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SYSTEMATIC RELATIONSHIPS OF THE RANUNCULACEAE BASED ON AMINO ACID SEQUENCE DATA

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Key Word Index—*Anemone*; *Aquilegia*; *Clematis*; *Consolida*; *Eranthis*; Ranunculaceae; phylogeny; amino acid sequence; plastocyanins; serology.

Abstract—Plastocyanin partial amino acid sequences (40 residues) of five members of the Ranunculaceae were used, together with many other flowering plant plastocyanin sequences already published, to construct dendrograms. On this basis the Ranunculaceae appear more closely related to the Rosaceae and Fabaceae than to the other families investigated. Dendograms constructed from amino acid sequence data and serological data of five members of the Ranunculaceae were similar.

INTRODUCTION

Comparisons of many diverse characters have been used to establish the natural relationships of the taxa within the Ranunculaceae. Among the investigations that have proven to be especially important have been the studies carried out on the karyotype [1, 2], secondary substances (such as benzylisoquinoline alkaloids, cyanogenic compounds and ranunculin [3, 4]), and the serological comparisons of the seed proteins [5]. Most of the recent schemes for the phylogenetical tree of the Ranunculaceae genera [4–9] are quite similar; only that of Zukova [6], based mainly on morphological characters, deviates significantly. For example, it is now generally accepted that the cyanogenic Thalictreae are well separated from the other genera, and that *Thalictrum* belongs to this tribe and not to the subfamily Anemonoideae.

Eranthis is of particular interest. It is normally regarded as being closely related to *Helleborus* [9] because of the similarity of their nectar-bearing petals. However, distribution of ranunculin [4] separates the two genera. Furthermore, the similarity of *Eranthis* to any other genus of the Ranunculaceae is extremely low, based on the results of serological investigations [5, 10, 11], so that the inclusion of *Eranthis* within the Ranunculaceae is presently in doubt.

Amino acid sequence data of plastocyanin have been shown recently to be another acceptable method of revealing the natural relationship of plants [12, 13]. Boulter *et al.* [13] suggested that these data should prove especially useful at the family and tribal level, but previous to this investigation only one partial amino acid sequence of plastocyanin had been determined (D. Boulter, unpublished data). This paper gives the results of an investigation into whether or not partial plastocyanin amino acid sequences confirm the results obtained from other data on the relationships of some species of the Ranunculaceae, especially of *Eranthis hyemalis*. Of special interest is the fact that the results have been obtained from two different types of proteins: plastocyanin and seed storage proteins. If agreement were found, this would not only confirm Jensen's taxonomic results, but also give greater confidence for the use of both serological and sequencing methods in systematics.

The number of taxa used was limited partly by the availability of adequate quantities of green plant material necessary for the extraction of sufficient amounts of plastocyanin for sequence determination, and partly by poor yields in many instances. However, the five taxa used represent different branches on the Ranunculaceae family tree. *Aquilegia vulgaris* represents the Thalictreae, *Consolida ajacis* and *Eranthis hyemalis* the Caltheae, *Clematis vitalba* and *Anemone nemorosa* the Ranunculeae [5].

Although not specifically investigated in this instance, previous work in our laboratory has shown that different samples of the same taxa give identical plastocyanin sequences.

RESULTS

Systematic relationships of five members of the Ranunculaceae

Table 1 gives a list of the first 40 N-terminal amino acid residues of plastocyanin from five members of the Ranunculaceae. A unique, most parsimonious dendogram was constructed from this data set by a total search procedure (T. Gleaves, unpublished) based upon a method [14] for counting the minimum number of substitutions required to account for a given dendrogram (see Fig. 1). This may be compared with the dendrogram given in Fig. 2, which shows the positions of these five species based on serological characters of their seed proteins [1].

Both dendrograms show *Anemone nemorosa* and *Clematis vitalba* close together; thus their partial plastocyanin sequences have only two differences (see Table 2). The placement of *Aquilegia vulgaris*, away from the *Anemone*/*Clematis* branch, is also the same in both dendrograms. Additionally, they agree in showing the three species *Aquilegia vulgaris*, *Consolida ajacis* and *Eranthis hyemalis* on long, well separated branches.

There is some difference in the results in the two figures for the relative positions of *Eranthis* and *Consolida*.

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* Visiting Fellow of the Deutsche Forschungsgemeinschaft.

Table 1. First 40 N-terminal amino acid sequence residues of five members of the Ranunculaceae

	1 5 10 15 20	25 30 35 40
Anemone nemorosa	Ile-Glu-Val-Leu-Leu-Gly-Ala-Ser-Asp-Gly-Gly-Leu-Ala-Phe-Val-Pro-Asn-Ser-Phe-Ser-	Val-Ala-Ala-Gly-Glu-Lys-Ile-Thr-Phe-Lys-Asn-Asn-Ala-Gly-Phe-Pro-His-Asn-Val-Val
Aquilegia vulgaris	Val-Glu-Val-Leu-Leu-Gly-Ser-Asp-Asp-Gly-Gly-Leu-Ala-Phe-Val-Pro-Ser-Asp-Phe-Ser-	Val-Ser-Ala-Gly-Glu-Lys-Ile-Val-Phe-Lys-Asn-Asn-Ala-Gly-Phe-Pro-His-Asn-Val-Val
Clematis vitalba	Ile-Glu-Val-Leu-Leu-Gly-Ser-Ser-Asp-Gly-Gly-Leu-Ala-Phe-Val-Pro-Asn-Ser-Phe-Ser-	Val-Ala-Ala-Gly-Glu-Lys-Ile-Val-Phe-Lys-Asn-Asn-Ala-Gly-Phe-Pro-His-Asn-Val-Val
Consolida ajacis	Leu-$\genfrac{}{}{0pt}{}{\text{Glu}}{\text{Asp}}$-Val-Leu-Leu-Gly-Ser-Asp-Asp-Gly-Gly-Leu-Ala-Phe-Val-Pro-Asn-Asn-Phe-Thr-	Val-Ser-Ala-Gly-Glu-Lys-Ile-Val-Phe-Lys-Asn-Asn-Ala-Gly-Phe-Pro-His-Asn-Val-Val
Eranthis hyemalis	Ile-Glu-Val-Leu-Leu-Gly-Ser-Asp-Asp-Gly-Ser-Leu-Ala-Phe-Val-Pro-Asn-Ser-Phe-Ser-	Val-Ala-Pro-Gly-Glu-Lys-Ile-Val-Phe-Lys-Asn-Asn-Ala-Gly-Phe-Pro-His-Asn-Ile-Val

However, since the exact position of *Eranthis* within the Ranunculaceae is uncertain from its serological characters [5], it is difficult to decide if this difference has any significance.

The relationships of the Ranunculaceae to other Angiosperm families

Seventy-seven species of 28 families of flowering plants ([15]; and unpublished data) have been separated into two groups on the basis of the difference between their partial plastocyanin amino acid sequences and those of five species of the Ranunculaceae (Table 2). Group 1 consists of those families in which one or more species show a 'similarity index' to the Ranunculaceae. Group 2 consists of those families whose species show no similarity index to the Ranunculaceae. The 'similarity index' given for a particular species is:

1*x*, *if it shows*: 2, 3 or 4 sequence differences to *one* species of the Ranunculaceae *or* 5 sequence differences to at least *two* species of the Ranunculaceae;

2*x*, *if it shows*: 2, 3 or 4 sequence differences to *one* species and 5 sequence differences to at least *two* species *or* 2, 3 or 4 sequence differences to *two* species of the Ranunculaceae; etc.

As a matter of interest, some species of Group 1 have a 2*x* or greater similarity index to the Ranunculaceae, e.g. *Senecio jacobaea* in the Asteraceae, *Cytisus battandieri*, *Daviesia latifolia* and *Lupinus polyphyllus* in the Fabaceae, *Lavatera arborea* in the Malvaceae, *Crataegus monogyna* in the Rosaceae and *Nicotiana tabacum* in the Solanaceae. Whereas in the Asteraceae and Solanaceae these cases of strong similarity appear to be exceptions to the non-similarity of the other members of these families, close connections to the Ranunculaceae are suggested for the Fabaceae, Rosaceae and Malvaceae.

The similarities of the Ranunculaceae sequences to those of species of other flowering plant families can also be examined by constructing the most parsimonious dendrograms from the amino acid sequence data sets. Twelve different dendrograms constructed by using a total search method (T. Gleaves, unpublished) are presented in Figs. 3–14; the species used for constructing the dendrograms are presented in Table 2 together with the numbers of amino acid differences in the sequences between these species and those of the Ranunculaceae. The dendrograms have been restricted to not more than 10 species by the limitation of the method, which cannot quickly 'handle' larger numbers. Often several different dendrograms were obtained using the same data set (see legends) but the one presented is quite representative since the alternatives differ in minor rearrangements only (i.e. the relationship of taxa on a branch).

The results in Fig. 3 show that on this evidence, the Rosaceae are related to the Ranunculaceae, e.g. *Crataegus monogyna* is closer to this family than is *Eranthis hyemalis*, whereas the Magnoliaceae are more distantly related. The results in Figs. 4 and 5 show that sequences of the Fabaceae intermingle with those of the Ranunculaceae. In Fig. 4 *Consolida ajacis* is next to *Cytisus battandieri* and *Lupinus polyphyllus* is next to *Aquilegia vulgaris*, whereas *Anemone nemorosa* is separated from *Clematis vitalba* and closer to *Daviesia latifolia*. Sixty-two different possible arrangements were found for the species in Fig. 4, emphasizing a strong similarity between the amino acid sequences of members of the two families.

In Fig. 5 *Crataegus monogyna* is placed next to *Eranthis hyemalis* as in Fig. 3. Figure 6 presents a dendrogram

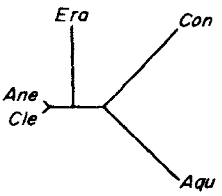

Fig. 1. Dendrogram for five species of Ranunculaceae based on plastocyanin amino acid sequence data. No. of alternative dendrograms: 1.

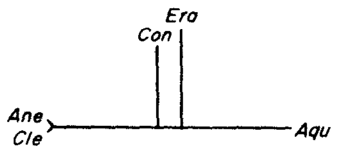

Fig. 2. Seed protein similarities detected by serological methods (after Jensen [5]).

Table 2. Similarities between the partial plastocyanin sequences of the Ranunculaceae species and species of other families

Family	No. of species*	No. of species belonging to group: 1	2	Species used for dendrograms in this paper	No. of differences to the Ranunculaceae taxa					Similarity Index†
					Ane	Aqu	Cle	Con	Era	
Ranunculaceae	5	5								
				Anemone nemorosa L.		7	2	7	6	*x*
				Aquilegia vulgaris L.	7		5	4	7	*x*
				Clematis vitalba L.	2	5		5	4	3*x*
				Consolida ajacis (L.) Schur.	7	4	5		7	*x*
				Eranthis hyemalis (L.) Salisb.	6	7	4	7		*x*
Families with species with strong amino acid sequence similarities to the Ranunculaceae										
Asteraceae	22	1	21							
				Inula magnifica Lipsky	12	12	11	13	12	
				Senecio jacobaea L.	5	7	3	7	5	2*x*
				Senecio vulgaris L.	6	6	5	7	8	
				Solidago virgaurea L.	11	12	10	13	12	
Caprifoliaceae	3	1	2							
				Lonicera periclymenum L.	8	9	8	8	7	
				Sambucus nigra L.	9	8	10	10	10	
				Viburnum tinus L.	7	7	6	7	4	*x*
Caryophyllaceae	1	1								
				Stellaria media (L.) Vill.	3	7	5	8	9	*x*
Cucurbitaceae	2	1	1							
				Cucumis sativus L.‡	7	10	8	7	6	
				Cucurbita pepo L.§	6	6	5	7	5	*x*
Dipeacaceae	1	1								
				Dipsacus fullonum L.	6	7	5	8	4	*x*
Fabaceae	9	4	5							
				Cytisus battandieri Maire	7	2	5	3	7	2*x*
				Daviesia latifolia R. Br.	3	8	5	8	5	2*x*
				Lupinus polyphyllus Lindl.	7	3	5	5	5	2*x*
				Pisum sativum L.	7	6	7	8	10	
Malvaceae	1	1								
				Lavatera arborea L.	6	3	4	4	4	4*x*
Oleaceae	1	1								
Rosaceae	2	2								
				Crataegus monogyna Jacq.	6	6	4	7	2	2*x*
				Prunus serrulata Lindl.	8	8	7	8	3	*x*
Rutaceae	1	1								
				Ruta graveolens L.	8	8	7	7	4	*x*
Solanaceae	5	2	3							
				Capsicum frutescens L.	7	8	8	9	7	
				Nicotiana tabacum L.	5	4	5	4	7	3*x*
				Solanum crispum Berd.	6	5	6	5	8	*x*
				Solanum tuberosum L.	9	8	10	6	10	
Urticaceae	1	1								
				Urtica dioica L.	5	7	5	8	9	*x*
Families without strong amino acid sequence similarities to the Ranunculaceae										
Apiaceae	4		4							
				Aegopodium podagraria L.	8	9	10	10	11	
				Anthriscus sylvestris (L.) Hoffm.	10	12	11	11	8	
				Heracleum sphondylium L.	8	11	9	9	9	
				Pastinaca sativa L.	8	11	9	9	9	
Balsaminaceae	1		1							
Boraginaceae	2		2							
Brassicaceae	2		2							
				Brassica oleracea L.	9	10	7	7	8	
				Capsella bursa-pastoria (L.) Medic.	7	8	6	9	6	
Chenopodiaceae	3		3							

Table 2. (*Continued*)

Family	No. of species*	No. of species belonging to group: 1	2	Species used for dendrograms in this paper	No. of differences to the Ranunculaceae taxa Ane	Aqu	Cle	Con	Era	Similarity Index†
				Beta vulgaris L.	11	11	10	12	12	
				Chenopodium album L.	11	9	10	7	9	
				Spinacia oleracea L.	10	8	9	11	9	
Convolvulaceae	1		1							
Euphorbiaceae	1		1							
				Mercurialis perennis L.	8	8	8	5	8	
Grossulariaceae	1		1							
				Ribes sanguineum Pursh.	11	9	11	10	8	
Lamiaceae	1		1							
Magnoliaceae	2		2							
				Magnolia × *soulangiana*	7	7	6	8	7	
				Liriodendron tulipifera L.	8	10	7	11	6	
Onagraceae	1		1							
Plantaginaceae	1		1							
Poaceae	1		1							
Polygonaceae	3		3							
				Fagopyrum esculentum Moench	10	7	9	7	7	
				Polygonum persicaria L.	10	7	9	8	7	
				Rumex obtusifolius L.	9	10	8	9	7	
Rubiaceae	1		1							
Scrophulariaceae	3		3							

* No. of species of which partial plastocyanin sequences are available.
† Similarity index explained in text.
‡Cus in Fig. 7.
§Cup in Fig. 7.
Group 1—species with strong similarities to the Ranunculaceae (see last column and text). Group 2—species without strong similarities to the Ranunculaceae

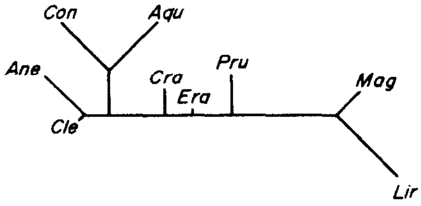

Fig. 3. Dendrogram for members of Ranunculaceae, Rosaceae and Magnoliaceae. No. of alternative dendrograms: 1.

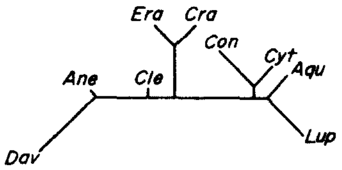

Fig. 5. Dendrogram for members of Ranunculaceae, Fabaceae and Rosaceae. No. of alternative dendrograms: 5.

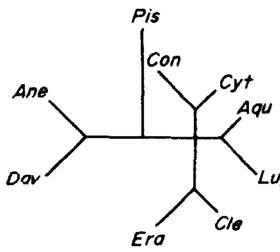

Fig. 4. Dendrogram for members of Ranunculaceae and Fabaceae. No. of alternative dendrograms: 62.

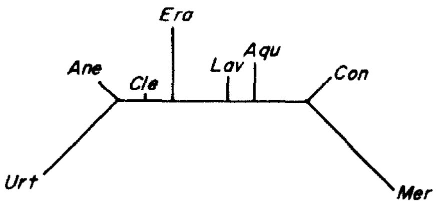

Fig. 6. Dendrogram for members of Ranunculaceae, Urticaceae, Malvaceae and Euphorbiaceae. No. of alternative dendrograms: 1.

which includes *Lavatera arborea* (Malvaceae), *Urtica dioica* (Urticaceae) and *Mercurialis perennis* (Euphorbiaceae). *Lavatera* is placed among the Ranunculaceae, whereas *Urtica* and *Mercurialis* are more separate. Figure 7 shows that on this evidence the Cucurbitaceae are nearer to the Ranunculaceae than to the Brassicaceae, but form a distinct family separated from the Ranunculaceae by two substitutions. Figure 8 shows *Dipsacus fullonum* (Dipsacaceae) closer to the Ranunculaceae than *Ruta graveolens* (Rutaceae) and *Viburnum tinus* (Caprifoliaceae). Figure 9 shows the Solanaceae clearly separated from the Ranunculaceae, even though the amino acid sequence of *Nicotiana tabacum* is similar to those of *Consolida ajacis* and *Aquilegia vulgaris* (see Table 2). Similarly, in spite of the sequence similarities between *Senecio jacobaea* and Ranunculaceae, *Senecio* is placed in the Asteraceae along with the other members of that family and not with sequences of the Ranunculaceae. Figures 11, 12, 13 and 14 show the Ranunculaceae as a distinct family (including *Eranthis*) with respect to sequences of the Caprifoliaceae, Polygonaceae, Apiaceae and Chenopodiaceae.

In summary, on the basis of plastocyanin amino acid sequence data, the Ranunculaceae appear more closely related to the Rosaceae and Fabaceae than to any other family discussed in this paper.

The position of Eranthis hyemalis

In general, the balance of sequence evidence places *Eranthis hyemalis* in the Ranunculaceae, but usually as its most extreme member (Figs. 11–14), although in some dendrograms (as in Figs. 4 and 9) its position is in the middle of the family. Furthermore, *Eranthis hyemalis* has a strong similarity to *Crataegus monogyna* (Figs. 3 and 5); both results suggest that *Eranthis hyemalis* is less closely related to members of the Ranunculaceae than they are to each other.

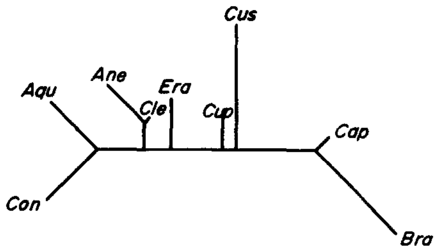

Fig. 7. Dendrogram for members of Ranunculaceae, Cucubitaceae and Brassicaceae. No. of alternative dendrograms: 10.

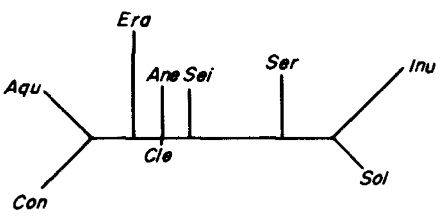

Fig. 10. Dendrogram for members of Ranunculaceae and Asteraceae. No. of alternative dendrograms: 3.

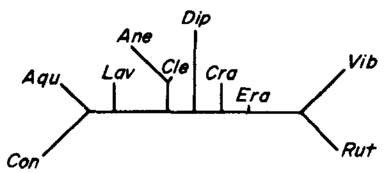

Fig. 8. Dendrogram for members of Ranunculaceae, Malvaceae, Dipsacaceae, Rosaceae, Caprifoliaceae and Rutaceae. No. of alternative dendrograms: 1.

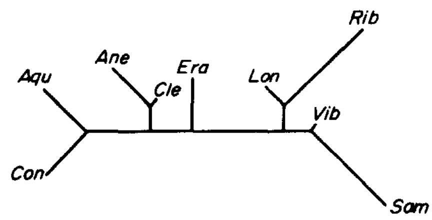

Fig. 11. Dendrogram for members of Ranunculaceae, Caprifoliaceae and Grossulariaceae. No. of alternative dendrograms: 1.

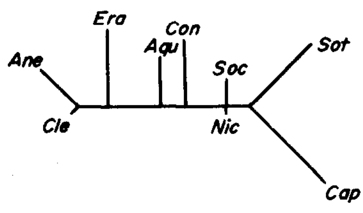

Fig. 9. Dendrogram for members of Ranunculaceae and Solanaceae. No. of alternative dendrograms: 9.

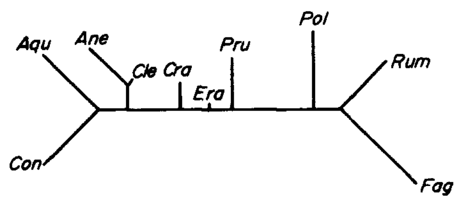

Fig. 12. Dendrogram for members of Ranunculaceae, Rosaceae and Polygonaceae. No. of alternative dendrograms: 2.

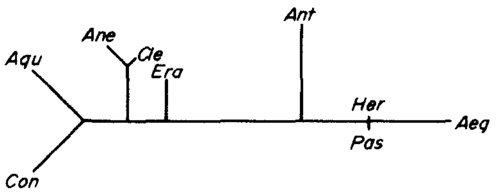

Fig. 13. Dendrogram for members of Ranunculaceae and Apiaceae. No. of alternative dendrograms: 3.

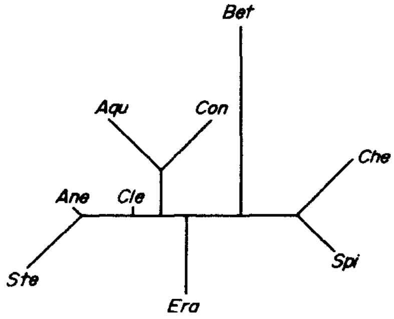

Fig. 14. Dendrograms for members of Ranunculaceae, Chenopodiaceae and Caryophyllaceae. No. of alternative dendrograms: 2.

DISCUSSION

Two different types of proteins were compared in this investigation, i.e. plastocyanin, an enzyme of the photosynthetic pathway, and the storage proteins of the seed. Furthermore, the taxonomic characters used were also different, i.e. partial amino acid sequence characters and antigenic determinants. Therefore, it is unlikely that similarities which were the result of convergent evolution would influence any conclusions drawn.

The great similarity in the two dendrograms (Figs. 1 and 2) constructed from the data of the two types of protein is a strong argument for accepting the evolutionary relationships of the Ranunculaceae depicted here. Furthermore, the results vindicate the use of protein characters in taxonomy, even though only five taxa were compared.

The amino acid sequence data place *Eranthis hyemalis* as a member of the Ranunculaceae, but support the suggestion, from serological data [5, 11], that it is the least related of the Ranunculaceae species studied (Figs. 3 and 12).

An important point emerges from consideration of the dendrograms in which the Ranunculaceae are compared with a range of other Angiosperm families. In some cases the sequence of only one member of an 'unrelated' family shows great similarity with one or a few sequences of Ranunculaceae species, e.g. *Nicotiana tabacum* with *Consolida ajacis* and *Aquilegia vulgaris* (Fig. 9, Table 2), and *Senecio jacobaea* with *Clematis vitalba* (Fig. 10 and Table 2). These similarities are presumably due to parallel substitutions and have little systematic significance. It is also not possible to suggest a close relationship between Ranunculaceae and Malvaceae therefore, since only one sequence of the latter family was available; however, the sequence similarity of *Lavatera arborea* to all members of the Ranunculaceae (Table 2) is remarkable. Similarly, the position of *Dipsacus fullonum* between *Anemone/Clematis* and *Crataegus* and *Eranthis* in the 'Ranunculaceae' (Fig. 8) is worth further investigation.

There are strong resemblances between some of the plastocyanin sequences of Ranunculaceae, Rosaceae and Fabaceae (Genisteae), although only two Rosaceae sequences were available and of the nine Fabaceae sequences examined only four showed great similarity to the Ranunculaceae. The plastocyanin data are in accord with the systems of earlier taxonomists, who derived the Rosales from the 'Polycarpicae' [16] or support Meeuse [17], who includes the Fabaceae in his Ranunculalean–Berberidalean–Rutalean line, on the basis of the distribution of certain alkaloids. Most other taxonomic data suggest that the Ranunculaceae are not closely related to the Rosaceae and Fabaceae. Also, serological comparisons of the major storage seed proteins [18] did not indicate determinant similarities between the two groups. It is often difficult to decide whether or not observed similarities are due to common ancestry or to convergence and there are examples where suspected convergent similarities later on have proved to be due to the expression of evolutionary relationship. In any case, even if the source of the plastocyanin similarities mentioned above are convergent, they nevertheless indicate the process and scale of parallel and back substitutions, and also those positions which are more involved than others, in relationship to their metabolic functions. However, further work is needed to determine the usefulness of different amino acid sequence positions in taxonomy.

EXPERIMENTAL

Materials. Aquilegia vulgaris L. leaves were obtained from Comp. Bornträger, 6521 Offstein, Germany. *Eranthis hyemalis* (L.) Salisb. was partly obtained from Gutshof Lehmkuhlen near Preetz, Germany, and partly from Comp. J. Bijl, Breezand, Holland. *Anemone nemorosa* L. was collected from the wild near Cologne, Germany, and *Clematis vitalba* L. near Cambridge, U.K. *Consolida ajacis* (L.) Schur. was grown from seed in the Durham Botanic Garden.

Methods. Plastocyanin was extracted and purified [19], except that 0.02 M Tris buffer was used instead of phosphate buffer and the leaf material had been frozen with liquid air soon after collection and stored at −20° for several weeks instead of using acetone-dried powders. The spectral ratios (A_{278}/A_{597}) of the purified protein were between 2.1 and 2.8 for different species. The yield was between 0.5 and 1.5 mg plastocyanin per kg leaves.

Edman degradation was carried out with a Beckmann 890C Automatic Sequencer using Beckmann's fast programme No. 122974. Protein (5 mg, 450 nmol) was applied to the sequencer in 450 μl of 70% formic acid and dried down using Beckmann's application sub-routine plus a single cleavage step and a butylchloride wash. The methods and criteria for identification of the phenylthiohydantoin derivatives were as in ref. [20]. A single analysis was used to determine each sequence.

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