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Chloroplast biogenesis 92: In situ screening for divinyl chlorophyll(ide) *a* reductase mutants by spectrofluorometry

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Abstract

Chlorophyll biosynthetic heterogeneity is rooted mainly in parallel divinyl (DV) and monovinyl (MV) biosynthetic routes interconnected by 4-vinyl reductases (4VRs) that convert DV tetrapyrroles to MV tetrapyrroles by conversion of the vinyl group at position 4 of the macrocycle to ethyl. What is not clear at this stage is whether the various 4VR activities are catalyzed by one enzyme of broad specificity or by a family of enzymes encoded by one gene or multiple genes with each enzyme having narrow specificity. Additional research is needed to identify the various regulatory components of 4-vinyl reduction. In this undertaking, *Arabidopsis* mutants that accumulate DV chlorophyllide *a* and/or DV chlorophyll [Chl(ide)] *a* are likely to provide an appropriate resource. Because the *Arabidopsis* genome has been completely sequenced, the best strategy for identifying 4VR and/or putative regulatory 4VR genes is to screen *Arabidopsis* Chl mutants for DV Chl(ide) *a* accumulation. In wild-type *Arabidopsis*, a DV plant species, only MV chlorophyllide (Chlide) *a* is detectable. However in Chl mutants lacking 4VR activity, DV Chl(ide) *a* may accumulate in addition to MV Chl(ide) *a*. In the current work, an in situ assay of DV Chl(ide) *a* accumulation, suitable for screening a large number of mutants lacking 4-vinyl Chlide *a* reductase activity with minimal experimental handling, is described. The assay involves homogenization of the tissues in Tris–HCl:glycerol buffer and the recording of Soret excitation spectra at 77 K. DV Chlide *a* formation is detected by a Soret excitation shoulder at 459 nm over a wide range of DV Chlide *a*/MV Chl *a* ratios. The DV Chlide *a* shoulder became undetectable at DV Chlide *a*/MV Chl *a* ratios less than 0.049, that is, at a DV Chlide *a* content of less than 5%.

Keywords: Metabolic tetrapyrroles; Chlorophyll biosynthesis; Chl mutants; Vinyl reductases; Spectrofluorometric screening

On the basis of an extensive body of experimental evidence, it has been proposed that the chlorophyll (Chl)[1] biosynthetic pathway consists of several parallel biosynthetic routes in both divinyl (DV) plant species (e.g., cucumber, *Arabidopsis*) and monovinyl (MV) plant species (e.g., corn, wheat, barley) [1]. DV plants accumulate DV protochlorophyllide (Pchlide) *a* in darkness, and in the light they form the bulk of their Chl via DV Chl biosynthetic routes [2,3]. MV plants accumulate MV Pchlide *a* in darkness, and in the light they form the bulk of their Chl via MV Chl biosynthetic routes [2,3]. It has also been proposed that the various DV and MV biosynthetic routes originate in Chl biosynthesis subcenters where Chl biosynthesis is coupled to the assembly of specific Chl–protein complexes [1,4,5]. It should be noted, however, that in green plants the end product of the DV and MV Chl biosynthetic routes in all Chl–protein complexes is MV Chl *a*.

Chlorophyll biosynthetic heterogeneity is rooted mainly in parallel DV and MV biosynthetic routes interconnected by 4-vinyl reductases (4VRs) that convert DV tetrapyrroles to MV tetrapyrroles by conversion of the

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[1] *Abbreviations used:* Chl, chlorophyll; DV, divinyl; MV, monovinyl; Pchlide, protochlorophyllide; 4VR, 4-vinyl reductase; Mpe, Mg-Proto monomethyl ester; Chlide, chlorophyllide; 4VCR, 4-vinyl Chlide *a* reductase; Chl(ide), Chlide and/or Chl.

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vinyl group at position 4 of the macrocycle to ethyl [1]. In this work, the Fischer terminology is used due to its simplicity. So far, five 4VR activities have been detected at the levels of DV Mg-protoporphyrin IX [6], Mg-Proto monomethyl ester (Mpe) (V.L. Kolossov and C.A. Rebeiz, unpublished), Pchlide *a* [7], chlorophyllide (Chlide) *a* [8], and Chl *a* [9].

What is not clear at this stage is whether the various 4VR activities are catalyzed by one enzyme of broad specificity or by a family of enzymes of narrow specificity encoded by one gene or multiple genes, as is the case for NADPH Pchlide oxidoreductases [1]. For example, Whyte and Griffiths [10] suggested that the various 4VR activities are catalyzed by one 4VR of very broad specificity, the activity of which is regulated by the plastid NADP/NADPH ratio. Although the pigment composition of the Nec 7 corn mutant [11] suggests that a single gene product is responsible for 4-vinyl reduction [12], a mutation in a gene that regulates the activities of various 4VRs cannot be excluded [13]. Indeed, involvement of the stromal fraction of plant plastids in the regulation of MV and DV tetrapyrrole formation has been well documented [14]. On the other hand, in *Rhodobacter capsulatus*, Suzuki and Bauer [15] observed that the disruption of the *bchJ* gene resulted in the accumulation of a large pool of DV Pchlide *a*. However, the formation of DV Pchlide *a* was still accompanied by the formation of significant amounts of MV Pchlide *a* and by the massive accumulation of MV Mpe. This in turn suggested that (i) the disruption of the *bchJ* gene was accompanied by the formation of MV Pchlide *a* via another biosynthetic route(s) and (ii) another 4VR was active in the *bchJ* mutant prior to MV Pchlide *a* formation, probably at the level of DV Mpe.

Other evidence pointing out that the 4VR activities at the level of DV Pchlide *a* and DV Chlide *a* may be catalyzed by two distinct enzymes is suggested by the observation that cucumber cotyledons subjected to three 2.5-ms light treatments, each separated by a 45-min dark incubation, accumulate exclusively massive amounts of DV Pchlide *a* in darkness [16]. A 2.5-ms actinic light flash converts the DV Pchlide *a* to DV Chlide *a*, and the latter is reduced to MV Chlide *a* within minutes after the light treatment [17]. It is tenuous to believe that these two 4VR activities are catalyzed by one 4VR isoform, although it is possible to invoke some regulatory mechanism at higher levels of plastid organization that allows the exclusive accumulation of DV Pchlide *a* in the presence of a very active 4VR capable of both DV Pchlide *a* and Chlide *a* vinyl reduction.

One way of resolving the issue of the multiplicity of 4VR activities is by cloning one of the 4VR genes and by studying the properties and substrate specificity of the expressed enzyme. The best 4VR activity for such an undertaking is the enzyme responsible for the reduction of DV Chlide *a* to MV Chlide *a*. This enzyme, named 4-vinyl Chlide *a* reductase (4VCR), is extremely potent and is carried out to completion within minutes of dark incubation, following an actinic flash of white light [8,17,18]. Also, because it has been proposed that 4VCR participates in several different Chl biosynthetic routes [1], detections of different mutants that accumulate DV Chlide and/or Chl [Chl(ide)] *a* may enable comparison of the genes and expression products of various 4VCRs that may belong to different biosynthetic routes. This in turn may enable the determination of whether 4VCRs belonging to different biosynthetic routes are identical or isozymic in nature.

The above literature review leads one to the conclusion that additional research is needed to identify the various regulatory components of 4-vinyl reduction. In this undertaking, mutants that accumulate DV Chl(ide) *a* are likely to provide an appropriate resource. Because the *Arabidopsis* genome has been totally sequenced, the best strategy for identifying 4VR and/or putative regulatory 4VR genes is to screen *Arabidopsis* Chl mutants for DV Chl(ide) *a* accumulation. In wild-type *Arabidopsis*, a DV plant species, only MV Chlide *a* is detectable. However, in mutants lacking 4VR activity, DV Chl(ide) *a* formed by various Chl biosynthetic routes [1] may accumulate in addition to MV Chl(ide) *a*.

Recently, an *Arabidopsis thaliana* mutant that exhibits a predominant replacement of MV Chl with DV Chl was isolated, and the gene presumably coding for 4VR was identified [13]. The putative *Arabidopsis* 4VR gene has no paralogs in the *Arabidopsis* genome and has scant homology with other higher plants. Moreover, it is absent in the complete genome sequences of the unicellular red algae *Cyanidiochyzon merolae*; the cyanobacteria *Synechocystis* sp. PCC6803, *Nostoc* sp. PCC7120, and *Gloedobacter violaceus* PCC7421; and some low light-adapted *Prochlorococcus* species [13]. Because all of these organisms synthesize MV Chl(ide), it is reasonable to assume that there may exist another 4VR gene(s) in the above organisms, as well as in *Arabidopsis*, that codes for 4VR that acts prior to the conversion of DV to MV Chli(de) *a*.

In this work, an in situ assay of DV Chl(ide) *a* accumulation, using cucumber as a model plant and suitable for screening a large number of mutants with minimal experimental handling, is described.

Materials and methods

Plant material

Cucumber (*Cucumis sativus* var. Beit alpha) seeds were purchased from Hollar Seeds (Rocky Ford, CO, USA). Germination was carried out in plastic trays containing wet vermiculate in darkness or in a growth

chamber illuminated with 1000-W metal halide lamps (211 W m^{-2}) at 28 °C.

Light and dark pretreatments

Four-day-old dark-grown cucumber seedlings were illuminated in the above-mentioned growth chamber for various lengths of time. Each light treatment was followed by 1 h dark incubation.

Acquisition of in situ emission and excitation spectra at 77 K for monitoring DV Chl(ide) a accumulation by greening cucumber cotyledons

In situ emission and excitation spectra were recorded on tissue homogenates as described below. At the end of 1 h dark incubation, 1 g of tissue was homogenized with mortar and pestle in 5 ml of homogenization buffer, consisting of 500 mM sucrose and 0.2 mM Tris–HCl (pH 7.7), under a low irradiance green light that does not photoconvert Pchlide *a* to Chlide *a*. The low irradiance light source had an output maximum at 503 nm, a bandwidth of 40 nm, and a photon density of approximately 0.01 μmol m^{-2} s^{-1}. The resulting homogenate was filtered through four layers of cheesecloth and one layer of Miracloth (Calbiochem, La Jolla, CA, USA), and 0.3 ml of the filtrate was mixed with 0.6 ml of glycerol. Essentially, aliquots were introduced into a 2.5-mm-diameter glass tube at room temperature in the dark room with a Pasteur pipette. This was followed by repeated shaking of the tube to drive the aliquot to the bottom of the long narrow tube. The tube was exposed to a 2.5-ms actinic white light flash, followed by immediate freezing in liquid nitrogen. After freezing, the tube was subjected to 77 K spectrofluorometric emission and excitation analysis. Emission spectra between 580 and 700 nm were elicited by excitation at 440 nm. Excitation spectra were recorded at the Chl(ide) *a* emission peak at 688 nm. Usually, 1 g of tissue was used, but as little as a few milligrams of tissue could be successfully used for screening purposes.

Pigment extraction from tissue homogenates and cotyledons

Homogenate (1 ml) prepared from cotyledons subjected to various light–dark treatments was introduced into a flat-bottomed glass tube (2 cm in diameter) and was subjected to a single 2.5-ms actinic white light flash that converts Pchlide *a* to Chlide *a* [19]. Immediately after the light treatment, the reaction was stopped by the addition of 7 ml acetone/0.1 N NH_4OH (9:1, v/v).

Determination of DV Chlide a/total Chl a ratio

After centrifugation, tetrapyrroles were partitioned between hexane and hexane-extracted acetone fractions as described elsewhere [20]. The hexane-extracted acetone fraction was used for determination of the amount of DV Chlide *a*, whereas the hexane fraction was used for the determination of Chl *a* [20]. The Chlide *a*/Chl *a* ratio was determined from the calculated Chlide *a* and Chl *a* values.

Spectrofluorometry

Fluorescence spectra were recorded on a fully corrected photon-counting, high-resolution SLM spectrofluorometer (model 8000 C) interfaced with an IBM model 60 microcomputer. Room temperature determinations were performed in cylindrical microcells, 3 mm in diameter at an emission and excitation bandwidth of 4 nm. Spectra at 77 K were recorded at emission and excitation bandwidths that varied from 0.5 to 4 nm depending on signal intensity. The photon count was integrated for 0.5 s at each 1-nm increment.

Results

Chlorophyll mutants usually are pale green in color and contain low amounts of Chl. Because the assay for DV Chl(ide) *a* will take place on whole tissue homogenates that may contain some MV Chl(ide) *a*, the assay must be able to distinguish between DV and MV Chl(ide) *a* in vivo.

To determine whether it is possible to distinguish between DV Chl(ide) *a* and MV Chl *a* in vitro, etiolated cucumber seedlings were partially greened by exposure to continuous illumination for various lengths of time, and then the seedlings were moved to a dark room for 60 min to induce the formation of DV Pchlide *a* [16]. After 60 min of darkness, the cotyledons were harvested and a tissue homogenate was prepared as described in Materials and methods. The homogenate was diluted with glycerol (1:2, v/v) and was subjected to an actinic white light flash to convert the accumulated DV Pchlide *a* to DV Chlide *a* [16]. To prevent the rapid conversion of DV Chlide *a* to MV Chlide *a* [8,16,17], the flashed homogenate was immediately cooled down to 77 K with liquid nitrogen.

After 2 h of greening and 1 h of dark incubation, the 77 K spectrofluorometric profile of the tissue homogenate exhibited short wavelengths of Pchlide *a* emission peaks at 627 and 640 nm and a strong long wavelength emission peak at 655 nm that corresponds to long-wavelength phototransformable Pchlide *a* (Fig. 1A, spectrum a) [21]. Extraction and analysis of the tetrapyrrole pools indicated that they consisted exclusively of DV Pchlide *a* and MV Chl *a* as well as smaller amounts of MV Chl *b*. The strong emission peak at 681 nm (Fig. 1A, spectrum a) corresponds to MV Chl(ide) *a* fluorescence [22]. The Soret excitation profile recorded at the 681-nm emission

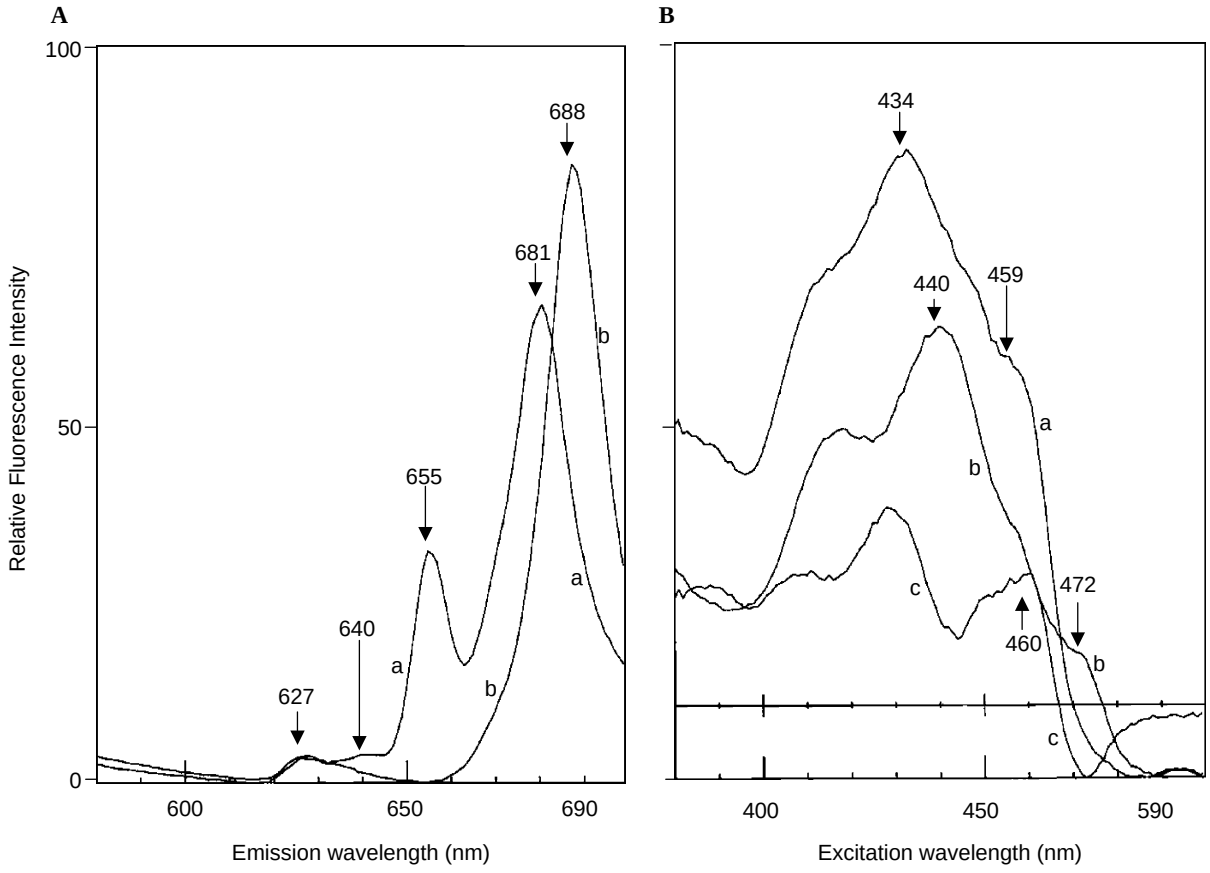

Fig. 1. Emission (A) and excitation (B) spectra of greening cucumber cotyledon homogenates recorded at 77 K before and after a flash of actinic white light. (Aa) Etiolated cucumber cotyledons were subjected to 2 h of continuous illumination and 1 h of dark incubation, followed by tissue homogenization in Tris–HCl/glycerol (1:2, v/v). (Ab) The tissue homogenate was subjected to one flash of actinic white light and then was immediately cooled down to 77 K. (Ba) = the same treatment as spectrum (Ab); (Bb) = same treatment as spectrum (Aa). Spectrum c is the difference spectrum of Ba–Bb, calculated via the SLM software by subtracting spectrum (Bb) from spectrum (Ba) in (B). Ordinate = relative fluorescence amplitudes. Emission was elicited by excitation at 440 nm. Excitation spectra were recorded at the respective emission maxima, that is, at 688 nm for spectrum Ba and at 681 nm for spectrum Bb. Abscissa = emission or excitation wavelengths. The upper abscissa is for the difference spectrum. Peaks of the difference spectrum above and below the upper abscissa are positive and negative peaks, respectively. Arrows point to wavelengths of interest.

maximum exhibited a strong Soret excitation maximum at 440 nm and weak excitation shoulders at 458–460 and 472 nm (Fig. 1B, spectrum b). The strong excitation maximum at 440 nm corresponds to the Soret excitation maximum of MV Chl(ide) *a*, whereas the weak excitation shoulder at 472 nm corresponds to the Soret excitation maximum of MV Chl *b* [4].

After exposure to an actinic white light flash and immediate cooling to 77 K, the pigment pools were extracted. Analysis of the extracted pigments indicated that they consisted of small amounts of unconverted DV Pchlide *a*, DV Chlide *a*, and MV Chl *a* as well as very small amounts of MV Chl *b*.

Spectrofluorometric analysis of the tissue homogenates following the 2.5-ms flash of actinic white light and immediate cooling to 77 K revealed that the Pchlide *a* and Chl(ide) emission and Soret excitation profiles underwent dramatic changes due to the photoconversion of Pchlide *a* to Chlide *a*. The long-wavelength phototransformable Pchlide *a* peak disappeared completely, whereas the emission intensity of the short-wavelength Pchlide *a* band between 620 and 645 nm decreased and underwent a blue shift (Fig. 1A, spectrum b). Also, the intense Chl(ide) *a* emission maximum at 681 nm underwent a red shift to 688 nm (Fig. 1A, spectrum b). Such a red shift corresponds to light-dependent spectral shift II, which was first described by Sironval et al. [23] and was attributed to the photoconversion of phototransformable long-wavelength Pchlide *a* to a Chlide *a* (E676 F688) pigment protein complex with red excitation and emission maxima at 676 and 688 nm, respectively [24]. Indeed, the Soret excitation profile recorded at the red-shifted Chl(ide) *a* emission maximum at 688 nm revealed the appearance of an intense Soret excitation shoulder at 459 nm (Fig. 1B, spectrum a) that corresponded to the red-shifted Soret excitation maximum of DV Chlide *a* in ether at 77 K [25]. The difference Soret excitation spectrum of the tissue homogenate after the light flash intensified the detection of the Soret excitation at 459 nm, which now appeared as a distinct peak at 459–460 nm

Table 1
Effects of various greening regimes on the appearance of a DV Soret excitation shoulder at 459 nm in greening cucumber cotyledons (ratios or picomoles/milliliter of tissue homogenate)

Exp	Tetrapyrroles	Greening regime					
		1 hL1 hD + F	2 hL1 hD + F	3 hL1 hD + F	4 hL1 hD + F	5 hL1 hD + F	6 hL1 hD + F
A	E459 Soret shoulder at 77 K	Yes	Yes	Yes	Yes	Yes	No
	DV Chlide *a*/MV Chl *a*	0.541	0.545	0.194	0.086	0.049	0.029
	MV Chl *a*	322	419	708	2346	4230	6726
B	E459 Soret shoulder at 77 K	Yes	Yes	Yes	Yes	Yes	No
	DV Chlide *a*/MV Chl *a*	0.595	0.349	0.228	0.082	0.052	0.028
	MV Chl *a*	297	421	622	2521	3992	6738

Note. The Soret excitation spectrum was recorded at 77 K, at an emission wavelength of 688 nm, on tissue homogenates diluted 1:2 (v/v) with glycerol. Exp, experiment; L, light; D, dark; F, one flash of actinic white light.

(Fig. 1B, spectrum c). The main MV Chl *a* Soret excitation maximum at 440 nm underwent a blue shift to 434 nm (Fig. 1B, spectrum a).

To correlate between the appearance of the DV Chlide *a* Soret excitation shoulder in tissue homogenates at 77 K and the DV Chlide *a*/MV Chl *a* ratio, cucumber cotyledons were induced to accumulate various DV Chlide *a*/MV Chl *a* ratios. To this effect, cucumber seedlings were greened under continuous illumination for 1–6 h prior to 1 h of dark incubation and exposure to a flash of actinic white light. Under continuous illumination, the tissue accumulated Chl *a* and Chl *b* [26]. During the 1-h dark incubation, the cotyledons accumulated DV Pchlide *a*, which was converted to DV Chlide *a* by the light flash. As shown in Table 1, detection of the DV Chlide *a* Soret excitation shoulder at 459 nm was possible over a wide range of DV Chlide *a*/MV Chl *a* ratios. The DV Chlide *a* shoulder became undetectable at DV Chlide *a*/MV Chl *a* ratios less than 0.049, that is, at a DV Chlide *a* content of less than 5%.

Discussion

The development of an in situ assay for screening Chl mutants for the formation of DV Chl(ide) *a* has been described. The assay is suitable for reasonably rapid screening purposes, and approximately 80 analyses can be readily performed in 8 h. Because the sensitivity of tetrapyrrole detection is in the picomole range [20], the method is well suited for the detection of DV Chlide *a* in tissue homogenates from single mutant lines consisting of only a few milligrams of tissue.

The analysis involves minimal tissue manipulation and is based on recording Soret excitation spectra on crude tissue homogenates at 77 K. DV Chl(ide) *a* is detected as a Soret excitation shoulder at 459 nm (Fig. 1B, spectrum a) over a wide range of DV Chlide *a*/MV Chl *a* ratios. At a DV Chl(ide) *a* content of less than 5%, the Soret excitation shoulder is no longer detectable (Table 1). It is well documented that greening cucumber cotyledons induced to accumulate exclusively massive amounts of DV Chlide *a* exhibit a Soret excitation maximum at 459 nm in diethyl ether at 77 K [16]. The 77 K Soret excitation shoulder observed in situ at 459 nm in cucumber tissue homogenates, induced to accumulate various amounts of DV Chlide *a* [16], matches perfectly the Soret excitation maximum of DV Chlide *a* at 77 K in diethyl ether [25]. This in turn suggests that the newly formed DV Chlide *a* may be very loosely bound to apoproteins.

It was also observed that etiolated cucumber cotyledons greened under continuous illumination exhibited a weak DV Chl(ide) *a* Soret excitation shoulder after 1 h of dark incubation (Fig. 1B, spectrum b). This in turn suggested that under these conditions, small amounts of DV Chlide *a* may have been formed via a light-independent conversion of DV Pchlide *a* to DV Chlide *a* [27].

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