4-VINYL-4-DESETHYL CHLOROPHYLL *a*: A NEW NATURALLY OCCURRING CHLOROPHYLL

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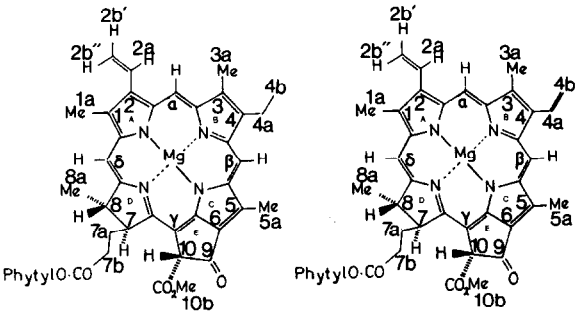
1. Introduction

In higher plants it had been assumed that there are only 2 chemicallly distinct chlorophylls (chl)s (*a* and *b*) actively involved in photosynthesis. However, the presence of 4 spectrally distinct, chl *a* and *b* species has been detected at 77 K [1,2] and 298 K [3] by fluorescence emission and excitation spectroscopy. Structures for these new species have been suggested on basis of the optical spectra [1,2] but such evidence is inconclusive in view of the wide variety of spectral modifications that reflect only changes in solvation and aggregation state rather than chemical structure [4,5]. Furthermore, optical spectra are incapable of distinguishing between the many biosynthetic precursors and the mature chlorophylls. Thus, modifications of the chlorophyll biosynthetic pathway proposed on the basis of these spectral forms [6] may be unjustified. Partial separation of 4 chl *a* chromophores by high pressure liquid chromatography has been reported [1] but their relationship to the spectral species detectable in unseparated pigment extracts is not clear. Many modification products of chlorophylls have been reported in the past but it is well-recognized that these can arise as artifacts of the extraction procedure [7,8].

A mutant of *Zea mays* (ON 8147) accumulates a new species of chl *a* in large amounts [9,10]. Here we show that this mutant may lack normal chl *a* altogether. We also rule out the possibility of artifactual origin of the new chlorophyll. The chlorophyll was isolated and structurally characterized by nuclear magnetic resonance (NMR) spectroscopy, and identified as 4-vinyl-4-desethyl chl *a*. This provides the first structural evidence for a new, naturally occurring chl *a* in the chloroplasts of photosynthetically active higher plants. These results have significant impact on current notions of the role of chl *a* in photosynthesis and on the present controversy over the biosynthetic pathway of chl *a*.



Structures and numbering system of (left) chl *a* and (right) 4-vinyl-4-desethyl chl *a*; phytyl = $C_{20}H_{39}$

2. Experimental

Seeds of mutant and normal plants were grown under identical conditions as in [11]. Chlorophylls were extracted from mutant and wild-type maize plants using a mild, high-yielding procedure that gave a chl *a*-enriched extract without pheophytinization or decomposition to allomers. This extract was suitable for NMR analysis without further purification. The procedure involved the extraction of pigments from freshly picked leaves of 2-week-old maize plants by homogenizing 100 g tissue for 2 min in a precooled Waring blender with 1500 ml cold methanol:pet.ether

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(b.p. 30–40°C) 2:1 (v/v) [8] followed by filtration through a Buchner funnel. The chlorophylls in the filtrate were transferred to the pet.ether phase by standard procedure of partition separation [8]. The chlorophylls in the dried pet.ether phase were separated further from colorless lipids and yellow pigment contaminants [12] to obtain fraction containing partially purified chlorophylls that is referred to here as 'crude extract'. The chlorophylls in crude extracts were separated from each other and purified by powdered sucrose column chromatography by standard procedure [8,12]. All extraction and purification steps were performed in the cold under dim light. All solvents were reagent grade and used without prior purification. Proton NMR spectra were recorded on Varian XL-100, in the Fourier transform mode. Other details are indicated in the legend of table 1.

3. Results

Using an XL-100 NMR spectrometer we were able to record the proton spectra of the crude extracts prior to chromatographic separation (fig.1). Although certain regions of the spectra are contaminated with impurities, the low field region consists largely of resonances arising from the 3 *meso*-protons (α,β,δ in structures). The shifts of these protons are highly characteristic of different chlorophyll structures, in identical solvents. We note that the 3 most intense peaks in this region of the spectrum are in different positions in the extracts from mutant and the wild-type maize. The positions of the peaks of wild-type maize are the same as those of chl *a* [12] (table 1). This demonstrates unequivocally that the extraction procedure used does not modify normal chl *a*. Hence the principal pigment in the mutant differs structurally from chl *a*, and the modification is not an artifact of the method of extraction.

Table 1
Chemical shift of *meso*-protons in the crude extracts

Meso	Mutant	Normal
β	9.84	9.67
α	9.46	9.34
δ	8.57	8.51

The chemical shifts are quoted in ppm downfield from TMS and are measured from internal d_6-acetone. The spectra were recorded in d_6-acetone at ambient probe temperature (~25°C). Uncertainties in shifts are ±0.02 ppm. The chemical shifts of the purified chlorophylls are identical within the uncertainties of measurement

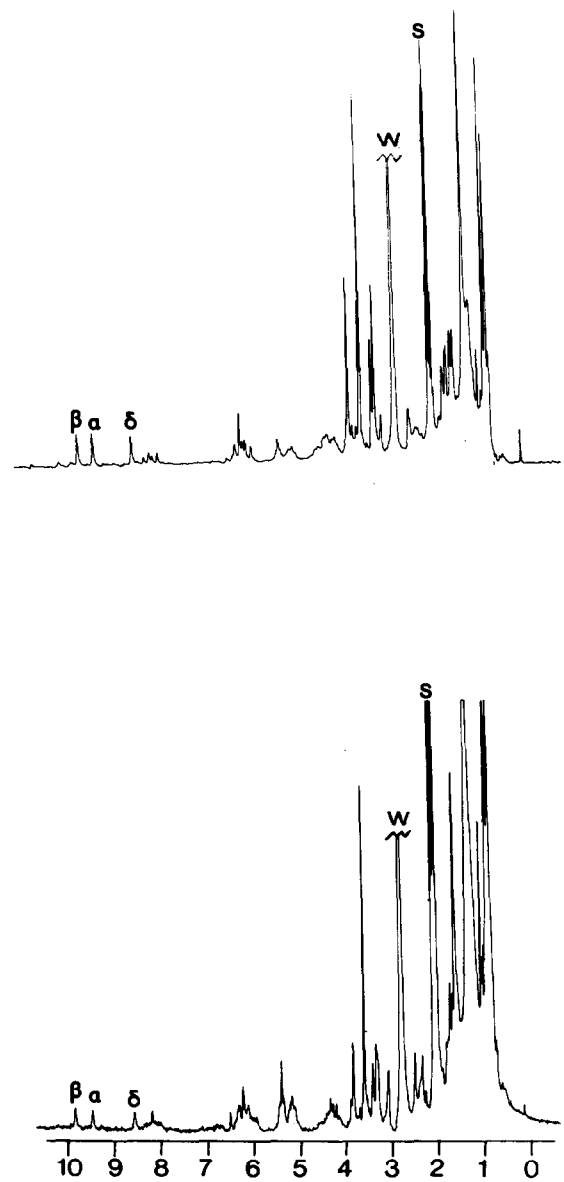


Fig.1. 100 MHz NMR spectra of crude extracts of chlorophylls from (top) normal maize and (bottom) mutant maize. The 3 *meso*-protons, α, β and δ are indicated; s, solvent peak (d_6-acetone); w, water peak.

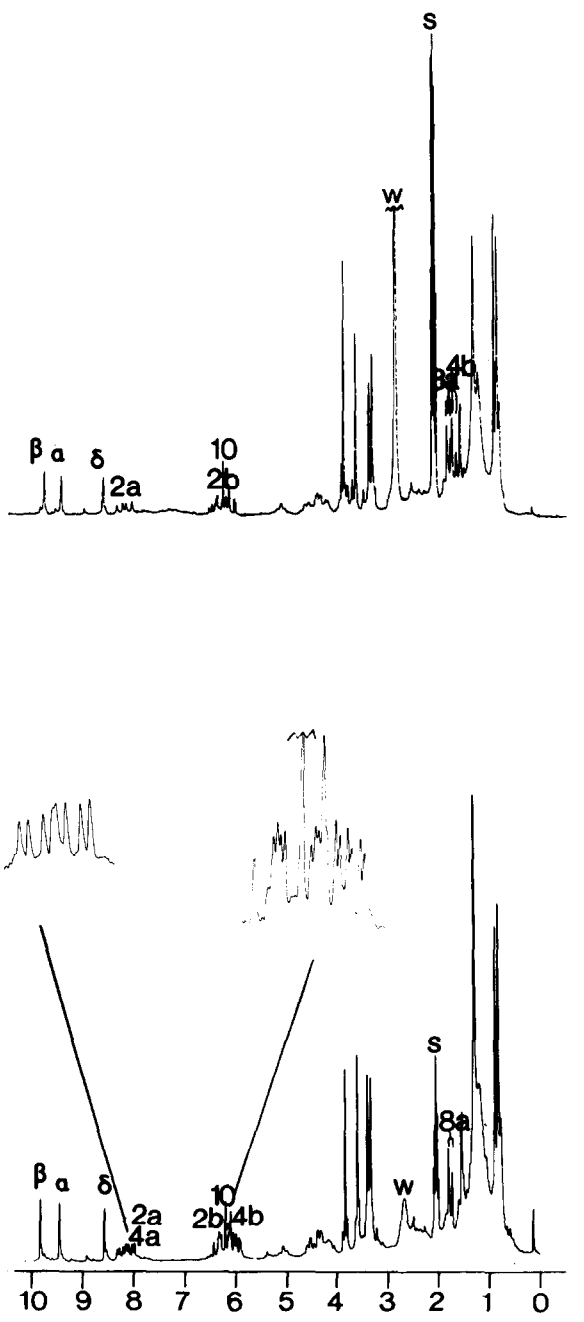


Fig.2. 100 MHz NMR spectra of the purified chlorophyll from (top) normal maize and (bottom) mutant maize. The 3 *meso*-protons, α, β and δ are indicated in both spectra; the protons, 2a, 2b, 10, 8a and 4b are indicated in the spectrum of chl *a*, whereas the protons 2a, 4a, 2b, 4b, 10 and 8a are indicated in the spectrum of 4-vinyl-4-desethyl chl *a*; some small impurity peaks are present in both spectra; s, solvent peak (d_6-acetone); w, water peak.

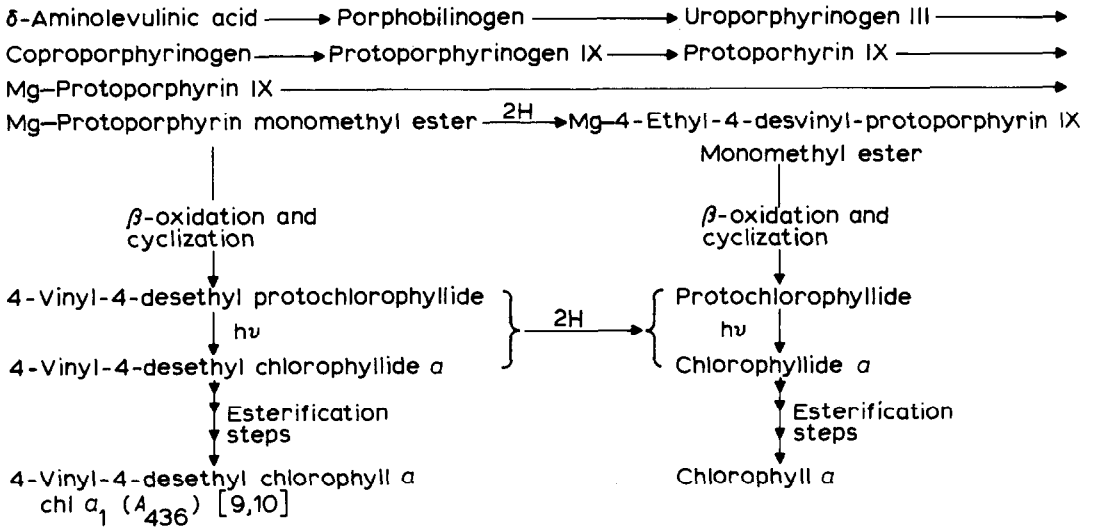
We chromatographically separated the chlorophyll pigments and isolated the major band. The NMR spectrum of this pigment differs considerably between the wild-type and mutant (fig.2). As well as chemical shift differences, we can also see that the low-field vinyl resonances between 5.5 and 8.5 ppm consist of twice as many peaks in the mutant; the resonances due to 4b and 4a are absent in the mutant. Furthermore, the largest chemical shift differences between the mutant and normal chl *a* occur around ring B. This is substantive evidence that the ethyl group on ring B has been replaced by a vinyl group as illustrated in the structure.

Hence we have shown that the structure of chl *a* from the mutant maize chloroplasts differs from normal chl *a* by substitution of a vinyl group for an ethyl group. The structure of the hydrocarbon tail has been characterized as phytol by using the techniques of field desorption, fast atom bombardment, and 'in beam' electron impact mass spectroscopy [13].

4. Discussion

This study provides the first structural evidence for a new, naturallly occurring chl *a* in the chloroplasts of a photosynthetically active higher plant.

In the chlorophyll biosynthetic pathway, the conversion of Mg–protoporphyrin IX monomethyl ester (Mg–PME), a divinyl species, into protochlorophyllide (PChlide) requires the reduction of the vinyl group at position 4 in addition to ring V formation through β-oxidation reaction. The existence of 4-vinyl-4-desethyl PChlide [14–17] however, has led to some disagreement over the sequence of events (reduction and cyclization) leading to the formation of PChlide [14,16]. Reduction of the vinyl group at position 4 may be non-specific for the rest of the macrocycle substituent [16]. The accumulation of large amounts of 4-vinyl-4-desethyl chl *a* in the ON 8147 mutant of maize, however, further suggests that the terminal sequence of enzymes may exhibit a broad specificity for the macrocycle and, in particular, be non-specific for the nature of the modification on position 4. Thus, a mutation affecting the activity of the reductive machinery for the vinyl group at position 4 leads to exclusive production of 4-vinyl-4-desethyl PChlide (M. B. B., unpublished) and forces the accumulation of divinyl chl *a* as the end product. In the normal chloroplast, such broad specificity of the enzymic machinery would produce branched pathways as follows:



Proposed biosynthetic pathways to 4-vinyl-4-desethyl chl *a* and normal chl *a*
The scheme is a modification of currently accepted pathway [10,11,16].

This scheme reconciles the divergent views concerning the order of reduction and cyclization leading to the protochlorophyllide species. A broad specificity of the terminal steps beyond the chlorophyllide stage provides for the observed accumulation of 4-vinyl-4-desethyl chl *a* in the mutant and would allow for appearance of various chl *a* forms speculated to exist in normal chloroplasts [1,2].

Chloroplasts isolated from the mutant (ON 8147) differ in many ways from the wild-type maize but have many characteristics similar to those of immature chloroplasts [11]: the photosynthetic unit is smaller in the mutant maize; the plastids have more intergranal lamellae; the ratio of 'chl *a*/*b*' is ~12:1 in the mutant, several times larger than usual. Thus the possibility arises that 4-vinyl-4-desethyl chl *a* is associated with a specific developmental stage in the maturation of the chloroplasts.

We propose, therefore, that at early stages of chloroplast development the activity of the divinyl pathway is predominant. This could imply absence or low activity of the necessary reductive steps leading to the monovinyl precursor. As the chloroplast matures the activity of the reduction at position 4 increases and the monovinyl pathway is enhanced, leading to formation of normal chl *a*. The divinyl products are, thus, suppressed in the mature system and a greater specificity for the monovinyl intermediates would accentuate this; however, low levels might be expected to occur. Since reaction centers are produced early in the development of chloroplasts, an enhancement of 4-vinyl-4-desethyl chl *a* in reaction centers might also be expected.

Acknowledgements

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