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Pea formaldehyde-active class III alcohol dehydrogenase: Common derivation of the plant and animal forms but not of the corresponding ethanol-active forms (classes I and P)

(pea enzyme structure/alcohol dehydrogenase origin/separate duplications/formaldehyde dehydrogenase/parallel evolution)

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ABSTRACT **A plant class III alcohol dehydrogenase (or glutathione-dependent formaldehyde dehydrogenase) has been characterized. The enzyme is a typical class III member with enzymatic parameters and substrate specificity closely related to those of already established animal forms. K_m values with the pea enzyme are 6.5 μM for NAD^+, 2 μM for *S*-hydroxymethylglutathione, and 840 μM for octanol versus 9, 4, and 1200 μM, respectively, with the human enzyme. Structurally, the pea/human class III enzymes are closely related, exhibiting a residue identity of 69% and with only 3 of 23 residues differing among those often considered in substrate and coenzyme binding. In contrast, the corresponding ethanol-active enzymes, the long-known human liver and pea alcohol dehydrogenases, differ more (47% residue identities) and are also in functionally important active site segments, with 12 of the 23 positions exchanged, including no less than 7 at the usually much conserved coenzyme-binding segment. These differences affect functionally important residues that are often class-distinguishing, such as those at positions 48, 51, and 115, where the plant ethanol-active forms resemble class III (Thr, Tyr, and Arg, respectively) rather than the animal ethanol-active class I forms (typically Ser, His, and Asp, respectively). Calculations of phylogenetic trees support the conclusions from functional residues in subgrouping plant ethanol-active dehydrogenases and the animal ethanol-active enzymes (class I) as separate descendants from the class III line. It appears that the classical plant alcohol dehydrogenases (now called class P) have a duplicatory origin separate from that of the animal class I enzymes and therefore a paralogous relationship with functional convergence of their alcohol substrate specificity. Combined, the results establish the conserved nature of class III also in plants, and contribute to the molecular and functional understanding of alcohol dehydrogenases by defining two branches of plant enzymes into the system.**

Different sets of dimeric zinc-containing alcohol dehydrogenases of the medium-chain dehydrogenase/reductase (MDR) (1) type have been characterized in animals and plants. One set encompasses animal alcohol dehydrogenases, including the classical, ethanol-active liver enzyme of class I with about 20 characterized enzymes (2–5), the apparently parent (6, 7) class III form [or glutathione-dependent formaldehyde dehydrogenase, present also in prokaryotes (8, 9)], and a total of minimally six classes (10) and some mixed-class lines (11, 12) in vertebrates. The other is the set of plant alcohol dehydrogenases, of which about 20 enzymes have been structurally characterized (3, 4, 13). Further, MDR alcohol dehydrogenases, but of a tetrameric type (including the yeast enzyme), as well as other alcohol dehydrogenases (including the short-chain dehydrogenase/reductase, SDR, forms), also exist (see ref. 14). The plant and animal alcohol dehydrogenases, although definitely related (4, 15), raise questions about the class III forms in plants and about the interrelationships of the ethanol-active forms in plants and animals.

The two sets of animal and plant ethanol-active enzymes were initially compared (15) before knowledge of the enzyme system at large and of the repeated gene duplications in the animal line. The latter have been traced to early vertebrate evolution, with class III as the ancestral type, as supported by estimates of the divergence rate (16), the presence of mixed-class vertebrate forms presumably reflecting the enzymogenesis (11, 12), and the presence of class III forms in invertebrates (17, 18) and prokaryotes (8, 9). In short, the animal enzyme system appears to originate from class III, which is hardly ethanol-active, and to have evolved into the other classes, including the ethanol-active class I liver enzyme, during vertebrate radiation. However, the structurally characterized plant enzymes exhibit reasonable ethanol activity, like the class I animal enzymes, and structural similarity to the class I proteins (4, 15). Furthermore, although a plant class III enzyme exists and has been partially purified as a formaldehyde dehydrogenase activity (19), no class III plant enzyme has been structurally characterized. The apparently ancestral class III line of the animal set has not been similarly defined in the plant set, in spite of the early class III origin. At the same time, the vertebrate ethanol-active classes of supposedly later origin appear to have equivalents in the plant line, which, if they reflect direct descendance from a common origin, would suggest an earlier origin than that postulated from the pattern of the animal classes alone. Clearly, this complicates the evolutionary scheme: either class III would be expected to be a distant ancestor also in the plant line and the vertebrate-specific enzyme classes not to be codescendants in one clade with the ethanol-active plant line, or the origin of the animal forms has to be reconsidered to include the conclusions from the plant enzymes. The unresolved relationships question interpretations on the origins and functions of the eukaryotic alcohol dehydrogenases at large, also making this complex enzyme system unclear in humans.

We have now characterized a plant class III enzyme from pea. Both its enzymatic and structural properties are clearly related to those of the class III enzyme of other sources, establishing that this class is universally constant in all life forms. The structure further establishes the relationships among the ethanol-active lines. They exhibit parallel evolu-

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Abbreviation: MDR, medium-chain dehydrogenase/reductase.
Data deposition: The sequence reported in this paper has been deposited in the Swissprot data base (accession no. P80572).

Table 1. Catalytic parameters of pea class III alcohol dehydrogenase

Substrate	K_m, μM	k_{cat}, min^{-1}
Ethanol	NS [NS]	
Octanol	840 [1200]	190 [220]
12-OH-dodecanoic acid	180 [60]	110 [170]
NAD^+	6.5 [9]	
HM-GSH	2 [4]	380 [200]

Measurements were made at pH 10.0 for the ordinary alcohols and at pH 8.0 for the glutathione-conjugated formaldehyde reaction. For comparison, corresponding values for the human class III protein (18, 20, 21) are also given within square brackets. NS, no saturation. HM-GSH (*S*-hydroxymethylglutathione) is a product of the spontaneous reaction between formaldehyde and glutathione (21).

tionary patterns in plants and animals and suggest a functional convergence toward ethanol dehydrogenase activity.

MATERIALS AND METHODS

Protein Purification. Dried peas (*Pisum sativum*; 100 g) were soaked in distilled water for 24 h and homogenized in 0.1 M Tris·HCl, pH 8.5/1 mM dithioerythritol. The homogenate was filtered through cheese cloth and centrifuged at 48,000 × *g* for 1 h. The supernatant was extensively dialyzed against 20 mM Tris·HCl, pH 8.5 (6 × 5 liters), and applied to a DEAE-Sepharose fast flow column (2.5 × 30 cm) equilibrated in the same buffer. After washing with the initial buffer, the material was eluted with a linear gradient of 0–0.5 M NaCl. Fractions with high specific activities were pooled, dialyzed against 25 mM sodium phosphate buffer (pH 7.5) with 0.1 mM dithioerythritol, and applied to an AMP-Sepharose column (2 × 7 cm). After washing with 50 mM of the same buffer, class III alcohol dehydrogenase was eluted with a linear gradient of 0–2 mM NAD^+. Class III active fractions were pooled and concentrated in an Amicon cell using a PM10 Diaflo membrane and finally purified by fast protein liquid chromatography (FPLC) on a Mono Q column (HR5/5) in 10 mM Tris·HCl (pH 8.0), with a linear gradient of 0–0.5 M NaCl.

Structural Analysis. The protein was reduced and ^{14}C-carboxymethylated as described for other alcohol dehydrogenases (16). Digestions with Lys-C protease, Glu-C protease, and Asp-N protease were performed in 0.1 M ammonium bicarbonate for 4 h at 37°C with protease/substrate ratios of 1:10–1:100, by weight. All digests were fractionated by reverse-phase HPLC on a Vydac C18 with a gradient of acetonitrile in 0.1 M trifluoroacetic acid. Peptides obtained were submitted to sequencer degradation with Applied Biosystems model 477A and MilliGen model 6600/6625 Prosequencer instruments equipped with on-line phenylthiohydantoin identifications. Total compositions were determined by amino acid analysis on a Pharmacia AlphaPlus instrument after hydrolysis at 110°C for 24 h with 6 M HCl and 0.5% phenol.

Enzymatic Measurements. Enzyme activities were determined by monitoring the formation of NADH at 340 nm with a Hitachi model 3000 spectrophotometer. K_m and k_{cat} values were measured for alcohols in 0.1 M glycine-NaOH at pH 10.0 (18, 20) and for glutathione/formaldehyde and NAD^+ in 0.1 M sodium pyrophosphate at pH 8.0 (21). Values were calculated with the program ENZYME (22). Values for the corresponding human enzyme were taken from refs. 18 and 20. Activity staining in gels used the phenazine methosulfate/Nitro Blue tetrazolium method.

Alignments, Functional Assignments, and Phylogenetic Trees. Alignments were constructed and phylogenetic trees were calculated, with corrections for multiple substitutions, by using the program CLUSTALW (23). The trees were drawn with the program TREETOOL (Genetic Data Environment, Cambridge, MA). Functional assignments of residue positions are from refs. 24–27 as originally determined by x-ray crystallography of the horse class I enzyme (24).

RESULTS

Plant Class III Alcohol Dehydrogenase. This protein was purified from peas by a three-step chromatographic procedure on DEAE-Sepharose, AMP-Sepharose, and MonoQ FPLC. The protein was obtained in a 16% yield after a 1400-fold purification, had a specific activity of 5.6 units/mg, and was homogeneous on SDS/PAGE with a subunit molecular mass of about 40,000 Da. These values are similar to those of an early purification which yielded an unstable product (19), although yields are lower and specific activity higher now than previously. Stability was now sufficient for enzymatic characterization. The enzyme showed selective activity toward glutathione-conjugated formaldehyde, reasonable activity toward pentanol and long-chain alcohols, and low activity toward ethanol, with turnover rates and K_m values closely similar to those of the human and animal class III enzymes (Table 1). Peptide sequence analysis of the ^{14}C-carboxymethylated protein (Fig. 1) also established the class III nature of this pea enzyme. It is far more related to the human (and animal) class III enzymes than to the pea (or other plant) ethanol-active alcohol dehydrogenase (Table 2). All regions of the protein chain were recovered by analysis of peptides from three digests. Formally, three segment connections were not obtained in overlapping peptides, but these segments are aligned from homology, and direct continuities are also supported by agreement with the total composition from acid hydrolysis of the whole protein chain. The N terminus is acetylated as in other alcohol dehydrogenases (28). The C terminus was proven as Asp-378 by identical ends of the C-terminal peptide from two different peptide sets (with Lys- and Glu-specific

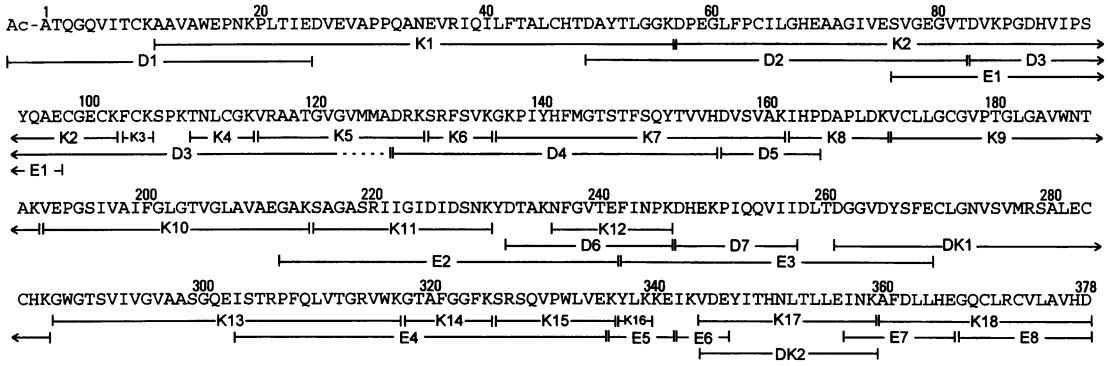

FIG. 1. Primary structure of the pea class III alcohol dehydrogenase now characterized by peptide analysis. Peptides purified are indicated by the line designations, marked K, E, D for generation by cleavages with Lys-C, Glu-C, and Asp-N protease, respectively, and by solid lines showing the extent of analysis by sequencer degradations.

Table 2. Relationship of the present plant enzyme (pea class III) to the previously known ethanol-active (pea class P) and human (class I) alcohol dehydrogenases and to the human glutathione-dependent formaldehyde-active class III form

	% residue identity		
	Pea class P	Human class I	Human class III
Pea class III	58	53	69
Pea class P		47	51
Human class I			63

For class I, calculations refer to the γ isozyme type.

proteases, respectively), none of which is expected to produce peptides with this end in appreciable yield. It was concluded that the class III alcohol dehydrogenase in plants was a 378-residue subunit (Fig. 1), closely related to the class III enzymes already known in animals (18) and yeast (29–31), thus establishing the universal occurrence and conserved nature of this enzyme in all eukaryotic lines.

Interrelationships of Ethanol-Active Plant and Animal Alcohol Dehydrogenase Classes. With the establishment of a common class III-type structure in animals and plants, it is possible to also evaluate the ethanol-active forms in these lines. It was noticed (Table 2) that the ethanol-active pea enzyme (this enzyme has been characterized from other plants) was more closely related to class III (animals or plants) than to the class I forms (from animals) with which it has hitherto been compared. Hence, while the animal and plant class III enzymes are closely related, the corresponding ethanol-active forms are not. These findings are still more pronounced when functional residues at the active sites are considered. Those residues are frequently conserved in substrate and coenzyme interactions as directly determined in the human/horse class I forms (24, 25) and the mixed-class bony fish form (26), and deduced to be valid also for the other classes (27), MDR alcohol dehydrogenases at large (32), and cinnamyl alcohol dehydrogenase (33, 34). Of 23 such residues, all but 3 are conserved between the plant and human class III forms (Fig. 2). Even these actual exchanges are minor; they affect residues with similar properties. This establishes a strict functional conservation of class III over all eukaryotes. In contrast, for the ethanol-active pea/human enzyme pair, the functional replacements are many (12 of 23 residues) and frequently affect residues with altered properties (Fig. 2). Furthermore, no less than seven of these exchanges affect the coenzyme-binding site, which is otherwise often the most conserved segment in alcohol dehydrogenases (5). Hence, the functional residues (Fig. 2) support the overall values (Table 2) in showing extensive differences between the plant and animal ethanol-active enzymes. These differences resemble the differences among classes in the animal line. The human (animal) and plant ethanol-active

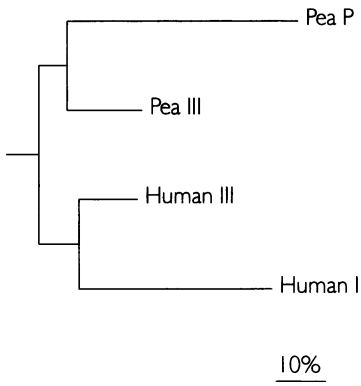

FIG. 3. Phylogenetic tree for the two sets of pea and human enzymes—i.e., the glutathione/formaldehyde-active class III and the ethanol-active P and I classes, respectively. Calculation with the program CLUSTALW (23) and using the prokaryotic *Haemophilus influenzae* enzyme as outgroup. Individual boostrap numbers are not significant, but identical topology is obtained with calculations based on the entire structures and those based on each of the two domains (catalytic and coenzyme-binding) separately.

enzymes, although related (4, 15), need therefore not belong to the same class (i.e., not be derived from the same ancestral gene duplication). The ethanol-active plant line may then be called class P to distinguish it from the animal class I line.

Scrutiny of the residue differences between classes P and I supports the separate derivation of the plant class P and animal class I enzymes. Several of the residue differences between these two enzyme types affect positions typical of the class distinctions also seen in the human/animal line (18). In particular, position 51 in class P is Tyr as in all known class III forms, but His in all class I vertebrate forms except bony fish, and position 48 is Thr in class P as in all class III forms, but Ser in all class I vertebrate, nonprimate forms. Moreover, and most importantly, residue 115 is Arg in all class III enzymes, and this residue has been ascribed an essential role in anion activation and substrate recognition (20, 35, 36), presumably constituting a glutathione binding site characteristic of the formaldehyde dehydrogenase activity [together with Asp-57 (37)]. Interestingly, this functionally important position also has Arg-115 in the pea class P enzyme, and in fact in all class P structures reported (3, 4), but never in the animal class I enzymes (Fig. 2). Hence, not only overall values but also critical residues show that class P exhibits extensive differences toward class I, but also resemblance with class III, suggesting that class P might be derived from class III through a gene duplication in a plant ancestor, as is already apparent for class I from class III in the human (animal) line. This conclusion is supported by calculation of a phylogenetic tree that encompasses all four

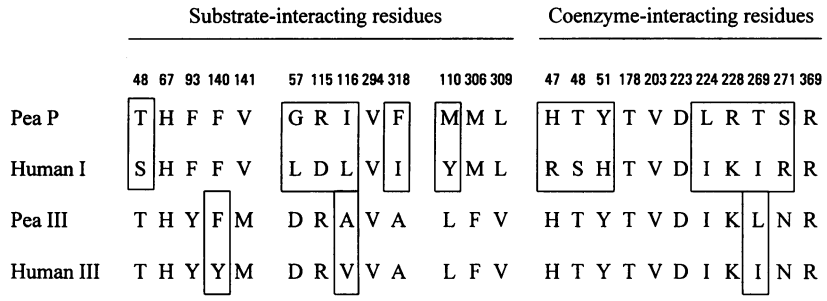

FIG. 2. Residues at functionally important positions in substrate binding and coenzyme binding for the two sets of pea and human enzymes of both types, class III (bottom) and ethanol active, classes P and I, respectively (top). Boxes indicate residue differences within each of the class P/I and class III enzyme pairs. The smaller differences in the class III pair, and the larger differences in the class P/I pair, which are also in the coenzyme-binding segment, are obvious. For class I, the γ isozyme type is the one shown. Positions of the interacting residues are derived from refs. 24–27 as originally determined by x-ray crystallography of the horse class I enzyme (24).

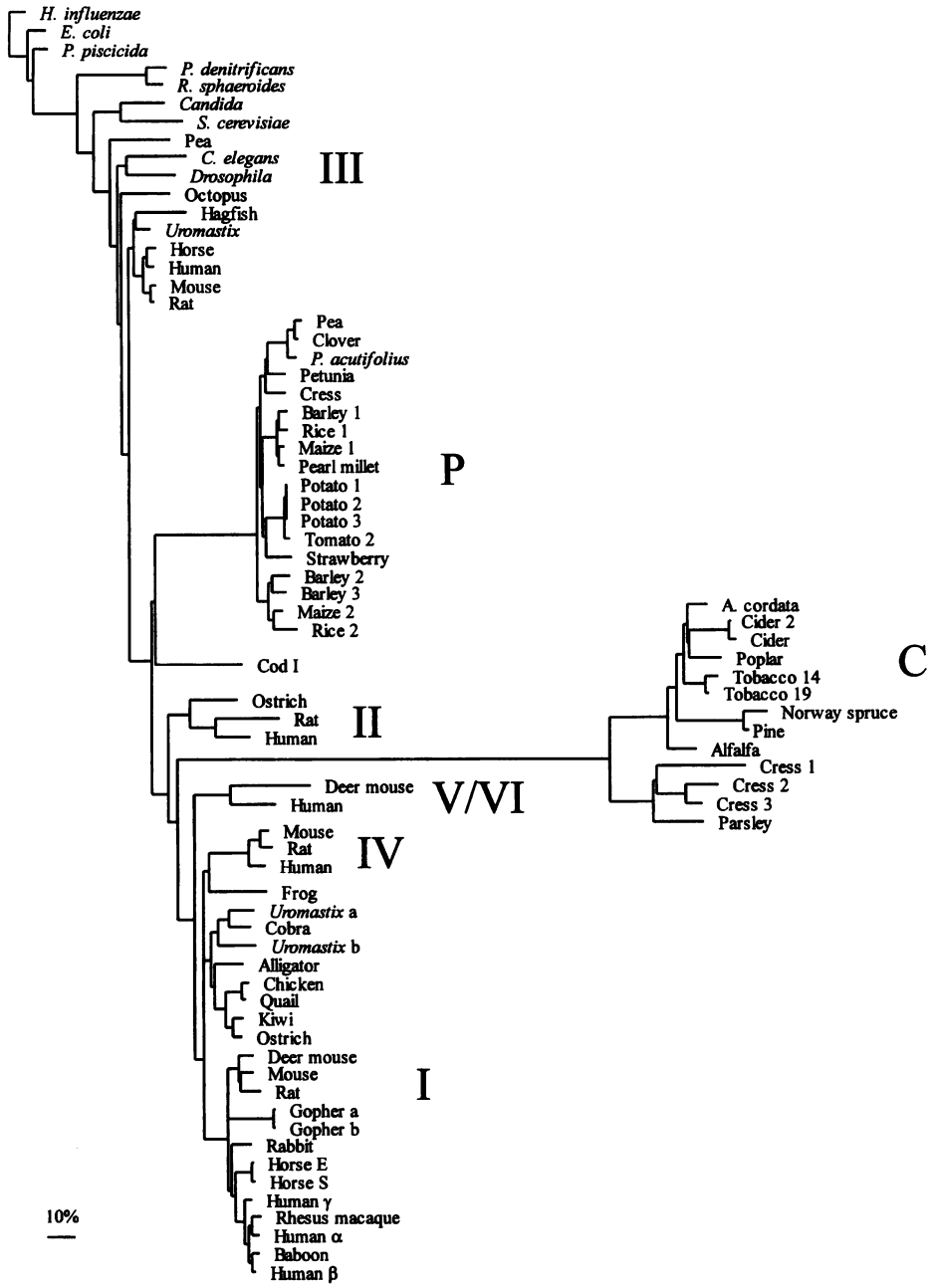

FIG. 4. Phylogenetic tree of all characterized forms of the plant and animal alcohol dehydrogenases, including the plant cinnamyl alcohol dehydrogenases. Four separate groupings are obvious, the animal classes I/IV/V/VI group, the plant class P group, the animal/plant class III group, and the plant cinnamyl alcohol dehydrogenase, group C. The mixed-class cod class I enzyme (11) and the class II forms, with a mixed-class class II form (12), are still not significantly resolved, presumably because of their rapid initial changes and present mixed-class properties. For these two subgroups and the group C forms, early branching is therefore still not considered final because of the closely positioned branch points.

enzymes concerned—i.e., the plant and animal forms of both class III and the major ethanol-active enzyme (Fig. 3). In this tree, class P originates separately from class I. Hence, they are concluded to be paralogously related and reflect different ancestral duplications in the plant and animal lines, respectively.

DISCUSSION

The present results establish the nature of a plant class III alcohol dehydrogenase. It is highly conserved versus other forms of the class III enzyme; therefore, in structural terms these data confirm (4, 19, 21) and quantify the universal presence of the glutathione-dependent formaldehyde dehydrogenase activity. Major functional segments in all eukaryotic lines from human/animals (18) to plants (this work) and fungi (29–31), and apparently also prokaryotes (8, 9), are structurally conserved. This establishes class III as an early form of common occurrence and structure, participating in basic defense mechanisms of formaldehyde elimination.

The results further interrelate the ethanol-active enzyme classes and suggest separate duplicatory origins (Fig. 3) for these classes in animals (class I) and plants (class P). In both cases they have apparently evolved from the diverging class III line by changes at active sites to acquire related substrate specificities. Hence, the class P and I lines are concluded to represent functional convergence.

Further additions of all characterized plant and human/animal alcohol dehydrogenases outline the separate subgroups (Fig. 4). The cinnamyl alcohol dehydrogenase (group

C in Fig. 4) in lignin biosynthesis also belongs to the MDR alcohol dehydrogenase family (38) and has been modeled into the same overall conformation (33, 34), proving the consistent pattern and giving at least four subgroups (Fig. 4). Interestingly, all four are structurally about equally well separated, although functionally they represent different stages of enzyme evolution. Thus, the alcohol dehydrogenase and cinnamyl alcohol dehydrogenase have separate specificities and are involved in different metabolic schemes. This applies to the alcohol and formaldehyde dehydrogenases as well; they also have some common substrates (long-chain aliphatic alcohols), whereas the plant class P and animal/human class I enzymes are more similar in substrate specificity.

The groupings do not yet allow judgement of some of the early details. This is expected, because two of the descendant forms still exhibit mixed-class properties, as evidenced in the cod class I (11) and ratite class II (12) forms, with presumably rapid initial evolutionary changes nonlinear with later changes, hence blurring the patterns. This is especially true for the positions of the origins of the cod class I enzyme, the vertebrate class II subgroup, and the plant cinnamyl alcohol dehydrogenase group, which do not appear significant in the present scheme, as obvious by short interbranch separations (Fig. 4). Their positions are especially sensitive to the alignments, which are not clear in all segments, and to the program used for evaluation of the interrelationships. Although further forms of early class I, II, and C interconnecting structures are therefore desirable, the present number of species is sufficient to establish the conserved nature of class III also in plants. The results contribute to the molecular and functional understanding of the alcohol dehydrogenase system, complicating it by adding one further gene duplication in the development of the plant ethanol-active enzyme (Fig. 3), but unifying concepts by bringing branches P and III of the plant alcohol dehydrogenases into the general system (Figs. 3 and 4).

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