Biochimica et Biophysica Acta, 975 (1989) 164–174
Elsevier

BBA 43013

Freeze-fracture studies on barley plastid membranes. VIII. In *viridis*-^{115}, a mutant completely lacking Photosystem II, oxygen evolution enhancer 1 (OEE1) and the α-subunit of cytochrome *b*-559 accumulate in appressed thylakoids

David J. Simpson, Olivier Vallon * and Diter von Wettstein

Department of Physiology, Carlsberg Laboratory, Copenhagen Valby (Denmark)

(Received 31 October 1988)
(Revised manuscript received 28 February 1989)

Key words: Absorption spectroscopy; Chlorophyll protein; Chloroplast; Electron microscopy; Fluorescence emission; Immunocytochemistry; P-700;

The barley nuclear gene mutant *viridis*-^{115} completely and specifically lacks Photosystem II (PS II)-dependent electron-transport activity. This was associated with the absence of variable fluorescence in room-temperature fluorescence induction, and a change in the 77 K fluorescence emission spectrum due to fluorescence from light-harvesting chlorophyll *a*/*b*-protein of Photosystem II (LHC II) unquenched by PS II reaction centres. The low-temperature absorption spectrum lacked chlorophyll *a* absorbing at 683 nm. Thin section electron microscopy showed that the grana were about 5 times larger in diameter than the wild type, with almost no inter-grana lamellae. Quantitation of the freeze-fracture ultrastructure of *viridis*-^{115} revealed a 96% reduction in the number of endoplasmic fracture face of thylakoid (EFs) particles, the presumed site of the PS II reaction centres. No change was seen in the protoplasmic fracture face of stacked (PFs) or unstacked (PFu) thylakoid particle densities, and the mutant contained 68% of the wild-type density of endoplasmic fracture face of unstacked thylakoid (EFu) particles, making it unlikely that they represent PS II located in the stroma lamellae (PS II$_\beta$). Calculations based on chlorophyll : P-700 and chlorophyll *a*/*b* ratios revealed a loss of between 60 and 70 molecules of chlorophyll *a* per PS II reaction centre. A double mutant, *clo-f2*^{2800} × *vir*^{115} contained grana in the absence of PS II and LHC II, both of which have been implicated in the maintenance of thylakoid adhesion. Three of the 'core' PS II polypeptides (CP47, CP43 and D1) were severely deficient, whereas the α-subunit of cytochrome *b*-559 was present in near normal levels, although the high potential form was not detectable by spectroscopy. The chlorophyll *a*/*b*-proteins CP29 and LHC I were present at normal levels, as was the extrinsic 33 kDa oxygen evolution enhancer 1 (OEE1).

Introduction

The thylakoid membrane consists of six major polypeptide complexes: Photosystem I, Photosystem II, a cytochrome f/b_6 complex, the ATP-generating coupling factor, the light-harvesting antenna of Photosystem I, and the light-harvesting antenna of Photosystem II [1]. These are present as functional and structural units within the membrane, and each is apparently stable in the absence of one or more of the other complexes. They correspond to distinct freeze-fracture particles visible on the different fracture faces of thylakoids. The nature of these freeze-fracture particles has been deduced from the ultrastructural changes seen in mutants specifically lacking a functional complex. It has been a

* Present address: Laboratoire de Microscopie Electronique, Institut Jacques Monod/CNRS, 2 Place Jussieu, Paris, France.

Abbreviations CP, chlorophyll-protein; DPC, 1,5-diphenylcarbazide; DCIP, 2,6-dichlorophenylindophenol; EFs/u, endoplasmic fracture face of stacked/unstacked thylakoids; ESs, endoplasmic surface of stacked thylakoids; F_0, fluorescence yield at onset of illumination; F_m, maximum yield of fluorescence; F_v, variable fluorescence; HP, high potential; LP, low potential; LHC I (II), light-harvesting chlorophyll *a*/*b*-protein of Photosystem I (II); LP, low potential; OEE, oxygen evolution enhancer; PAGE, polyacrylamide gel electrophoresis; PFs/u, protoplasmic fracture face of stacked/unstacked thylakoids; PS I (II), Photosystem I (II); SDS, sodium dodecyl sulphate.

Correspondence: D.J. Simpson, Department of Physiology, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark.

frequent finding that the absence of one polypeptide results in the loss of many or all of the polypeptides associated with it in a functional complex [1–5]. This is due either to their failure to assemble, or because they assemble to form a non-functional complex, with a subsequent rapid turnover of the component polypeptides.

The loss of all Photosystem II reaction centres from thylakoids should result in the removal of the chlorophyll *a*-binding polypeptides D1, D2, CP43 and CP47 [6–11]. Since these are associated with only about 60 molecules of chlorophyll per reaction centre [8,11], and assuming, on a chloroplast basis, about 500–600 chlorophyll molecules per photochemically active Photosystem II reaction centre, a mutant specifically deficient in Photosystem II would not be visibly different from wild type. It would, however, exhibit a high level of red fluorescence at room temperature from the LHC II, which is no longer quenched by the reaction centres. We therefore used the screening procedure of Miles [12] to select high chlorophyll, high fluorescence barley mutants in M2 spikes from Bonus barley seeds mutagenised with sodium azide/oxygen [13]. This paper reports the characterisation of one of these mutants, designated *viridis*-^{115}, with respect to spectral properties, polypeptide and cytochrome composition, and thin section and freeze-fracture ultrastructure of the thylakoids. In addition, results from investigations of a double mutant, *clo*-$f2^{2800} \times vir^{115}$, are reported. This recombinant was produced to determine if thylakoid stacking occurred in the absence of both PS II and LHC II.

Materials and Methods

Plant material. Seeds were germinated in plastic trays containing vermiculite moistened with tap water. Seedlings were harvested after 7 days at 22°C growing under continuous white light at 1700 lx (85 $\mu E \cdot m^2 \cdot s$) provided by Sylvania Gro-lux tubes. Mutant seedlings were identified by their strong red fluorescence when illuminated by UV light (approx. 350 nm) from Osram L20W/73 tubes at a distance of 30–40 cm [12,14].

Electron microscopy. Leaves were processed for thin section electron microscopy, as described in Ref. 14. Thylakoids were isolated and prepared for freeze-fracture and freeze-etching according to Ref. 4. Quantitation of particle size and density was carried out according to Ref. 14 using a Summagraphics Microgrid II digitiser interfaced to a Hewlett Packard 9836S personal computer. Immunocytochemistry of thin sections of Lowicryl K4M-embedded isolated thylakoids was done according to the schedule of Ref. 15. The distribution of different antigens between stacked and unstacked thylakoids was expressed as the ratio of the labelling density, determined from the number of gold particles over stacked appressed regions and unstacked regions, and the stacking ratio.

Spectral measurements. Room temperature fluorescence induction kinetics and 77 K fluorescence emission spectra of whole leaves were recorded using the equipment described in Ref. 14. Solid dilution 77 K fluorescence emission spectra were recorded according to Ref. 16, which involved grinding a small amount of leaf with silica spheres in liquid nitrogen, to avoid self-absorption due to high chlorophyll concentrations. Low-temperature absorption spectra were recorded on an Aminco DW-2a spectrophotometer, using the low-temperature attachment. Seedlings leaves were stuck onto clear adhesive tape and carefully abraded with a razor blade to reduce the absorbance at 675 nm to about 0.3 A. The spectra were recorded on a Nicolet Explorer III digital oscilloscope and transferred to the HP 9836S for further analysis. P-700 and cytochromes were measured with the Aminco DW-2a, as described in Ref. 17.

Antibodies. Monoclonal antibodies to the α-subunit of cytochrome *b*-559, Chl *a*-Pl, CP29 and LHC I from barley were kindly provided by Ms. Lisbeth Hønberg [18] and Dr. Gunilla Høyer-Hansen and co-workers [17,19] and the polyclonal antibodies against *Chlamydomonas* thylakoid polypeptides (equivalent to CP47, CP43 and the 33 kDa oxygen-evolving enhancer) were the generous gift of Professor N.-H. Chua, Rockefeller University. The polyclonal antibody against D1 was kindly provided by Dr. J. Hirschberg, The Hebrew University, Jerusalem.

Other. Photosystem II activity was determined spectrophotometrically with DCIP as the acceptor and water or DPC as electron donors, as described in Ref. 17. Chlorophyll concentration was determined by extraction into 80% acetone, and the chlorophyll *a*/*b* ratio calculated from the equations given in Ref. 17. SDS-PAGE and immuno-blotting was carried out as described in Ref. 19. Thylakoids for SDS-PAGE, biochemical measurements and immunocytochemistry, were isolated in 0.4 M sucrose, 50 mM Tricine, pH 7.9, 5 mM $MgCl_2$, centrifuged for 5 min at $1400 \times g$ and washed twice in 10 mM tricine (pH 7.9), 5 mM $MgCl_2$.

Results

Of the 69 high chlorophyll, high fluorescence barley mutants found (designated *viridis*-^{101} to -^{169}), seven showed fluorescence induction kinetics characteristic of severe or complete deficiency of Photosystem II function. These are the mutants *viridis*-^{102}, -^{115}, -^{123}, -^{132}, -^{133}, -^{143}, -^{168}. The seedlings segregated in a wild type/mutant ratio of 3:1, and the mutants were extremely difficult or impossible to distinguish from wild type by eye unless fluorescence emission was observed under UV light. Diallelic crosses indicate that they

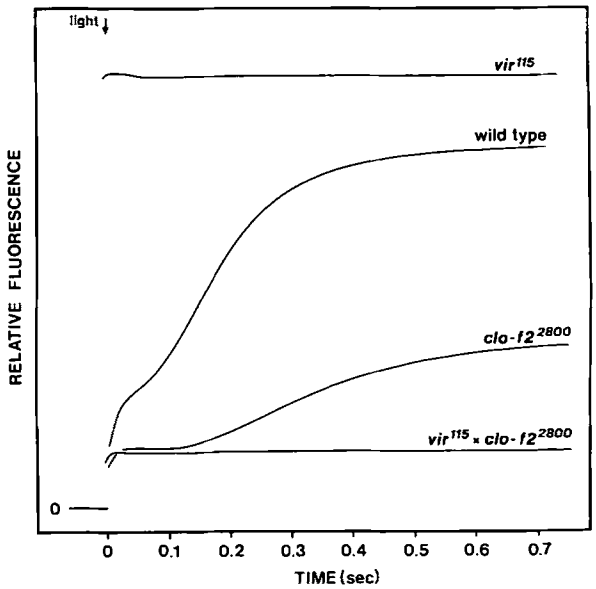


Fig. 1. Room temperature fluorescence induction kinetics of dark-adapted barley seedling leaves. Illumination of a constant leaf area with high intensity (450 $\mu E \cdot m^2 \cdot s^{-1}$) blue light (Corning 4-96 filter) was controlled by an electronic shutter with an opening time of 0.6 ms.

represent mutations in seven different genes, none of which are allelic with the previously designated genes giving rise to Photosystem II deficiency [14], namely *viridis-c*, *-e*, -1, *-v*, *-zd* and *-ze*.

The fluorescence induction kinetics of the mutant *viridis-*^{115} is shown in Fig. 1. The initial level of fluorescence (F_0), measured after 0.6 ms of illumination, was about 6 times that of wild type on a leaf area basis, equivalent to the maximum level of fluorescence (F_m) of wild type. The absence of variable fluorescence (F_v) is diagnostic for mutants completely lacking Photosystem II (PS II) activity [12,14,20]. The low-temperature absorption spectrum, shown in Fig. 2, was very similar to wild type. Computer subtraction of the *vir*^{115} spectrum from that of wild type, showed a peak at 683 nm, indicating the specific loss from *vir*^{115} of a significant amount of chlorophyll *a* absorbing at 683 nm. The 77

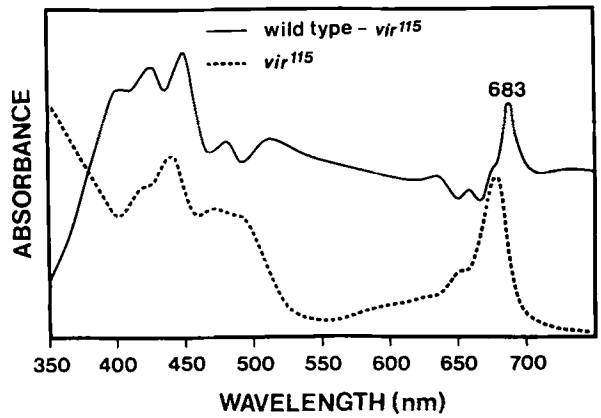
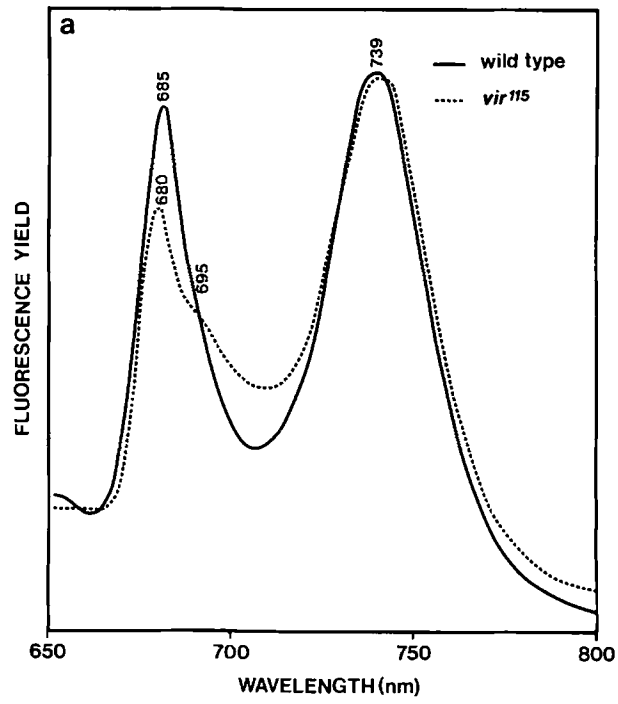


Fig. 2. Low temperature (77 K) absorption spectrum of an abraded leaf of *viridis-*^{115}, together with the difference spectrum (at 2×scale) with respect to wild type. Note the peak at 683 nm, indicating a deficiency of this species of chlorophyll *a* in the mutant.



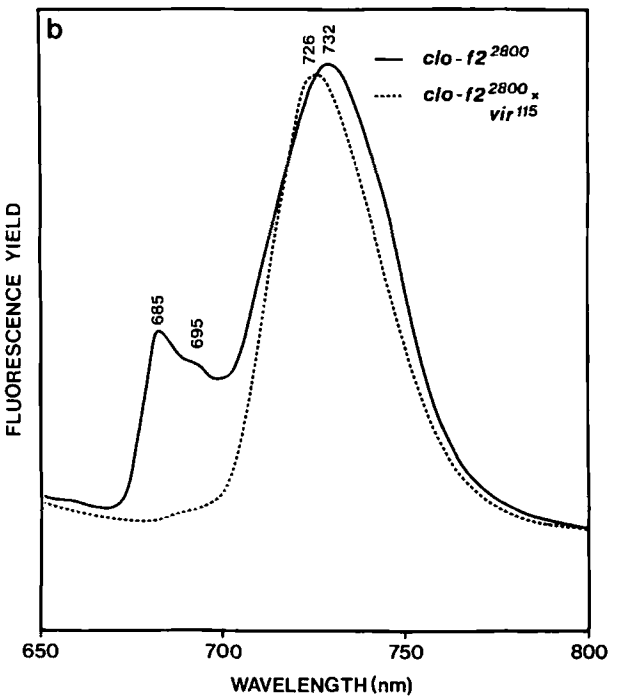


Fig. 3. Solid dilution fluorescence emission spectra at 77 K of (a) wild type and *vir*^{115} seedling leaves. The 685 nm peak in the wild type is shifted to 680 nm and the 695 nm peak is more pronounced in the mutant, both features arising from LHC II unquenched by PS II. (b) The chlorophyll *b*-less mutant *clo-f2*^{2800} and the double mutant *clo-f2*^{2800} × *vir*^{115}. In *clo-f2*^{2800} the fluorescence at 685 nm must come from the PS II reaction centre, since in the double mutant there is only a single peak at 726 nm arising from the PS I reaction centre.

TABLE I

Composition of thylakoid membranes

Wild-type data are from Ref. 17. Values in nmol per mg Chl ± S.D., except for Chl *a*/*b* ratio; electron transport values are in μmol DCIP reduced per mg Chl per h.

Component	Wild type	*Viridis*-115
Cytochrome *f*	1.6 ± 0.2	1.9 ± 0.3
Cytochrome *b*-563	3.3 ± 0.2	4.8 ± 0.9
Cytochrome *b*-559 LP	3.2 ± 0.5	3.1 ± 0.9
Cytochrome *b*-559 HP	2.4 ± 0.3	0.12 ± 0.01
P-700	2.2 ± 0.3	1.9 ± 0.1
Chlorophyll *a*/*b*	3.5 ± 0.1	2.43 ± 0.08
$H_2O \rightarrow$ DCIP	33.1	< 0.03
DPC → DCIP	33.8	< 0.03

K fluorescence emission spectrum of *vir*[115] leaves differed from that of wild type in two respects. The 685 nm peak was shifted to 680 nm, and the fluorescence yield at 695 nm was higher in *vir*[115]. This was also seen in the solid dilution fluorescence emission spectrum (Fig. 3a), where the yield of 680–685 nm fluorescence relative to that at 740 nm was reduced in *vir*[115] compared to wild type. The room temperature fluorescence spectra of *vir*[115] and wild-type leaves were also similar, except that the fluorescence yield per leaf area of the mutant was about 4 times higher (data not shown).

The PS II-dependent electron-transport activity of isolated *vir*[115] thylakoids (Table I) was less than 0.1% of the wild-type rate, both with water and DPC as electron donors. The P-700 content, measured both chemically and photochemically, was slightly lower for *vir*[115] (Table I), giving a chlorophyll-to-P-700 ratio of 596 ± 43, compared with 518 ± 56 for wild type. The level of cytochrome *f*, *b*-563 and *b*-559 LP were at least as high in *vir*[115] as in wild type, on a chlorophyll basis. The high potential (HP) form of cytochrome *b*-559, which is associated with a functional PS II reaction centre, was reduced to 5% of the wild-type level.

Thin section electron microscopy of *vir*[115] chloroplasts showed that the thylakoid system was organised into grana with an extremely long diameter (Fig. 4). They had an average diameter of 2.43 ± 0.54 μm, with 5.0 ± 1.4 discs per granum, compared with wild-type grana which have an average diameter of 0.42 ± 0.05 μm, with 10.4 ± 4.8 discs [4]. Measurement of the stacking ratio (percent appressed thylakoids) gave a value of 76 ± 3%, compared with 65% for wild type [17]. Since most grana profiles were almost as long as the long axis of the chloroplast, it was unusual to see stroma lamellae connecting grana. As a result, grana end discs represented most of the unappressed membranes. Occasionally, a single thylakoid membrane was seen running parallel to the grana.

Thylakoid ultrastructure was further investigated by freeze-fracture electron microscopy of unfixed cryoprotected membranes. The appearance of the four different freeze-fracture faces is shown in Fig. 5. The PFu and PFs faces resembled those of wild type thylakoids, as did the EFu face, with its low number of particles on a heavily pitted, uneven surface. In contrast, the EFs face showed a dramatic loss of the characteristic large particles which is the proposed location of the PS II reaction centres. These impressions were confirmed by measurement of freeze-fracture particle densities (Table II), which showed that *vir*[115] thylakoids contained normal levels of PFu and PFs particles, 68% of the EFu particles per μm^2 and only 4% of the EFs particles present in the wild type. Determination of freeze-fracture particle sizes showed that the remaining EFs par-

Fig. 4. Thin section electron micrograph of *vir*[115] chloroplast showing the presence of extremely long grana with almost no interconnecting stroma lamellae. Magnification, 26000×.

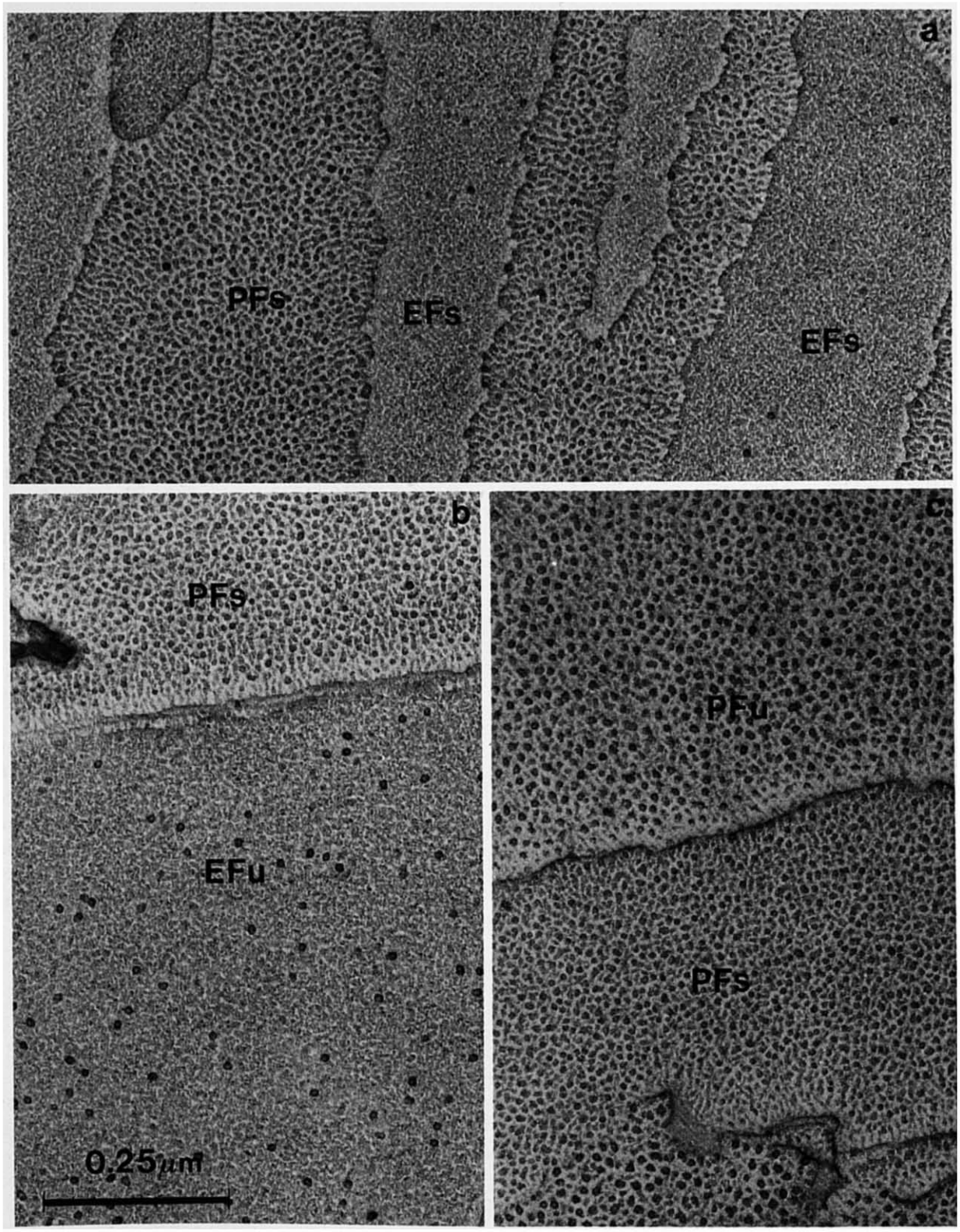


Fig. 5. Freeze-fractured, rotary shadowed replicas of isolated *vir*^{115} thylakoids. Note the almost complete absence of particles from the EFs face (a), while other faces (b and c) resemble those of wild type. Magnification, 125000×.

ticles were significantly smaller than wild-type EFs particles (Table III). No significant differences were found for the other fracture faces. The inner surface of grana membrane regions, the ESs, was almost smooth (Fig. 6) and devoid of the tetrameric particles which characterise wild-type thylakoids.

The polypeptide composition of *vir*^{115} was analysed by denaturing SDS-PAGE (Fig. 7). Comparison of the

TABLE II

Freeze-fracture particle density data

Values are numbers per $\mu m^2 \pm$ S.E. Wild-type data are from Ref. 4, except for PFs and PFu large, which are from Ref. 5.

Face	Wild type	*Viridis*-115
EFu	347± 6	244±16
PFu	4553±25	4527±37
EFs	1624±11	69± 3
PFs	6525±30	6697±34
PFu large	3290±27	2930±37
PFu small	1439	1597

relative intensities of Coomassie-staining bands revealed a loss of polypeptides in the regions corresponding to D1, CP47 and CP43, which are chloroplast-coded intrinsic polypeptides of the PS II reaction centre core. The latter two chlorophyll-proteins were identified by immune blot analysis (Fig. 7) using polyclonal antibodies raised against the corresponding proteins (5 and 6) in *Chlamydomonas*. While CP47 and CP43 were detectable in wild type at 15 μg, 3 μg and 0.6 μg chlorophyll, the reaction is weak, as previously shown for thylakoids [17]. These denatured polypeptides of barley thylakoids cross-react poorly with antibodies raised against *Chlamydomonas*, so the immune blots cannot be used to evaluate the presence or absence of CP43 and CP47 in *vir*[115]. In contrast, the extrinsic 33 kDa polypeptide involved in enhancement of oxygen evolution (OEE1), was present at a significant level (between 1/3 and 1/2 of wild type), as was the α-subunit of cytochrome *b*-559 (over 1/2 of the wild-type level). The chlorophyll *a*/*b*-binding antenna proteins of PS II, CP29 and LHC II, were present at normal levels (Fig. 7).

The localisation of specific thylakoid polypeptides was investigated by immuno-gold labelling of isolated *vir*[115] thylakoids embedded in Lowicryl K4M, using monoclonal and polyclonal antibodies whose monospecificity had been demonstrated [15,17,19]. It has been shown [17] that in wild-type thylakoids, the polyclonal antibodies against D1, CP43 and CP47 reacted with polypeptides located in the grana and stroma membranes. It is apparent that the latter two antibodies recognise epitopes in the fixed polypeptides, but not after denaturation by SDS-PAGE. In all three cases, 80% of the label was over appressed membranes and 20% over non-appressed stroma membranes. In the mutant *vir*[115], no significant labelling was seen with a polyclonal antibody to D1 (Fig. 8a), while antibodies to CP43 and CP47 produced significant levels of labelling (Fig. 8e). However, there was no preference of label over the appressed regions (Table IV), as found in the wild type. High labelling densities were seen with monoclonal antibodies to CP29 and the α-subunit of cytochrome *b*-559 and a polyclonal antibody to the 33 kDa extrinsic polypeptide of the oxygen-evolving complex (OEE1) (Fig. 8b–d). The distribution of gold particles over appressed and non-appressed thylakoids was measured (Table IV), and showed that 90% of CP29 and OEE1 was in appressed lamellae, while 80% of cytochrome *b*-559 was found over appressed regions. Since more appressed profiles than non-appressed profiles are present in the mutant, the patterns for the different PS II components are as follows: CP43 and CP47 are concentrated in non-appressed lamellae, CP29, OEE1 and cytochrome *b*-559 are enriched in the giant grana, while the reaction centre of Photosystem I (Chl *a*-P1)

TABLE III

Freeze-fracture particle sizes

Wild-type data are from Ref. 4.

Face	Axis	Average size ± S.D. (Å)	
		wild type	*viridis*-115
EFu	small	105±15	88± 9
	large	126±18	102±11
PFu	small	93±15	82±15
	large	112±18	100±19
EFs	small	117±13	88±12
	large	155±20	102±16
PFs	small	74±12	74± 9
	large	93±14	86±10

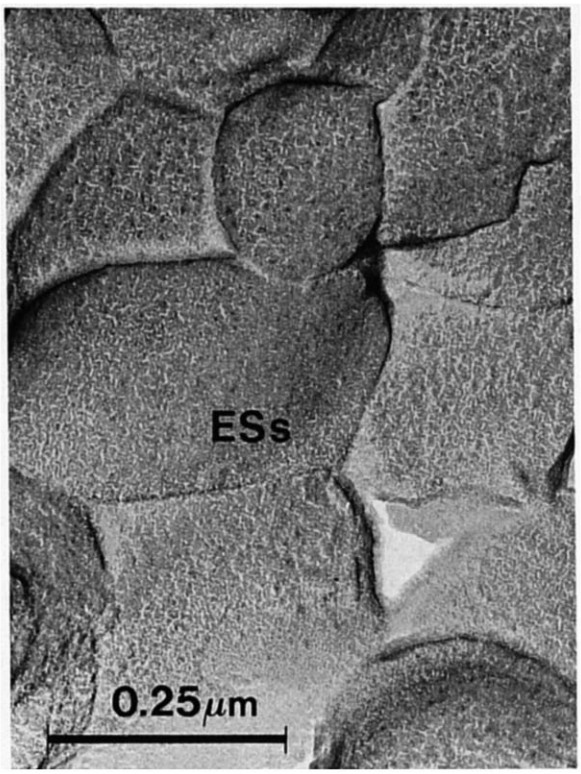


Fig. 6. Freeze-etched *vir*[115] thylakoids showing the absence of the characteristic tetrameric ESs particles containing the oxygen evolving complex from the inner surface of grana. Magnification, 125000×.

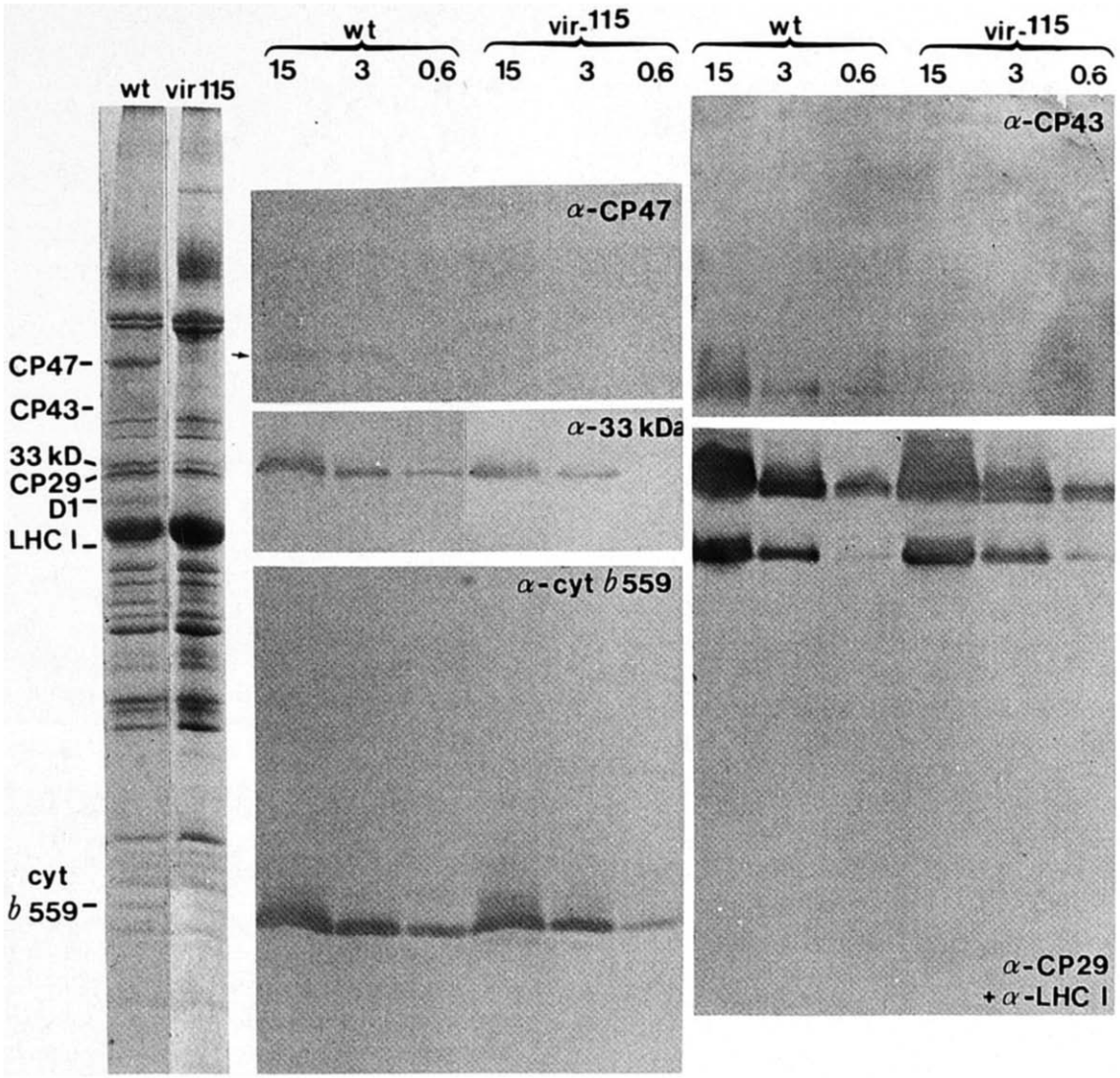


Fig. 7. Analysis of the polypeptides of *vir*^{115} by SDS-PAGE and immuneblot assay. Each lane has been loaded with 15, 3 or 0.6 μg of chlorophyll. The mutant contains near normal levels of the extrinsic 33 kDa oxygen enhancing polypeptide (OEE1), CP29, LHC I and the α-subunit of cytochrome *b*-559. cyt, cytochrome.

(Fig. 8f) was completely excluded from appressed regions (Table IV).

A double mutant lacking both PS II and chlorophyll *b* was isolated by crossing *clo-f2*^{2800} × *vir*^{115}. It was considered of interest to produce such a double mutant, in which the major grana components (PS II and LHC II) were genetically removed. Recombinant seedlings were readily distinguished from *clo-f2*^{2800} due to their visibly paler colour. The fluorescence induction kinetics of the double mutant showed a low F_0, equivalent to *clo-f2*^{2800}, and no F_v, as for *vir*^{115} (Fig. 1). The 77 K solid dilution fluorescence emission spectrum showed a single peak at 726 nm, resulting from Photosystem I, with no fluorescence at 685 or 695 nm (Fig. 3b). Thin section electron microscopy revealed the presence of grana (Fig. 9). There were fewer grana per chloroplast than in *vir*^{115}, and they had a diameter of 1.72 ± 0.65 μm with 3.8 ± 1.4 discs per granum.

TABLE IV.

Distribution of label in immunocytochemistry of viridis-^{115} *thylakoids*

The unstacked/stacked value gives the relative labelling densities of gold particles on unappressed vs. appressed membranes, using the formula:

$$\frac{100-x}{x} \cdot \frac{y}{100-y}$$

where x = percent label over stacked regions and y = stacking ratio. Wild-type data are from Ref. 17.

Antibody to	% label on stacked regions	unstacked/stacked	
		vir^{115}	wild type
CP47	58.3	2.26	0.45
CP43	41.3	4.51	0.35
CP29	92.3	0.26	0.09
33 kDa OEE1	90.9	0.32	0.37
Cytochrome *b*-559	81.7	0.71	0.18
CPI	4.9	61.3	22.4

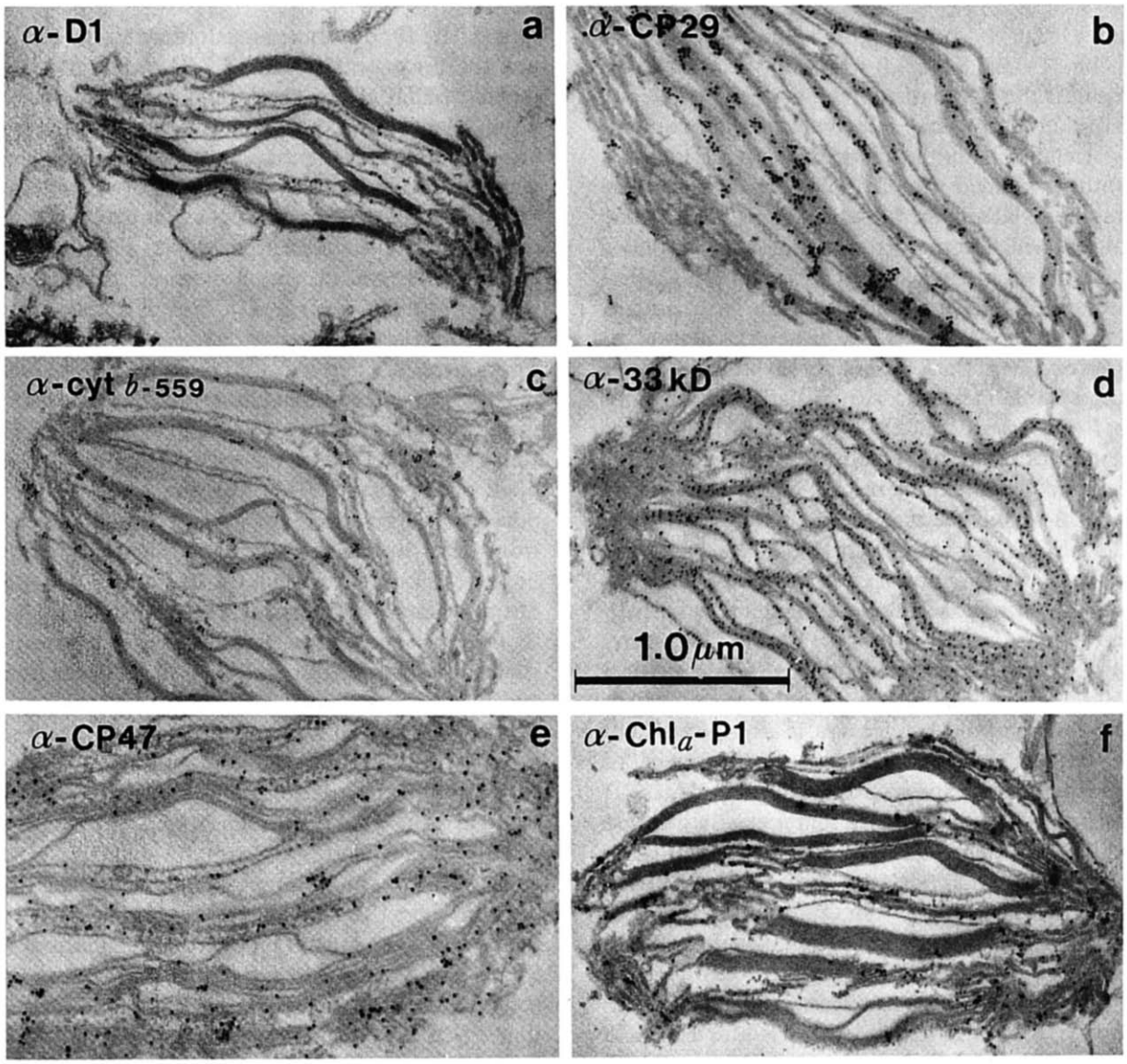


Fig. 8. Immunocytochemistry of isolated, Lowicryl-embedded thylakoids of *vir*^{115}. After incubation with the antibody indicated, thin sections were reacted with colloidal gold (dark spots on micrograph) bound either to protein A (a, d and e) or rabbit anti-mouse IgG (b, c and f). Magnification, 30000×. cyt, cytochrome.

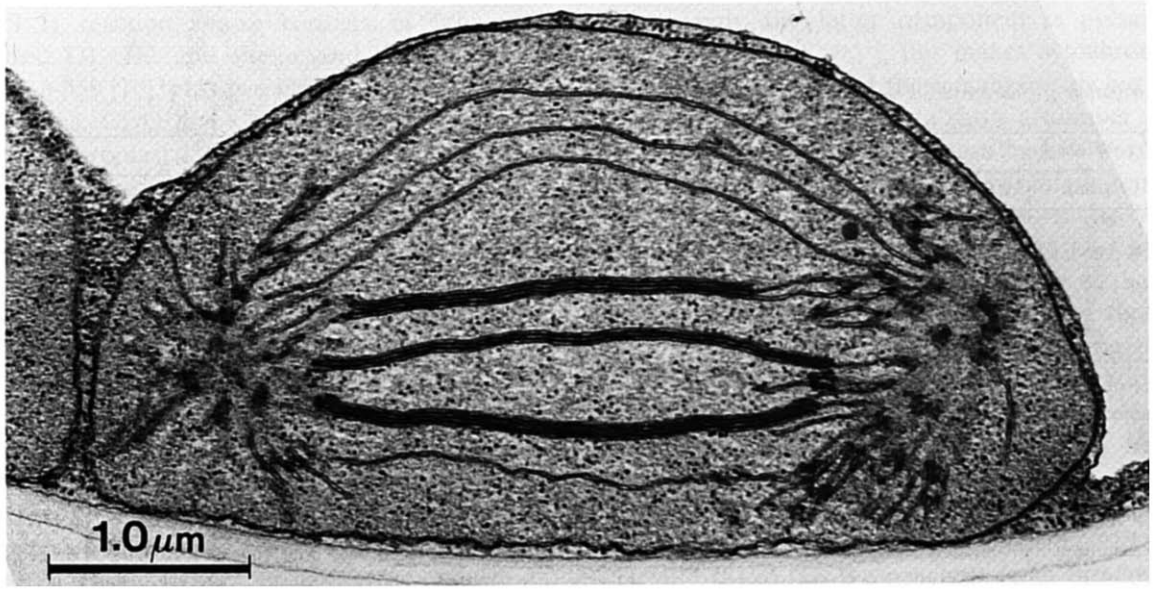


Fig. 9. Thin section electron micrograph of the double mutant *clo-f2*^{2800} × *vir*^{115} showing the presence of grana, even though PS II and LHC II are both severely depleted. Magnification, 26000×.

Discussion

Viridis-^{115} is the first barley mutant which completely lacks PS II activity, yet has a high chlorophyll content. This is correlated with the disappearance of the EFs particles, the presumed site of the PS II reaction centre in appressed thylakoid membranes [1], as well as the ESs particles, which contain the extrinsic polypeptides involved in enhancement of oxygen evolution [21]. Previously described PS II mutants of barley have either a partial loss (e.g., *vir-m*^{29}, Ref. 1), or severe chlorophyll deficiency (e.g., *vir-c*^{12}, Ref. 14), or exhibit extensive pleiotropic effects, including partial deficiency in Photosystem I (e.g., *vir-zd*^{69}, Ref. 14). The PS II-deficient NC95 mutant of tobacco [2] is visibly chlorophyll deficient. *Viridis*-^{115} most closely resembles the maize PS II mutant *hcf*-3*, which has been extensively characterised by Miles and co-workers [12,22–27]. As found for *vir*^{115}, the *hcf*-3* mutation causes a complete loss of PS II activity, without visible loss of chlorophyll [22], and a decrease in the chlorophyll *a/b* ratio [25].

The fluorescence induction kinetics of *vir*^{115} shows a high F_0 level with a constant yield, roughly equivalent to the F_m level in wild-type leaves. This is consistent with the pattern found during the development of manganese deficiency and concomitant loss of PS II activity in spinach leaves, resulting in a steady increase in F_0, while F_m remains constant, so that F_v progressively decreases [28]. In *vir*^{115}, the loss of PS II has been complete ($F_v = 0$), so that in the thylakoids, LHC II is not connected to PS II reaction centres. This is reflected in the 77 K fluorescence emission spectrum, where the 685 nm peak has shifted to 680 nm, typical of 'free' LHC II, and the 695 nm peak, which is also found in Mg^{2+}-aggregated LHC II in vitro, has a higher amplitude. The same features have been reported for the *hcf*-3* mutant [24]. These results imply that in wild type, LHC II contributes much more to the 685 and 695 nm fluorescence than the PS II reaction centre. Removal of LHC II by combining *vir*^{115} with the chlorophyll *b*-less homozygous mutant *clo-f2*^{2800} results in the loss of both of these peaks. Loss of LHC II alone causes a large decrease in the amplitudes of the 685 and 695 nm fluorescence emission peaks, and about a 50% reduction in the fluorescence yield on a chlorophyll basis at room temperature and a decrease in the F_m/F_0 ratio (see *vir-k*^{23}, Ref. 29, *clo-f2*^{101}, Ref. 30). Loss of both PS II and LHC II produces a low F_0 with no F_v (Fig. 1). The source of F_0 in *vir*^{115} × *clo-f2*^{2800} is not known, but may come from residual chlorophyll *a/b*-proteins binding only chlorophyll *a* (e.g., CP29 and LHC II – see Refs. 19 and 31), or from some of the antenna chlorophyll of Photosystem I. Clearly most of the room-temperature fluorescence in wild type thylakoids comes from LHC II, which is normally quenched by PS II.

An obvious ultrastructural feature of *vir*^{115}, and all of the other six PS II barley mutants, is the organisation of the thylakoids into giant grana, with most of the stroma lamellae corresponding to the unappressed granal end membranes. This is also true of the chlorophyll-deficient PS II mutants *vir-c* and *vir-1* mutants, but not of the partially PS II-deficient mutants *vir-m*^{29} and *vir-zd*^{69} [14], nor of the maize *hcf*-3* mutant [22]. We find similar giant grana with appressed thylakoids in the double mutant *clo-f2*^{2800} × *vir*^{115} which lacks both PS II and LHC II. The chlorophyll *b*-less mutant of barley contains grana in vivo [4] and stacking in vitro can be maintained with high (25 mM) concentrations of $MgCl_2$, which also precipitate the PS II core from detergent solubilised thylakoids [31]. This led to the conclusion that PS II might play a role in thylakoid appression [31]. Our finding that thylakoid appression occurs in vivo in the absence of both LHC II and PS II raises the possibility that yet another membrane component can

TABLE V

Calculated distribution of chlorophylls in wild type and viridis-^{115} *thylakoids*

	Wild type [a]			*Viridis*-^{115} [a]			*Viridis*-^{115} [b]		
	grana	stroma	total	grana	stroma	total	grana	stroma	total
Chl *a*	223	180	403	241	180	421	141	106	247
Chl *b*	85	30 [c]	115 [d]	145	30 [c]	175 [d]	85	18	103
Chl *a* + *b*	308	210 [c]	518 [d]	386	210 [c]	596 [d]	226	124	350
Chl *a/b*	2.67 [3]	6.0 [c]	3.5 [d]	1.66 [e]	6.0 [c]	2.4 [d]	1.66 [e]	6.0 [c]	2.4 [d]
Area (%)	67 [f]	33	–	80	20 [g]	–	80	20	–

[a] Per molecule of P-700.
[b] Per 0.586 molecules of P-700 (i.e., 85/145).
[c] From Ref. 33.
[d] From Table II.
[e] Calculated.
[f] From Ref. 4.
[g] Calculated, assuming the same concentration of P-700 per unit area of stroma lamellae as in wild type, based on density of large PFu particles (= 0.33 × 85/145).

be involved. It will be interesting to see if the 26 kDa polypeptide associated with LHC II in grana, but not stroma lamellae [32] is also present in the double mutant.

The reduction in the amount of stroma lamellae is the probable cause of the decrease in the P-700 concentration in *vir*^{115} (Table I). From the chlorophyll : P-700 and chlorophyll *a/b* ratios of thylakoids (Table I) and stroma lamellae [33], the distribution of chlorophyll *a* and *b* between appressed and non-appressed membranes can be calculated (Table V). This produces a chlorophyll *a/b* ratio of 2.67 for wild type grana, which is a little higher than found for detergent isolated grana membranes (2.53, Ref. 34; 2.3, Ref. 35) and 1.66 for *vir*^{115} grana, which is not consistent with a chlorophyll *a/b* ratio of 1.37 for isolated barley LHC II [36], and 2.5 for CP29 [6].

When the figures for *vir*^{115} are normalised for the chlorophyll *b* content of grana (assuming no loss of chlorophyll *b* from grana, and no change in stroma lamellae composition), so that chlorophyll is expressed per 0.586 molecules of P-700, there is a loss of 82 molecules of chlorophyll *a* per 308 molecules of chlorophyll *a* + *b* in grana (Table V). This represents 56–69 molecules of chlorophyll *a* associated with the PS II core polypeptides D1, D2, CP43 and CP47, if one assumes 220–260 molecules of chlorophyll per PS II reaction centre [7,9]. This is consistent with values of 66 ± 4 [11] and 65 ± 4 [8] molecules of chlorophyll *a* per isolated PS II reaction centre core. Since 4–6 molecules of chlorophyll *a* are associated with D1 and D2 [10], this means that CP47 and CP43 each bind about 30 molecules of chlorophyll. With molecular masses of 52 and 56 kDa, respectively, this gives a high chlorophyll/protein ratio, but is similar to that of LHC II, which binds 14 chlorophylls per 25 kDa polypeptide [36]. It should also be noted that the calculated percentage (80%) of thylakoid area accounted for by appressed lamellae (Table V), is close to that measured (76%).

The PS II reaction centre consists of four core polypeptides, D1, D2 and the α and β subunits of cytochrome *b*-559 [10], plus two closely associated chlorophyll-proteins, CP43 and CP47 [6], all of which are encoded by chloroplast DNA. Three extrinsic subunits involved in oxygen evolution, OEE1, OEE2 and OEE3, are encoded in the nucleus. They are regulated independently of the core polypeptides, since they are present at normal levels in PS II-deficient mutants [37,38]; and their presence is not strictly necessary for the stability of the PS II reaction centre (Refs. 39 and 40; see also Wollman, F.A., personal communication).

In *vir*^{115}, the PS II polypeptides fall into two categories. Analysis of the thylakoid polypeptides by SDS-PAGE revealed that D1, CP47 and CP43 were absent or greatly reduced, while OEE1, CP29 and the α-subunit of cytochrome *b*-559 were present at normal or near normal levels. Studies in another laboratory (Mullet, J., personal communication) have shown that *vir*^{115} has normal mRNA levels for the *psbA, -B, -C* and *-D* genes, but that translation of *psbA* mRNA is deficient, suggesting that the *vir*^{115} gene product is involved in the regulation of D1 translation. It therefore differs from *hfc*-3*, which shows an accelerated turnover of D1, the *psbA* gene product [27]. The accumulation of CP43 seems to be independent of that of CP47 [41,42], while the accumulation of D2 is dependent on that of D1 [43]. Genetic deletion of D1 [44], or D2 [38], and genetically engineered deletion of CP43, CP47 [41,42] and cytochrome *b*-559 [45] show that each of these five proteins is required for the assembly of functional PS II reaction centres.

The fate of cytochrome *b*-559 has not been fully investigated in most PS II mutants, and we present the first evidence for the accumulation of an intrinsic PS II polypeptide at near normal levels in a mutant highly deficient in the other components. Apparently the α-subunit of cytochrome *b*-559 moves from its site of synthesis on chloroplast ribosomes to the appressed membranes independently of D1 and D2, where it is stably integrated into the thylakoid membrane.

The presence of normal levels of CP29 and OEE1 in *vir*^{115} with the same distribution as found in wild type (Table IV) indicates that that their synthesis on cytoplasmic ribosomes and subsequent migration into appressed lamellae is independent of the intrinsic PS II components. Furthermore, the presence of PS II is not required for the efficient exclusion of PS I reaction centres from appressed thylakoid regions. The OEE2 polypeptide is also present at near normal levels in *vir*^{115} [18], but the absence of ESs tetrameric particles indicates that a typical oxygen-evolving complex does not form. The extrinsic nature of OEE1 implies an association with a membrane 'anchor'. It has recently been shown [46] that it can bind to a PS II reaction centre containing only D1, D2 and cytochrome *b*-559. Since only the latter component is present in near normal levels in *vir*^{115}, this makes cytochrome *b*-559 a possible candidate for the membrane anchor for OEE1.

The freeze-fracture data show a number of interesting features. The 96% decrease in EFs particles (from 1622 to 69 per μm^2) is the most dramatic reported for a PS II mutant (cf. 872 → 104 for maize *hcf*-3* [3], 1250 → 350 for *Chlamydomonas* F34 [20] and 1558 → 190 for tobacco NC95 [2]). The nature of the remaining EFs particles is not known. They may represent partially assembled PS II centres, containing the residual amounts of PS II polypeptides detected immunologically.

The number of EFu particles in *vir*^{115} represents a high proportion of the wild type level, as found for maize *hcf*-3* (74%) [3] and NC95 tobacco (58%) [2], as well as manganese-deficient spinach (91%) [28] and *vir-m*^{29} (81%) [1]. We conclude from the drastic reduc-

tion of 4 of the PSII core polypeptides (Fig. 7 and Mullet, J., personal communication), that the EFu particles in wild type thylakoids are unlikely to represent PS II reaction centres. We also find that the number of PFs particles per μm^2 is not significantly higher in *vir*[115], as found for other barley PS II mutants, such as *vir-m*[29] and *vir-zd*[69] [1], manganese-deficient spinach [28] and tobacco NC95 [2]. This contrasts with *Chlamydomonas* mutant F34 [20] and maize *hcf*-3* [3], where large increases in PFs particles were reported (4500 → 7600 and 2903 → 4069, respectively). Our data, therefore, do not support the hypothesis that some LHC II is located in EFs particles, giving rise to extra PFs particles in the absence of the PS II reaction centre.

Acknowledgements

We thank Drs John Mullet and Francis-André Wollman for communicating results prior to publication, and Kirsten Kristiansen and Bente Jensen for for propagating the mutant seed stocks and for producing the double mutant. We are grateful to Ann-Sofi Steinholtz and Nina Rasmussen for photographic work and for drawing the figures, and to Jean Sage for expert technical assistance with the electron microscopy.

References

1 Simpson, D.J. (1986) in Encyclopedia of Plant Physiology. New Series, Photosynthesis III. (Staehelin, L.A. and Arntzen, C.J., eds.), Springer-Verlag Berlin, Vol. 19, pp. 665–674.
2 Miller, K.R. and Cushman, R.A. (1979) Biochim. Biophys. Acta 546, 481–497.
3 Leto, K., Keresztes, A. and Arntzen, C.J. (1982) Plant Physiol. 69, 1450–1458.
4 Simpson, D.J. (1979) Carlsberg Res. Commun. 44, 305–336.
5 Simpson, D.J. (1983) Eur. J. Cell Biol. 31, 305–314.
6 Bassi, R., Høyer-Hansen, G., Barbato, R., Giacometti, G.M. and Simpson, D.J. (1987) J. Biol. Chem. 262, 13333–13341.
7 Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1984) Biochim. Biophys. Acta 765, 388–398.
8 Millner, P.A., Marder, J.B., Gounaris, K. and Barber, J. (1986) Biochim. Biophys. Acta 852, 30–37.
9 Murata, N., Miyao, M., Matsunami, H. and Kuwabara, T. (1984) Biochim. Biophys. Acta 765, 363–369.
10 Namba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
11 Yamagishi, A. and Katoh, S. (1985) Biochim. Biophys. Acta 807, 74–80.
12 Miles, D. (1980) in Methods Enzymol. 69, 3–22.
13 Jende-Strid, B. (1978) Barley Genet. Newsl. 8, 55–57.
14 Simpson, D.J. and Wettstein, D. Von (1980) Carlsberg Res. Commun. 45, 283–314.
15 Vallon, O., Wollman, F.A. and Olive, J. (1985) FEBS Lett. 183, 245–250.
16 Weis, E. (1985) Photosynth. Res. 6, 73–86.
17 Vallon, O., Høyer-Hansen, G. and Simpson, D.J. (1987) Carlsberg Res. Commun. 52, 405–421.
18 Hønberg, L. (1984) Carlsberg Res. Commun. 49, 703–719.
19 Høyer-Hansen, G., Bassi, R., Hønberg, L.S. and Simpson, D.J. (1988) Planta 173, 12–21.
20 Wollman, F.A., Olive, J., Bennoun, P.and Recouvreur, M. (1980) J. Cell Biol. 87, 728–735.
21 Simpson, D.J. and Andersson, B. (1986) Carlsberg Res. Commun. 51, 467–474.
22 Miles, D. and Daniel, D.J. (1974) Plant Physiol. 53, 589–595.
23 Leto, K. and Miles, D. (1980) Plant Physiol. 66, 18–24.
24 Leto, K. and Arntzen, C. (1981) Biochim. Biophys. Acta 637, 107–117.
25 Metz, J.G. and Miles, D. (1981) Biochim. Biophys. Acta 681, 95–102.
26 Metz, J.G., Krueger, R.W. and Miles, D. (1984) Plant Physiol. 75, 238–241.
27 Leto, K., Bell, E. and McIntosh, L. (1985) EMBO J. 4, 1645–1653.
28 Simpson, D.J. and Robinson, S.P. (1984) Plant Physiol. 74, 735–741.
29 Von Wettstein, D., Møller, B.L., Høyer-Hansen, G. and Simpson, D. (1979) EC Solar Energy R&D Programme. 3rd Coordination Meeting of Contractors, June 18–20, Brussels, Commission of the European Community XII/750/79.
30 Simpson, D.J., Machold, O., Høyer-Hansen, G. and Von Wettstein, D. (1985) Carlsberg Res. Commun. 50, 223–238.
31 Bassi, R., Hinz, U. and Barbato, R. (1985) Carlsberg Res. Commun. 50, 347–367.
32 Bassi, R., Giacometti, G. and Simpson, D.J. (1988) Biochim. Biophys. Acta 935, 152–165.
33 Bassi, R. and Simpson, D.J. (1987) Eur. J. Biochem. 163, 221–230.
34 Henry, L.E.A. and Møller, B.L. (1981) Carlsberg Res. Commun. 46, 227–242.
35 Hinz, U.G. (1985) Carlsberg Res. Commun. 50, 285–298.
36 Hinz, U.G. and Welinder, K.G. (1987) Carlsberg Res. Commun. 52, 39–54.
37 Delepelaire, P. (1984) EMBO J. 3, 701–706.
38 Kuchka, M.R., Mayfield, S.P.and Rochaix, J.-D. (1988) EMBO J. 7, 319–324.
39 Mayfield, S., Bennoun, P. and Rochaix, J.-D. (1987) EMBO J. 6, 313–318.
40 Mayfield, S., Rahire, M., Frank, G., Zuber, H. and Rochaix, J.-D. (1987) Proc. Natl. Acad. Sci. USA 84, 749–753.
41 Vermaas, W.F.J., Williams, J.G.K., Rutherford, A.W., Mathis, P. and Arntzen, C.J. (1986) Proc. Natl. Acad. Sci. USA 83, 9474–9477.
42 Vermaas, W.F.J., Ikeuchi, M. and Inoue, Y. (1988) Photosynth. Res. 17, 97–113.
43 Erickson, J.M., Rahire, M., Malnoë, P., Girard-Bascou, J., Pierre, Y., Bennoun, P. and Rochaix, J.-D. (1986) EMBO J. 5, 1745–1754.
44 Bennoun, P., Spierer-Herz, M., Erickson, J., Girard-Bascou, J., Pierre, Y., Delosme, M. and Rochaix, J.-D. (1986) Plant. Mol. Biol. 6, 151–160.
45 Pakrasi, H.B., Williams, J.G.K. and Arntzen, C.J. (1988) EMBO J. 7, 325–332.
46 Gounaris, K., Chapman, D.J. and Barber, J. (1988) FEBS Lett. 234, 374–378.