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IMMUNOCHEMICAL COMPARISONS OF RIBULOSEBISPHOSPHATE CARBOXYLASES USING ANTISERA TO TOBACCO AND SPINACH ENZYMES

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Key Word Index—*Nicotiana tabacum Spinacia oleracea*; Solanaceae; Chenopodiaceae; microcomplement fixation; phylogenetic survey; ribulosebisphosphate carboxylase.

Abstract—Ribulosebisphosphate carboxylase molecules from over 50 species of angiosperms and gymnosperms have been compared by quantitative microcomplement fixation, using antisera prepared against tobacco and spinach enzymes. There were close antigenic similarities between tobacco enzyme, enzymes from other members of the Solanaceae, and enzymes from members of the Nolanaceae, Cuscutaceae, and Convolvulaceae. There were relatively close similarities between spinach enzyme and enzymes from two other members of the Chenopodiaceae. There were relatively great differences between tobacco enzyme, spinach enzyme, and most other enzymes tested. The enzymes from most of the angiosperms tested were as different from tobacco enzyme and almost as different from spinach enzyme as were the enzymes from the gymnosperms.

INTRODUCTION

Ribulosebisphosphate (RuBP) carboxylase catalyzes an essential carbon-fixation step of photosynthesis in all types of green plants, from lower algae to the most advanced C-3 and C-4 angiosperms [1,2]. A determination of the ancestral relationships between enzymes from a selection of plant species, obtained through a comparison of the enzymes' structures, could help unravel the evolutionary relationships between the species themselves.

RuBP carboxylase is a good antigen and is well suited for comparisons by immunochemical techniques [3–6]. Champion *et al.* [7] and Wallace and Boulter [8] have shown that for many proteins there is quantitative relationship between an immunochemical measure of difference, microcomplement fixation, and measures of primary structure difference. In this report, I present comparisons by microcomplement fixation of RuBP carboxylases from 48 angiosperm and 5 gymnosperm species (representing 42 families) using antiserum prepared against the enzyme from *Nicotiana tabacum* var Xanthi and of enzymes from 53 angiosperm and 5 gymnosperm species (48 families) using antiserum to the enzyme from *Spinacia oleracea*.

RESULTS

Comparing tobacco with closely related species

With the antiserum prepared to tobacco enzyme, the values of 'immunological distance' (I.D.) for extracts of 5 different species of the Solanaceae were very low, ranging from 1.2 to 8.4 (Table 1). In fact, the values for two species, *Lycopersicon esculentum* and *Petunia hybrida*, were not significantly different from zero, indicating that the RuBP carboxylases in these species were antigenically identical to that in tobacco. In contrast, I.D. values were much higher for members of other families. representatives of 39 families (all but three of those tested with anti-tobacco antisperm) had I.D. values above 50 (mean 83.1 ± 12.2—Tables 1–3). I conclude that the antigenic structure of RuBP carboxylase from plants of the Solanaceae is a unique family characteristic: the immunochemical comparisons using antiserum to tobacco RuBP carboxylase can be considered a powerful tool for detecting a close relationship between the Solanaceae and other families.

Table 1 shows the values of I.D. obtained using extracts from representatives of families grouped in a single order with the Solanaceae according to taxonomic schemes of Cronquist [9], Takhtajan [10], Dahlgren [11] and Thorne [12]. The value for *Nolana humifusa*, a member of the Nolanaceae, was not significantly different from those found with the Solanaceae. The values for *Cuscuta campestris* and *Convolvulus arvenis* were lower than those for other families listed except the Solanaceae and Nolanaceae. In contrast, representatives of the Polemoniaceae, Hydrophyllaceae, Scrophulariaceae, Gesneriaceae, Myoporaceae, and Fouquieriaceae gave I.D. values that were no lower (and possibly even higher) than those observed with families traditionally thought to be less closely related.

The data suggest the conclusion that the Solanaceae are closely related to the Nolanaceae and fairly closely related to the Cuscutaceae and Convolvulaceae. The very low I.D. found for *Nolana* strongly supports the suggestion [12] that the Nolanaceae should be placed in the same family as the Solanaceae; the moderately low I.D. values found for *Cuscuta* and *Convolvulus* support the ordinal grouping of their families with the Solanaceae [9, 11, 12]. The data provide no support for any of the other alliances mentioned in Table 1.

Comparing spinach with closely related species

The antiserum against spinach enzyme also showed high specificity. The I.D. for crude spinach extract was −3.0 (indistinguishable from the purified enzyme used as a standard), while representatives of 47 families

Table 1. Reactions of anti-tobacco RuBP carboxylase with extracts from plants of the Solanaceae and related families

Species, I.D.*	Mean I.D. for species
Solanaceae	
Lycopersicon esculentum Mill., −3.0, 10.3; 0.0, 0.8, −2.1	1.2
Solanum tubersum L., 5.6	5.6
Petunia hybrida Vilm., −0.8, 3.4	1.3
Capsicum frutescens L. var grossum, 8.2; 8.6	8.4
Datura candida (Pers.) Safford, 5.8	5.8
Families possibly related to Solanaceae †‡§	
Nolana humifusa (Gouan) I. M. Johnston (Nolanaceae)†‡§‖ 3.0, 7.9	5.5
Cuscuta campestris Yunk. (Cuscutaceae)†§‖ 19.8; 12.0, 20.2	17.3
Convolvulus arvensis L. (Convolvulaceae)†§‖ 21.5, 24.5; 27.1, 30.5	25.9
Phlox drummondii Hook. (Polemoniaceae)†§‖ 78.7; 82.6	80.6
Nemophila menziesii Hook. and Arn (Hydrophyllaceae)†§ 86.0	86.0
Antirrhinum majus L. (Scrophulariaceae)‡ 49.0; 56.7	52.9
Digitalis purpurea L. (Scrophulariaceae)‡ 87.8; 99.5, 107.3	98.2
Paulownia tomentosa Steud (Scrophulariaceae)‡ 78.7; 98.9	88.8
Aeschynanthus lobbiana Hook. (Gesneriaceae)‡ 99.8, 108.4	104.1
Myoporum acuminatum R. Br. (Myoporaceae)‡ 100.2, 101.1	100.6
Fouquieria diquetii (Van Tieghem) I. M. Johnston (Fouquieriaceae)‖ 74.4, 83.2	78.8

*I.D. = 'immunological distance'. Each value represents a separate experiment. Values separated by a comma represent separate assays of the same extract; values separated by a semicolon represent assays of different extracts.

†Family ordinally grouped with Solanaceae by Cronquist [9].

‡Family ordinally grouped with Solanaceae by Takhtajan [10].

§Family ordinally grouped with Solanaceae by Dahlgren [11].

‖Family ordinally grouped with Solanaceae by Thorne [12]

(all but Chenopodiaceae) had I.D. values over 25 (mean 56.7 ± 16.3) (Tables 2 and 3). The specificity of spinach antiserum was perhaps slightly less than that of tobacco antiserum: the I.D. for tobacco measured using spinach antiserum was 74.5, less than the I.D. for spinach measured using tobacco antiserum, 80.8 The difference appears to be minor and can be easily considered in the interpretation of the data.

Values of I.D. measured for two species of Chenopodiaceae, *Salsola iberica* (14.0) and *Beta vulgaris* (25.2), were lower than that of any other species except spinach itself. However, the values were higher than corresponding values of members of the Solanaceae measured with tobacco antiserum. Low I.D. values were also found with *Dianthus* (27.4) and *Petroselinum* (25.8): the first is traditionally considered closely related to spinach; the second is not [9, 10]. Taken together, these results suggest that *S. oleracea*, *S. iberica* and *B. vulgaris* do not form as closely related a family as do the Solanaceae.

Comparing tobacco and spinach with plants in more distant taxa

Tables 2 and 3 give the I.D. values obtained with extracts from plants in all the major dicot subclasses and from some monocots and gymnosperms. Dicot subclasses follow assignments of Cronquist [9], except that the Asteridae have been divided into two sections following the results with tobacco antiserum discussed above.

With tobacco antiserum the average values for all groups, including all the dicot subclasses (but excluding the Solanaceae, Nolanaceae, Cuscutaceae, and Convolvulaceae), the monocots considered as a single group, and the gymnosperms considered as a single group, were very similar. Pairwise comparisons by Student's t test indicated a significant difference ($P < 5\%$) between the value for the Rosidae and the value for the Asteridae (including families such as the Polemoniaceae, Scrophulariaceae, etc.) but no significant difference ($P > 10\%$) between any other pair of values. The best conclusion is that these values were drawn from a single population of values and that the RuBP carboxylase molecules of the groups were equally distantly related to that of tobacco.

With spinach antiserum, the means for the Rosidae, Hamamelidae, Dilleniidae, Magnoliidae, and the monocots could not be distinguished from one another by t test ($P > 10\%$). In contrast, the mean I.D. values for the group containing the Solanaceae, Nolanaceae, Cuscutaceae and Convolvulaceae and for the gymnosperms were significantly higher than the means of the former groups ($P < 0.6\%$). The mean for the Asteridae (excluding the Solanaceae, etc.) could not be distinguished from any of the other groups with the data available.

DISCUSSION

One way to interpret the above data uses the 'evolutionary clock' hypothesis, which assumes that mutation, selection and genetic drift change the amino acid sequence [13] and the antigenic structure [14] of a protein at a fairly regular and constant rate in different ancestral lines over evolutionary time. If this holds true for RuBP carboxylase, I.D. values measured for proteins in different species will be approximately proportional to the length of time that has elapsed since the ancestral lines leading to these species diverged from the line leading to the species to which the antiserum was directed. In applying this hypothesis to my data, I assume that sets of plants mentioned below (e.g. dicot subclasses, monocots, gymnosperms) represent natural groups, each of which diverged from the line leading to tobacco or spinach at a particular time. Under this assumption, it is appropriate to measure the difference between tobacco or spinach and each group by using the mean I.D. for the group. This procedure minimizes unreasonable conclusions based on individual species, the proteins of which may have changed at abnormal rates (see below). The procedure risks error, however, in the choice of natural groups: for instance, Thorne [15] has summarized evidence that the Hamamelidae is not a monophyletic group This particular error

Table 2. Reactions of anti-RuBP carboxylase (tobacco and spinach) with extracts from dicots

Species	Mean I.D. for species† Tobacco a.s.	Spinach a.s.
Rosidae		
Prunus amygdalus Batsch (Rosaceae)	NT	64.8
Chaenomeles sp. (Rosaceae)	NT	57.8
Petroselinum crispum Nym. (Umbelliferae)	61.1	25.8
Tropaeolum majus L. (Tropaeolaceae)	67.5	39.0
Euphorbia pulcherrima Willd. (Euphorbiaceae)	82.6	39.1
Maytenus boaria Molina (Celastraceae)	88.1	45.1
Phaseolus vulgaris L. (Leguminosae)	77.3	43.1
Aesculus californica Nutt (Hippocastanaceae)	65.2	36.1
Pittosporum tobira Ait. (Pittosporaceae)	NT	52.8
Oenothera speciosa var childsii Munz (Onagraceae)	NT	40.0
Hedera helix L. (Araliaceae)	NT	60.7
Mean for Rosidae (±SD)*	73.6 ± 10.7	44.3 ± 11.1
Magnoliidae		
Michelia champaca L. (Magnoliaceae)	89.6	71.6
Liriodendron tulipifera L. (Magnoliaceae)	71.8	66.5
Persea americana Mill. (Lauraceae)	80.1	47.7
Eschscholzia californica Cham. (Papaveraceae)	68.2	50.2
Berberis aquifolium Pursh. (Berberidaceae)	NT	50.9
Mean for Magnoliidae (±SD)*	76.3 ± 7.0	54.5 ± 9.8
Caryophyllidae		
Spinacea oleracea L. (Chenopodiaceae)	80.8	−3.0
Salsola iberica Sennen and Pau (Chenopodiaceae)	63.6	14.0
Beta vulgaris L. (Chenopodiaceae)	76 5	25.2
Dianthus sp. (Caryophyllaceae)	NT	27.4
Rumex crispus L. (Polygonaceae)	NT	46.4
Pereskia grandifolia Haw. (Cactaceae)	81.2	86.4
Phytolacca dioica L. (Phytolaccaceae)	87.5	99.0
Mean for Caryophyllidae (±SD)*	80.8 ± 7.0	54.3 ± 37.4
Dilleniidae		
Brassica nigra Koch (Cruciferae)	108.3	62.8
Brassica Kaber (D.C.) Wheeler (Cruciferae)	NT	63.6
Camellia saluenensis Stapf. (Theaceae)	86.4	44.6
Cucurbita pepo L. (Cucurbitaceae)	78.5	36.1
Viola cornuta L. (Violaceae)	73.3	59.3
Salix babylonica L. (Salicaceae)	NT	51.5
Althaea rosea Cav. (Malvaceae)	NT	48.2
Mean for Dilleniidae (±SD)*	85.1 ± 13.8‡	50.5 ± 9.9
Hamamelidae		
Ficus benjamina L. (Moraceae)	76.5	44.5
Quercus suber L. (Fagaceae)	78.3	39.6
Alnus rhombifolia Nutt. (Betulaceae)	94.4	NT
Betula pendula Roth (Betulaceae)	78.2	62.7
Liquidambar styraciflua L. (Hamamelidaceae)	90.3	61.3
Juglans hindsii (Jeps.) Jeps. (Juglandaceae)	NT	39.6
Mean for Hamamelidae (±SD)*	82.8 ± 6.5	49.5 ± 11.6
Asteridae		
Nicotiana tabacum var xanthi (Solanaceae)	cf Table 1	74.5
Petunia hybrida Vilm. (Solanaceae)	cf Table 1	79.7
Solanum tuberosum L. (Solanaceae)	cf Table 1	93.7
Convolvulus arvensis L. (Convolvulaceae)	cf Table 1	76.9
Mean for above section of Asteridae (±SD)*	—	79.8 ± 4.0
Antirrhinum majus L. (Scrophulariaceae)	cf Table 1	50.9
Paulownia tomentosa Steud. (Scrophulariaceae)	cf Table 1	66.5
Coleus blumei Benth. (Labiatae)	89.0	82.8
Chrysanthemum frutescens L. (Compositae)	107.7	47.3
Calendula officinalis L. (Compositae)	92.8	47.0
Helianthus annuus L. (Compositae)	NT	63.2
Fraxinus holotrica Koehne. (Oleaceae)	81.1	NT
Mean for second section of Asteridae (± SD)*	92.0 ± 10.8§	64.7 ± 16.0

*Mean and standard deviation for families within subclass. If a family was represented by more than one species, the values for those species were averaged, and the single value used in the calculation.

†Standard deviation for individual determinations was *ca* ±6.0. Values shown are means of one to four determinations.

‡Includes values for Fouquieriaceae from Table 1.

§Includes values for Polemoniaceae, Hydrophyllaceae, Scrophulariaceae, Gesneriaceae, and Myoporaceae from Table 1.

NT. not tested.

would have minimal effect on the conclusions, but others might not.

Under the evolutionary clock hypothesis, the low I.D. values observed among the Solanaceae suggest that this is a young family. It would be younger than the Chenopodiaceae, since the intra-family I.D. values relative to gymnosperm values were much lower for antiserum to tobacco enzyme (4.5 ± 3.1 relative to 79.4 ± 16.3) than they were for antiserum to spinach enzyme (19.6 ± 7.9 relative to 68.0 ± 12.2). The relatively low values observed for *Nolana, Cuscuta and Convolvulus* suggest that the ancestors of these diverged

Table 3. Reactions of anti-RuBP carboxylase (tobacco and spinach) with extracts of monocots and gymnosperms

Species	Mean I.D. for species† Tobacco a.s.	Spinach a.s
Monocots		
Narcissus pseudo-narcissus L. (Amaryllidaceae)	98.8	51.1
Hordeum vulgare L. (Gramineae)	77.4	61.3
Collima elegans Liebn. (Palmae)	73.9	44 1
Zebrina pendula Schnizl. (Commelinaceae)	94.6	78 8
Elodea densa (Planch.) Casp. (Hydrocharitaceae)	NT	59.1
Typha domingensis Pers. (Typhaceae)	NT	44.2
Scirpus acutus Muhl. (Cyperaceae)	NT	40.7
Allium cepa L (Liliaceae)	NT	52.8
Mean for Monocots (±SD)*	86.2 ± 12.4	50.0 ± 12 4
Gymnosperms		
Ginkgo biloba L. (Ginkgoaceae)	65.2	51.0
Juniperus chinensis L. (Cupressaceae)	90.1	65.5
Zamia floridana A. DC (Zamiaceae)	98.0	85.3
Taxus baccata cv fastigiata Loud. (Taxaceae)	83.9	70.4
Ephedra chilensis Presl. (Gnetaceae)	59.8	67.9
Mean for Gymnosperms (±SD)*	79.4 ± 16.3	68.0 ± 12.2

*Mean and standard deviation for families within subclass. If a family was represented by more than one species, the values for those species were averaged, and the single value used in the calculation.

†Standard deviation for individual determinations was *ca* ±6.0. Values shown are means of one to four determinations.

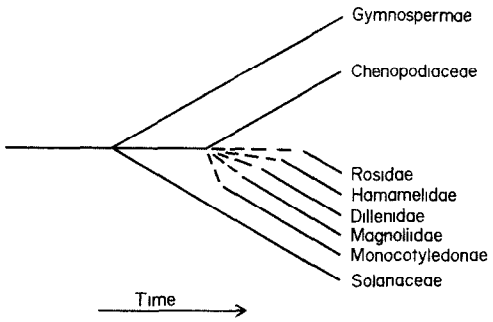

Fig. 1. Evolutionary tree representing an interpretation of the data in Tables 1–3. The number and nature of the divergence events leading to the separation of the Rosidae, Hamamelidae, Dilleniidae, Magnoliidae, and Monocotyledonae from the line leading to the Chenopodiaceae was purposely left vague.

from ancestors of the Solanaceae relatively late. The high values observed for other angiosperm families with tobacco antiserum imply that these diverged from the Solanaceae relatively early; the fact that the mean I.D. values for the major angiosperm groups were equal suggest that the groups diverged from the Solanaceae at about the same time; and the finding that the means for angiosperms were the same as for the gymnosperms suggests that the major angiosperm groups and the ancestors of the Solanaceae split very soon after the angiosperms diverged from the gymnosperms. The identical mean I.D. values observed for the Rosidae, Hamamelidae, Dilleniidae, Magnoliidae and the monocots using the spinach antiserum suggest that these groups all diverged from the line leading to spinach at about the same time; the higher mean I.D. values of the Solanaceae, Convolvulaceae, etc. and of the gymnosperms suggest an earlier divergence time.

The conclusions drawn from the two sets of data obtained with the tobacco and the spinach antisera were very consistent. Fig. 1 presents these conclusions in diagrammatic form. The main discrepancy between this Figure and more traditional plant phylogenies lies in the early divergence of the ancestors of the Solanaceae from the other angiosperms.

An alternative interpretation of the data makes the assumptions (a) that the variable amino-acid positions (or antigenic determinants) represent surface characteristics of RuBP carboxylase that have functional significance, (b) that the rate of evolutionary change of RuBP carboxylase depends primarily on selection and (c) that some surface characteristics for which this protein is selected may be different in different ancestral lines and at different times. Under these assumptions, the lower intrafamily I.D. values of the Solanaceae relative to the Chenopodiaceae suggest that stabilizing selection has worked more strongly in the Solanaceae to conserve characteristics of RuBP carboxylase that contribute to its antigenic structure. The high I.D. values of other angiosperm families would represent the effects of selection for surface structures different from those of the tobacco and spinach enzymes. The observation that average values for different groups are equal suggests changes in surface structures representing an equal number of antigenic determinants, but implies nothing about the times at which the changes occurred.

It seems likely that the differences between RuBP carboxylases in different species have accumulated in response both to clocklike mechanisms and to extraordinary selective pressures that occurred sporadically. This would explain why most of the I.D. values for plants in a taxon are closely clustered and why there are exceptions. Selective pressure applied to an ancestor of the Solanaceae could explain why the RuBP carboxylases of this family are so different from those of other angiosperms, even though the family appears young from the low intra-family I.D. values and from other evidence [16]. From this, one might predict that RuBP carboxylase of the Solanaceae would have some functional property significantly different from those of enzymes of other plants. This property would probably not involve the active site, which should be selectively conserved during evolution; in fact, Kawashima and Wildman [4] have shown that tobacco and spinach RuBP carboxylases have nearly equal affinities for HCO_3^- and RuBP. The distinguishing property might involve allosteric sites [17]. It might be related to the

ability of tobacco enzyme to crystallize at low ionic strengths [18] or to its cold-lability [19].

EXPERIMENTAL

Preparation of antisera. RuBP carboxylase was purified from fr. spinach leaves as in ref. [20]. The final product gave a single band containing >96% of aniline-blue-black-stainable material when subjected to electrophoresis through 5% polyacrylamide gels. Enzyme was purified and crystallized from fr. tobacco leaves by F. Daley (Dept. of Biochemistry, University of California, Davis), as in ref. [21]. For each antigen, two New Zealand white rabbits were injected 7–8 times, each time with 7–10 mg of purified enzyme divided among intravenous and several subcutaneous sites. The injection series lasted 60–90 days. Sera were collected 4 times starting one week after the last injection, pooled and stored frozen. Each serum pool gave a single line of pttn when tested against crude extracts of leaves of its corresponding species, using both double diffusion and immunoelectrophoresis in agar gel. Spinach antiserum inhibited spinach RuBP carboxylase activity, as assayed by $H^{14}CO_3^-$ incorporation into acid-stable material [20]. 20μl of serum inhibited over 70% of the activity observed in 30 μg of spinach extract protein. Antisera prepared against the protein from tobacco have been shown to ppt. RuBP carboxylase enzyme activity [22].

Extracts for assays. Live plant material was purchased commercially, obtained from the greenhouses of the Botany Department or from the UCD Arboretum, or collected in the immediate region of Davis. Fr. green tissue (1 g) was mixed with 0.5 g of insoluble PVP and 6 ml of 0.2 M Tris–SO_4 buffer, pH 8. The mixture was ground at moderate speed (setting '6') for 10 sec with a Polytron tissue homogenizer (Brinkmann) and centrifuged at 31000 *g* for 20 min. If necessary, the supernatant was further clarified by filtration or centrifugation. The final soln was tested for protein by the procedure of ref. [23] with BSA as standard and after appropriate dilution was used in complement fixation assays. This soln seldom showed non-specific anticomplementarity in amounts below 100 μg of protein.

Complement fixation assays. Quantitative microcomplement fixation was performed according to ref. [24]. With the spinach antiserum, purified spinach enzyme was included in each assay as a standard: *ca* 0.5 μg of enzyme and 1/6000 ml of antiserum gave a maximum fixation of about 0.6. With the tobacco antiserum, fr tobacco extract was included in each assay as standard. Amounts of antigen and antiserum giving maximum fixation between 0.1 and 0.9 were determined empirically by titration. The difference in immunochemical reactivity between the standard antigen and a cross-reacting antigen ('immunological distance'—I.D.) depends upon the antiserum concns used with the standard and cross-reacting antigens (X_H, X_h) and the degrees of maximum complement fixation observed with the antigens (Y_H, Y_h): I.D. = 100 ($\log_{10} X_h/X_H + 1/m(Y_H - Y_h)$). Using standard antigens, the constant m had an average value of 2.33 for tobacco antiserum and 2.64 for spinach antiserum. I assumed m to be the same with cross-reacting antigens as it was with standard antigens. Titrations with sample cross-reacting antigens confirmed this.

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