Distribution of Chlorophylls, Carotenoids and Quinones in Chloroplasts of Higher Plants

K. H. Grumbach

Botanisches Institut der Universität Karlsruhe, Kaiserstraße 12, D-7500 Karlsruhe, Bundesrepublik Deutschland

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Leaves, cotyledons, isolated chloroplasts and subplastid fractions (thylakoids and envelopes) of radish (*Raphanus sativus* L. cv. Saxa) and spinach (*Spinacia oleracea* L. cv. Matador) were assayed for their pigment and quinone content and composition. Virtually all the chlorophylls, carotenoids and quinones were contained in the thylakoids. Envelopes prepared by the method described contained very low amounts of chlorophyll *a* and *b*, violaxanthin and neoxanthin, but no β-carotene, lutein, zeaxanthin and antheraxanthin. Among the quinones trace amounts of plastoquinone and α-tocopherol but no plastohydroquinone, α-tocoquinone and phylloquinone were detected. Presented data may be taken as evidence that *in vivo* the chloroplast envelope is not a location site of carotenoids and quinones as generally accepted. Possible implications for the biosyntheses of quinones and pigments are discussed.

Introduction

The structure, chemical composition and biological importance of the chloroplast envelope has been investigated extensively during the last ten years [1–6]. It is now generally accepted that the envelope membrane consists of protein and acyllipids and plays an important role in plastid galactolipid biosynthesis [1, 3, 6]. However, the presence of carotenoids and quinones in this membrane has still been subject to argument.

There has never been any doubt, that envelopes do not contain chlorophylls, although they were detected in significant amounts in earlier envelope preparations [2]. Besides chlorophylls, all carotenoids and quinones normally contained in the thylakoid membrane were also detected in envelope fractions [2, 7]. The quinone composition of the envelope was reported to be very similar to that of the thylakoids [7]. Among the carotenoids particularly violaxanthin was detected in envelope fractions in high amounts [2] but the qualitative carotenoid composition was virtually identical to that of the thylakoids.

In the present report evidence will be presented, suggesting that *in vivo* chlorophylls, carotenoids and quinones are not constituents of the envelope membrane. It is shown for the first time that the detectability of chlorophylls and certain carotenoids and quinones in the prepared envelope fractions was negligible and far below any physiological importance of the individual terpenoid. It is proposed that the trace amounts of pigments and quinones obtained in the envelope fractions are caused by an nonspecific binding of chlorophylls. carotenoids and quinones to the envelope membrane during chloroplast isolation.

Materials and Methods

Radish (*Raphanus sativus* L. cv. Saxa) was grown for 6 or 12 days in continuous white light (23 ± 2 °C, 90 ± 5% relative humidity, 6.4 W/m^2) in a greenhouse. Spinach (*Spinacia oleracea* L. cv. Matador) was grown for 1 or 2 month under natural conditions in the botanical gardens. From the 6 days old radish and the one month old spinach, plants, cotyledons, leaves and chloroplasts were assayed for their pigment and quinone composition. Thylakoids and envelope fractions were prepared from intact chloroplasts isolated from 2 month old spinach leaves and 12 days old radish cotyledons. Highly purified intact chloroplasts were isolated from the plants according to the methods of Nakatani and Barber [8] and Haas *et al.* [9]. Radish cotyledons and spinach leaves were disrupted for 7 s in an isolation

Reprint requests to Dr. K. Grumbach.

Abbreviations: A.U., absorption unit; A.U.F.S., absorption unit full scale; S, solvent peak; K_1, phylloquinone; PQ-9, plastoquinone-9; α-T, α-tocopherol; N, neoxanthin; V, violaxanthin; A, antheraxanthin; L, lutein; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; βC, β-carotene; *E*, absorbance; MES, morpholino-ethane-sulfonic acid.

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medium consisting of 0.33 M sorbitol, 0.2 mM $MgCl_2$ and 20 mM MES and adjusted to pH 6.5 with tris-hydroxymethylaminomethane. The crude slurry was filtered through 10 layers of nylon cloth and the filtrate centrifuged at $100 \times g$ for 60 s. The pellet was discarded and the supernatant centrifuged again at $660 \times g$ for 4 min. The green pellet was resuspended in 0.33 M sorbitol (adjusted to pH 7.5 with 0.5 mM tris-hydroxymethyl-aminomethane), filtered through 10 layers of nylon cloth and centrifuged again at $660 \times g$ for 4 min. The resulting pellet was resuspended in resuspension medium prepared according to Jensen and Bassham [10], Chloroplasts obtained by this method with an intactness of 80 to 90% were further purified on linear gradients of Percoll in suspension medium. Gradients were prepared from equal volumes of Percoll containing 0.33 M sorbitol (and adjusted to pH 7.5) and resuspension medium. Aliquots of the chloroplast suspension were layered on top of the Percoll gradient and centrifuged at $3500 \times g$ for 20 min. The lower main green band consisting of intact chloroplasts was removed from the gradient diluted with resuspension medium and chloroplasts sedimented using standard techniques. The chloroplast pellet was gently resuspended in swelling medium (10 mM tricin/NaOH containing 5 mM $MgCl_2$ and adjusted to pH 7.5) and incubated at 6 °C for 15 min. Aliquots of the swelling medium containing the subplastid fractions were layered on top of a discontinuous gradient and thylakoid and envelope fractions prepared by the method of Douce *et al.* [2].

Envelope and thylakoid fractions were removed from the gradient and resuspended in water. Before extracting the pigments and quinones aliquots of both fractions were solubilized with 2% desoxycholate and the precipitated and redissolved protein assayed according to the method of Lowry *et al.* [10]. Pigments and quinones were extracted from the leaves, cotyledons, chloroplasts and subplastid fractions using standard techniques [11]. Chlorophylls were assayed as described by Ziegler and Egle [12], carotenoids by the methods of Hager and Meyer-Bertenrath [13] and Britton and Goodwin [14] and quinones according to Grumbach [11]. Pigment and quinone contents and compositions of the thylakoid and envelope fractions were assayed by using high performance liquid chromatography (Figs. 1 and 2). Presented data are means of 5 (cotyledons, leaves, chloroplasts) or 3 (thylakoids, envelopes) independent analyses ± S.D.

Results

Chlorophylls, carotenoids and the quinones plastoquinone-9, plastohydroquinone-9, α-tocopherol, α-tocoquinone and phylloquinone are contained in chloroplasts of all higher plants (Table I–III, [15, 16]). Within the chloroplast these terpenoids are located virtually exclusively in the thylakoid membrane, but carotenoids were also obtained in envelope fractions [2, 17] and quinones

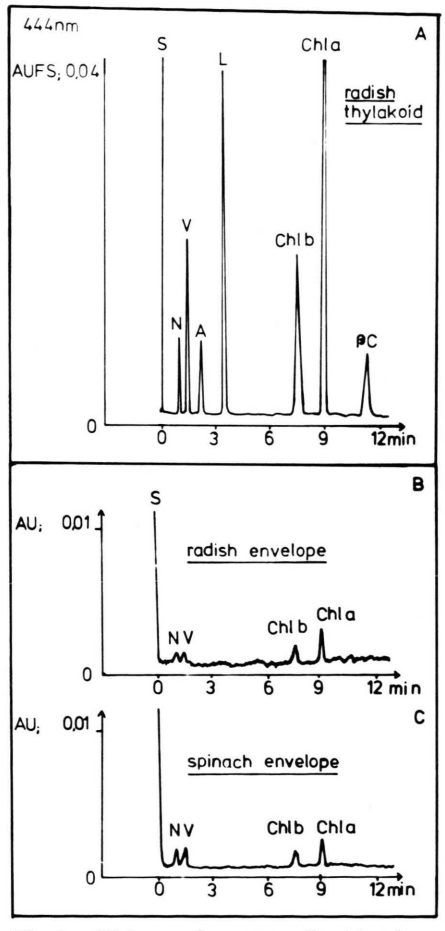

Fig. 1. High performance liquid chromatograms of chlorophylls and carotenoids from radish thylakoids and spinach and radish envelope fractions. Column: 250 × 3 mm I.D., Li-Chrosorb R.P. 18; 5 µm; pressure: 120 bar; methanol-water gradient: 90 to 100% methanol in water; flow: 1.5 ml/min; UV-VIS detector, detection at 444 nm, 0.04 A.U.F.S., 50 µl.

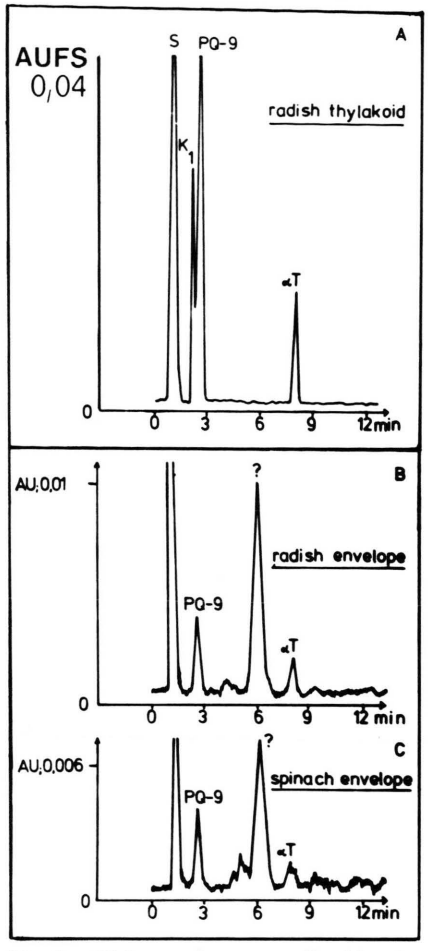

Fig. 2. High performance liquid chromatograms of quinones from radish thylakoids and spinach and radish envelope fractions. Column: 250 x 3 mm I.D., Li-Chrosorb Si-60; 5 µm; pressure: 80 bar; solvent: 0.6% dioxane in hexane; flow: 2.3 ml/min, UV-VIS detector; detection: plastoquinone-9 and phylloquinone at 260 nm, α-tocopherol at 290 nm, 0.16 A.U.F.S., 20 µl.

in thylakoids [15] envelopes [7] and plastiglobuli [18–21].

The terpenoid composition of the thylakoid membrane prepared from radish and spinach leaves was similar (Table I). Quinones, carotenoids and chlorophylls were contained in the thylakoid in a ratio of 1:3:18. The chlorophyll, carotenoid and quinone composition of spinach or radish leaves, chloroplasts and thylakoids is shown in Tables II and III. Chlorophyll a and b were contained in the thylakoid in a ratio of 3:1. Among the carotenoids lutein and β-carotene were the main pigments besides lower amounts of violaxanthin, antheraxanthin and zeaxanthin. The main quinone contained in chloroplasts and their thylakoid membranes was the electron carrier and proton translocator redox system plastoquinone-9/plastohydroquinone-9 [22]. α-Tocopherol, suggested to serve as a structural component and antioxidative agent [23] was contained in leaves and chloroplasts in similar amounts as plastoquinone-9 (Tables II, III). α-Tocoquinone and phylloquinone were also obtained in thylakoids, although in much smaller amounts (Table II). As can be deduced from Tables I–III the overall pigment and quinone composition of chloroplasts from different plant species grown in the same natural environment does not change very much, but considerable changes can be observed between plants grown under different intensities of light ([24, 25], Tables II, III). An analysis concerning the distribution of chlorophylls, carotenoids and quinones in subplastid fractions revealed that virtually all pigments and quinones were contained exclusively in the thylakoid membrane and not in the envelope (Table IV). Although 5 mgs (spinach) and 4.1 mgs (radish) envelope protein were assayed, the amount of chlorophylls, carotenoids and quinones contained in these fractions was in the range of detectability and could only be estimated quantitatively by using high performance liquid chromatography (Figs. 1, 2). As compared to recent envelope preparations no specific enrichment in violaxanthin was obtained but most surprisingly more polar terpenoids like chlorophyll *a* and *b*, violaxanthin and neoxanthin were still detected. β-carotene, lutein and antheraxanthin were completely absent. These results most likely suggest that the very small amounts of pigments obtained in the isolated envelope fractions may derive from a nonspecific binding of these more polar terpenoids to the envelope membrane during chloroplast isolation.

Among the quinones α-tocoquinone, plastohydroquinone and phylloquinone were not detected in the envelope fractions, but α-tocopherol and plastoquinone were obtained in small amounts (Table IV, Fig. 2). Whether these two quinones are constituents of the envelope membrane *in vivo* is still uncertain since it is well known that they are contained in large amounts in the osmiophilic plastoglobuli [19, 20]. Therefore they may appear in trace amounts in envelope preparations during their isolation (Tables IV, V).

Table I. Terpenoid content of radish and spinach leaves and their plastid and thylakoid fractions. Presented data are given as μg terpenoid.

Terpenoids	Plants assayed					
	Radish			Spinach		
	Cotyledons	Chloroplasts	Thylakoids	Leaves	Chloroplasts	Thylakoids
Chlorophylls	24461	12376	28600	60000	5170	17300
Carotenoids	4692	2322	5631	11101	1204	2497
Quinones	1473	704	1706	3643	473	936

Table II. Pigment and quinone content of radish and spinach leaves, chloroplasts and thylakoid fractions. Presented data (μg total terpenoid) are means of three to five independent preparations ± standard deviation.

Terpenoid	Plants assayed					
	Radish			Spinach		
	Cotyledons	Chloroplasts	Thylakoids	Leaves	Chloroplasts	Thylakoids
Chlorophyll *a*	18773 ± 156	9536 ± 85	21400 ± 178	46300 ± 150	3900 ± 148	12900 ± 215
Chlorophyll *b*	5688 ± 61	2840 ± 53	7200 ± 48	13700 ± 85	1270 ± 25	4400 ± 58
β-Carotene	1122 ± 10	598 ± 14	2146 ± 37	2834 ± 218	286 ± 6	594 ± 16
Lutein	1850 ± 31	880 ± 21	2744 ± 56	3171 ± 278	412 ± 6	1081 ± 168
Zeaxanthin	180 ± 19	76 ± 9	38 ± 4	92 ± 6	12 ± 2	10 ± 3
Antheraxanthin	396 ± 13	211 ± 16	166 ± 8	920 ± 27	108 ± 3	90 ± 5
Violaxanthin	562 ± 16	256 ± 13	393 ± 11	2540 ± 315	246 ± 5	586 ± 14
Neoxanthin	582 ± 12	301 ± 28	149 ± 16	1544 ± 134	140 ± 10	136 ± 17
Plastoquinone-9	428 ± 16	201 ± 17	832 ± 24	1652 ± 95	213 ± 9	630 ± 25
Plastohydroquinone-9	350 ± 18	151 ± 10	127 ± 10	460 ± 82	58 ± 9	63 ± 3
α-Tocopherol	544 ± 35	286 ± 24	180 ± 7	1192 ± 234	160 ± 18	94 ± 2
Phylloquinone	110 ± 10	48 ± 7	510 ± 25	250 ± 88	30 ± 8	101 ± 5
α-Tocoquinone	41 ± 7	18 ± 5	57 ± 4	89 ± 29	12 ± 2	48 ± 2

Table III. Percentage pigment and quinone composition of radish and spinach leaves, chloroplasts and thylakoid fractions. Data are calculated from Tab. II.

Terpenoid	Plants assayed					
	Radish			Spinach		
	Cotyledons	Chloroplasts	Thylakoids	Leaves	Chloroplasts	Thylakoids
Chlorophylls						
Chlorophyll *a*	76.7	77.0	74.8	77.2	75.4	74.6
Chlorophyll *b*	23.3	23.0	25.2	22.8	24.6	25.4
Carotenoids						
β-Carotene	23.9	25.8	38.1	25.5	23.8	23.8
Lutein	39.4	37.9	48.7	28.6	34.2	43.3
Zeaxanthin	3.8	3.3	0.7	0.8	1.0	0.4
Antheraxanthin	8.4	9.1	2.9	8.3	8.9	3.6
Violaxanthin	12.0	11.0	7.0	22.9	20.4	23.5
Neoxanthin	12.5	12.9	2.6	13.9	11.7	5.4
Quinones						
Plastoquinone-9	29.0	28.5	48.8	45.3	45.0	67.3
Plastohydroquinone-9	23.8	21.4	7.4	12.6	12.3	6.9
α-Tocopherol	36.9	40.6	10.5	32.7	33.8	10.0
Phylloquinone	7.5	6.8	29.9	6.9	6.3	10.7
α-Tocoquinone	2.8	2.7	3.4	2.5	2.6	5.1

Table IV. Distribution of chlorophylls, carotenoids and quinones in thylakoid and envelope fractions of green radish and spinach plants.

Terpenoid	Radish			Spinach		
	µg in Thylakoids + Envelopes	% in Thylakoids	% in Envelopes	µg in Thylakoids + Envelopes	% in Thylakoids	% in Envelopes
Chlorophyll *a*	21404	99.981	0.019	12903	99.974	0.026
Chlorophyll *b*	7201	99.982	0.018	4401	99.977	0.023
β-Carotene	2146	100.000	–	594	100.000	–
Lutein	2744	100.000	–	1081	100.000	–
Zeaxanthin	38	100.000	–	10	100.000	–
Antheraxanthin	166	100.000	–	90	100.000	–
Violaxanthin	393	99.996	0.004	586	99.983	0.017
Neoxanthin	149	99.987	0.013	136	99.928	0.072
Plastoquinone-9	834	99.736	0.264	633	99.589	0.411
Plastohydroquinone-9	127	100.000	–	63	100.000	–
α-Tocopherol	184	98.044	1.956	96	98.021	1.979
Phylloquinone	510	100.000	–	101	100.000	–
α-Tocoquinone	57	100.000	–	48	100.000	–

Table V. Pigment and quinone content of thylakoid and envelope fractions isolated from intact radish and spinach chloroplasts. Data are presented as µg terpenoid per mg membrane protein; b.d. = below detectability.

Terpenoid	Plastid membranes			
	Radish		Spinach	
	Thylakoids	Envelopes	Thylakoids	Envelopes
Chlorophyll *a*	142.70	0.68	146.60	1.00
Chlorophyll *b*	48.00	0.75	50.00	0.32
β-Carotene	14.30	b.d.	6.75	b.d.
Lutein	18.50	b.d.	12.28	b.d.
Zeaxanthin	0.25	b.d.	0.11	b.d.
Antheraxanthin	1.11	b.d.	1.02	b.d.
Violaxanthin	2.62	0.02	6.66	0.003
Neoxanthin	0.99	0.02	1.55	0.005
Plastoquinone-9	5.55	0.52	7.16	0.54
Plastohydroquinone-9	0.85	b.d.	0.72	b.d.
α-Tocopherol	1.20	0.38	1.07	0.88
Phylloquinone	3.40	b.d.	1.15	b.d.
α-Tocoquinone	0.38	b.d.	0.09	b.d.

On a protein basis approximately 200 µg of chlorophyll, 33 µg carotenoid and 10 µg quinones per mg were detected in the thylakoid membrane (Table V). As compared to the thylakoid the pigment content of the envelope membranes was much lower.

Discussion

Chlorophylls, carotenoids and quinones are constituents of the thylakoid membrane. Within the membrane chlorophylls and carotenoids are bound to protein and integrated in a highly specific orientation [26], Photosystem I and II particles contain chlorophyll *a* and *β*-carotene as light-harvesting pigments [27–29]. Besides chlorophyll *a* all the xanthophylls and chlorophyll *b* appear to be located in the light-harvesting chlorophyll *a/b* protein [30]. In the thylakoid membrane both photosystems are connected by a large proton carrier-like plastoquinone pool [22]. However, the distribution and biological importance of α-tocopherol, α-tocoquinone and phylloquinone are still a matter of investigation. Sufficient evidence has now been

presented that quinones and particularly plastoquinone and α-tocopherol are contained also in the osmiophilic plastoglobuli. All carotenoids but notably violaxanthin were also obtained in chloroplast envelope fractions [2] but they were never identified properly. Besides carotenoids quinones were also detected in envelope preparations [7].

As compared to recent envelope isolations, envelope membranes prepared from chloroplasts isolated according to the methods of Nakatani and Barber and Haas *et al.* contained pigments only in very small amounts (Table IV), and notably more polar terpenoids like chlorophylls, violaxanthin and neoxanthin suggesting that chlorophylls and carotenoids are not contained in the envelope membrane. The very small amounts detected may rather derive from a nonspecific binding of pigments to the envelope membrane during chloroplast isolation. That envelope preparations may be contamined by chlorophylls and carotenoids is further supported by the observation that the carotenoid and particularly the violaxanthin content of envelopes prepared from irradiated green leaves was much higher than that of envelopes isolated from darkened green leaves [17].

It is well known that depending on the light intensity a plant received, rapid changes in the xanthophyll composition and particularly in the amount of violaxanthin, antheraxanthin and zeaxanthin occur [31]. This interconversion of xanthophylls (xanthophyll-cycle) is, however, restricted exclusively to the thylakoid membrane [24] and may be the reason why the carotenoid content of different envelope preparations varies considerably. Most surprisingly α-tocoquinone, plastohydroquinone-9 and phylloquinone were not detected in the envelope fractions although the experimental conditions for their detectability were in the range of 10 nanograms (Fig. 2). Only plastoquinone-9 and α-tocopherol were detected in significant amounts (Tables IV, V). Whether these two quinones are constituents of the envelope membrane is rather doubtful since a contamination of envelope preparations with plastoglobuli containing high amounts of plastoquinone-9 and α-tocopherol can not be excluded.

Different functions of the envelope membrane have been proposed. There should be no real doubt that the envelope is a location site of galactolipid biosynthesis [32, 1]. That the envelope membrane is also involved in certain stages of pigment and quinone biosynthesis has been proposed [33, 34]. However, only recently sufficient evidence has been presented that the esterification of chlorophyllide with geranylgeranylpyrophosphate [35] as well as the biosynthesis of carotenoids from isopentenylpyrophosphate [36] may proceed only at the thylakoid membrane and not at the envelope. Whether this is also the case for the biosynthesis of plastidic quinones is under investigation.

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