His-tagged protein and purified using nickel-affinity and size-exclusion chromatographies. All three isoforms were isolated as homodimeric forms (~65 kDa; monomer, ~32.6 kDa) for biochemical characterization.

Each AtCM isoform converted chorismate to prephenate but with clear differences in kinetic behavior (Fig. 3; Table 2). Both AtCM1 and AtCM2 followed Michaelis-Menten kinetics (Fig. 3, A and B). In contrast, AtCM3 displayed positive cooperativity with a Hill coefficient of 2.1 (Fig. 3C; Table 2), indicating that substrate binding at one active site of the homodimer enhanced interaction at the second active site. Weak cooperativity ($n = 1.2–1.5$) has been reported for chorismate mutases isolated from yeast, Nicotiana silvestris (flowering tobacco), and Solanum tuberosum (potato) (13, 19, 20). The catalytic efficiency ($k_{cat}/K_m$) of AtCM2 was 11- and 22-fold higher than that of AtCM1 and AtCM3, respectively. This results from a combination of a more rapid turnover rate and a lower $K_m$ value for chorismate displayed by AtCM2 compared with the other two isoforms. In earlier studies (15, 16), the inability to measure AtCM protein levels in cell extracts precluded estimation of the $k_{cat}$ value for each isoform. The turnover rates of purified AtCM1 (16.1 s$^{-1}$), AtCM2 (38.7 s$^{-1}$), and AtCM3 (13.0 s$^{-1}$) were up to 20-fold slower than the $k_{cat}$ of the yeast enzyme (387 s$^{-1}$) (13).

**Differential Regulation and Identification of New Effectors of Arabidopsis Chorismate Mutases—**

The previously reported differential feedback effects of aromatic amino acids on the Arabidopsis chorismate mutases were based on single concentrations of each effector in yeast cell extracts (15, 16). Using purified proteins, the effector regulation of each AtCM isoform was re-examined. None of the aromatic amino acids at concentrations up to 10 mM altered AtCM2 activity. Both AtCM1 and AtCM3 were sensitive to effector control but with different sets of amino acids.

To determine the effect of aromatic amino acids on AtCM1, the $EC_{50}$ values for tryptophan, phenylalanine, and tyrosine.
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were determined (Fig. 4A; Table 3). Tryptophan enhanced AtCM1 activity from 19.4 to 55.0 μmol min⁻¹ mg⁻¹ with an EC₅₀ of 2.6 μM (n = 0.8). Both tyrosine and phenylalanine reduced AtCM1 activity by roughly 20-fold. Although there were similar effects on turnover rates, tyrosine (EC₅₀ = 10.5 μM; n = 0.7) binds 5-fold better than phenylalanine (EC₅₀ = 49.8 μM; n = 1.2). Both of these aromatic amino acids were weaker effectors than tryptophan. Screening of the other 17 amino acids as possible effectors of AtCM1 showed no alterations in prephenate production. Similar results were also observed with AtCM2.

Binding of tryptophan to AtCM3 had a much larger effect than that observed for AtCM1 and led to a 6-fold increase in activity from 4.0 ± 0.1 to 25.0 ± 1.5 μmol min⁻¹ mg⁻¹ with an EC₅₀ of 5.50 ± 0.03 μM (n = 1.2) (Fig. 4B). In contrast to their negative effects on AtCM1, neither tyrosine nor phenylalanine altered AtCM3 activity. Surprisingly, screening of other amino acids as effectors of AtCM3 revealed that cysteine (EC₅₀ = 123 ± 1 μM; n = 1.6) and histidine (EC₅₀ = 31.6 ± 0.2 μM; n = 1.0) each activated enzymatic activity by ~3-fold (Fig. 4B).

Three-dimensional Structure of Arabidopsis Chorismate Mutase I—To understand effector regulation in the Arabidopsis chorismate mutases, we determined the x-ray crystal structures of AtCM1 in complex with phenylalanine and tyrosine at 2.4 and 2.3 Å resolution, respectively (Table 1). AtCM1 crystallized with one monomer in the asymmetric unit, which forms a crystallographic symmetry-related dimer (Fig. 5A). Eight α-helices comprise the core of each AtCM1 monomer. The symmetric dimer interface is made of four helices (α1, α2, α4, and α7) from monomer A interacting with the same helices from monomer B. The overall structure of AtCM1 is comparable with that of the yeast enzyme (6) with a root mean square deviation of 1.63 Å for 262 Cα atoms.

Comparison of AtCM1 and ScCM complexed with the transition state analog 8-hydroxy-2-oxa-bicyclo[3.3.1]non-6-ene-3,5-dicarboxylic acid (TSA) shows that the active site features of both enzymes are highly conserved (6). Fig. 5A shows the position of TSA modeled into AtCM1 based on structural alignment with ScCM. A cluster of α-helices (α1, α4, α7, and α8) surrounds the active site to position a set of highly conserved residues around the analog (Fig. 5B). Two catalytic residues (Arg-157 and Lys-168 in ScCM; Arg-229 and Lys-240 in AtCM1) are invariant across the AtCM isoforms (Fig. 2). These two basic residues are essential for substrate binding, orient the two negatively charged carboxylic acids of chorismate, and provide transition state stabilization during catalysis (6). In addition, Val-236, Thr-327, and Lys-328 in AtCM1 are retained in the other Arabidopsis isoforms and ScCM (Figs. 2 and 5B). Compared with ScCM, subtle side-chain differences in AtCM1 occur at Leu-264 (Ile-192 in ScCM), Met-324 (Ile-239 in ScCM), and Gln-331 (Glu-246 in ScCM).

Crystallization of AtCM1 with either phenylalanine or tyrosine yielded excellent electron density for each ligand (Fig. 5, C and D). The position of these ligands in AtCM1 clearly identifies the effector binding site at the dimer interface (Fig. 5A). In each structure, the N-terminal loop (residues 79–91) interacts with the side-chain guanidinium group of Arg-79 (2.7 Å), the backbone nitrogens of Gly-213 (3.1 Å), and Ser-214 (2.7 Å), and the carbonyl oxygen of Val-148 (3.3 Å). The side-chain oxygens of Asn-211 (2.9 Å) and Ser-214 (2.8 Å) hydrogen bond to the amine group of the bound amino acid. These contacts position the phenylalanine R-group into a space delineated by Gly-213, Val-217, Val-148, Gly-149, and Arg-150 with Asn-211, Gly-213, Ser-214, and Val-217 coming from chain B.

The structure of the AtCM1-phenylalanine complex reveals a set of hydrogen bond interactions that lock the ligand in the effector site (Fig. 6A). The carboxylate of phenylalanine interacts with the side-chain guanidinium group of Arg-79 (2.7 Å), the backbone nitrogens of Gly-213 (3.1 Å), and Ser-214 (2.7 Å), and the carbonyl oxygen of Val-148 (3.3 Å). The side-chain oxygens of Asn-211 (2.9 Å) and Ser-214 (2.8 Å) hydrogen bond to the amine group of the bound amino acid. These contacts position the phenylalanine R-group into a space delineated by Gly-213, Val-217, Val-148, Gly-149, and Arg-150. In the structure of AtCM1 complexed with tyrosine (Fig. 6B), a similar set of interactions are formed but with the addition of a hydrogen bond between the ligand hydroxyl group and Nε of Arg-83 (3.3 Å).

Although the structures of AtCM1 complexed with phenylalanine and tyrosine (Fig. 6, A and B) are similar to those of ScCM with tyrosine and tryptophan bound (Fig. 6, C and D) (10), differences exist in the effector sites of these two proteins. In AtCM1, Val-148 and Val-217 replace Ile-74 and Thr-145 from the yeast enzyme. Two striking differences between the plant and yeast enzymes were also observed. First, Gly-149 in AtCM1 replaces Arg-75, which interacts with the carboxylate group of amino acid effectors in ScCM. Second, the N-terminal loop of AtCM1 contributes an alternative basic residue (i.e. Arg-79) to interact with bound effector molecules.

Comparison of residues in the AtCM1 effector site with the corresponding positions in the unregulated AtCM2 and the differentially regulated AtCM3 suggests possible amino acid changes that lead to differences in regulation (Figs. 2 and 6A). Across the three AtCM isoforms, the residues corresponding to Val-148, Arg-150, Asn-211, and Ser-214 are invariant. The res-
the effector site, Arg-79, His-145, and Gly-213 of AtCM1 are replaced by aspartate, glutamine, and proline, respectively, in AtCM2. The effector site residues of AtCM3 are nearly identical to those of AtCM1 with the exception of an aspartate substitution for Gly-149.

To examine the effector site differences, we generated a series of site-directed mutants of Arabidopsis Chorismate Mutases—AtCM2. The effector site residues of AtCM3 are nearly identical to those of AtCM1 with the exception of an aspartate substitution for Gly-149.

Functional Analysis of Effector Site Differences on Regulation of Arabidopsis Chorismate Mutases—To examine the effector site differences, we generated a series of site-directed mutants for kinetic analysis. The first set of AtCM1 mutants probed changes to Arg-79, His-145, Gly-213, and Val-217. The R79K, H145Q, and V217T mutants had varied effects on the EC50 values for phenylalanine and tyrosine by 7- and 10-fold, respectively, but did not change either positive or negative effects on enzymatic activity (Table 3).

The subtle mutation of Arg-79 to a lysine increased the EC50 for phenylalanine and tyrosine but did not change values for the aromatic amino acid effectors but did not change either positive or negative effects on enzymatic activity (Table 3). The subtle mutation of Arg-79 to a lysine increased the EC50 for phenylalanine and tyrosine but retained an EC50 for tryptophan comparable to wild type. The H145Q mutant led to a

![Figure 3](image-url)  
**FIGURE 3.** Steady-state kinetic analysis of *A. thaliana* chorismate mutase isoforms. Velocity versus substrate curves are shown for AtCM1 (A), AtCM2 (B), and AtCM3 (C). A and B, data were fit to the Michaelis-Menten equation. C, the Hill equation for cooperative kinetics was used for data fitting. The dashed line shows a fit to the Michaelis-Menten equation. Values shown are the average ± S.E. for an n = 3.
The crystallographically determined position of phenylalanine in the active site was modeled in AtCM1 based on comparison with the yeast ScCM-TSA complex (green). C, electron density of phenylalanine in the AtCM1-phenylalanine complex is shown as a 2Fo − Fc omit map (1.5σ). D, electron density of tyrosine in the AtCM1-tyrosine complex is shown as a 2Fo − Fc omit map (1.5σ).

The ratio of effector/no effector specific activities for wild-type and mutant AtCM1 and AtCM3 are shown. All values are normalized to the no effector ratio. For each protein, bars correspond to ratios for tryptophan (white), phenylalanine (black), tyrosine (orange), cysteine (green), and histidine (blue). Concentrations for assays were 0.5 mM chorismate and 10 mM effector. Ratios were calculated from data with n = 3 and standard errors less than 10% of the mean.

DISCUSSION

Chorismate lies at an important branch point in the synthesis of aromatic amino acids and multiple specialized metabolites that contain aromatic groups (1–5). The enzymes that function at this branch point, including chorismate mutase, are tightly regulated. In contrast to the bacterial and yeast chorismate mutases, which have been extensively studied as models for allosteric control (6–13, 29, 30), the plant chorismate mutases are not well understood.

Earlier reports describe three isoforms in Arabidopsis, two of which are plastid-localized and regulated by aromatic amino acids (14–16). These previous studies of the Arabidopsis chorismate mutases relied on the analysis of proteins in yeast cell extracts, which contain residual phenylalanine, tyrosine, and tryptophan and complicated accurate assessment of the biochemical properties of the plant proteins. Kinetic analyses of purified AtCM1–3 revealed distinct biochemical and regulatory properties of each enzyme (Table 2; Figs. 3 and 4). For example, AtCM3 showed strong positive cooperativity, whereas the other isoforms followed Michaelis-Menten kinetics.
TABLE 4
Effect of aromatic amino acids on mutant AtCM1 and AtCM3

| Ligand | EC50 | $V_{max}$ | Fully bound $V_{max}$ |
|--------|------|----------|-----------------------|
|        | µM   | µmol min⁻¹ mg protein⁻¹ | µmol min⁻¹ mg protein⁻¹ |
| AtCM1 mutant | | | |
| G149D | Trp | 7.9 ± 0.1 | 1.49 ± 0.32 | 20.0 ± 0.5 |
| G149A | Trp | 11.7 ± 0.2 | 1.76 ± 0.12 | 14.9 ± 0.2 |
| AtCM3 mutant | | | |
| D132G | Trp | 1.4 ± 0.1 | 2.52 ± 0.42 | 9.65 ± 0.44 |
| D132G | Phe | 3.2 ± 0.1 | 2.85 ± 0.10 | 0.35 ± 0.06 |
| D132G | Tyr | 30.9 ± 2.0 | 2.63 ± 0.73 | 0.42 ± 0.10 |

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130 million years ago, do not encode AtCM2-like chorismate mutases (32).

The second clade of plant chorismate mutases can be divided into three sub-clades (Fig. 8, light green, blue, and dark green). AtCM1 homologs in the first sub-clade contain glycines at positions 149 and 213, and most members contain a putative N-terminal plastid localization sequence. This sub-clade only contains sequences from eudicot species with some species containing multiple isoforms. For example, soybean encodes five members of this sub-clade. The sequence homology suggests that members of this sub-clade will be regulated by tryptophan as an activator and with phenylalanine and tyrosine acting as negative regulators.

The second sub-clade includes AtCM3. Although phylogenetically this clade does not appear distinct, chorismate mutases grouped here consistently have an aspartate instead of a glycine at position 149, while retaining a glycine at 213 and the plastid localization sequence. This suggests that the members of this clade likely share the distinct effector control of AtCM3. Interestingly, only members of the family Brassicaceae contain AtCM3-like isoforms, which suggests a specialized role for these proteins in this group of plants. The regulation of AtCM3...
by tryptophan, cysteine, and histidine, all of which are synthesized in the chloroplast (1, 33, 34), may provide additional control and/or integration with sulfur and nitrogen metabolism. For example, the synthesis of indole glucosinolates requires both indole and sulfur-containing amino acids and activation of AtCM3, and related isofoms may support specialized metabolism (35). The structural and functional studies presented here suggest that AtCM1-like isoforms are essential for basal phenylalanine/tyrosine biosynthesis and that AtCM3-like isoforms may play a role in specialized metabolite production and stress responses in the Brassicaceae.

Chorismate mutases of the third sub-clade, which contains \textit{P. patens} and \textit{S. moellendorffii}, share effector sites that retain the two critical glycines like AtCM1 and have the putative plastid localization signal; however, these homologs are phylogenetically different from the other AtCM1-like sub-clades. This group is interesting as it contains species that diverged quite distantly, including the basal angiosperm \textit{Amborella}, along with monocots, such as rice and maize. Although the effector site sequences suggest members of this clade would be regulated like AtCM1, the lower ~50% amino acid sequence identity makes it interesting to see whether they share biochemical properties or whether they behave differently.

A final question remains. What is the role of the unregulated cytosolic chorismate mutase isoforms in plants? Since aromatic amino acid biosynthesis is localized in the plastid, AtCM2 could be involved in an alternative pathway (15). Besides tyrosine and phenylalanine biosynthesis, there is no other known role for prephenate. Moreover, the enzymes that use prephenate are localized to the plastid (31). Recent work has identified another route to phenylalanine in the cytosol that requires conversion of prephenate to phenylpyruvate followed by transamination to phenylalanine with tyrosine as a donor (36). Interestingly, while the first step of this alternative route appears to be plastidic, the final step is cytosolic. It is possible that AtCM2-like proteins could be essential for this alternative pathway, thus being linked to cytosolic phenylalanine synthesis. Moreover, the high catalytic efficiency of AtCM2 may be required in the cytosol where chorismate levels are likely lower than concentrations in the plastid. Ultimately, our studies of the three Arabidopsis chorismate mutase isoforms suggest that subtle changes may result in evolution of specialized regulation of these enzymes in plants.

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