AN ANALYSIS OF TEMPERATURE AND LIGHT SENSITIVITY IN *tigrina* MUTANTS OF BARLEY

by

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Quantitative determinations of chlorophylls, carotenes and xanthophylls were carried out by high pressure liquid chromatography on the nuclear gene mutant *tigrina-o*³⁴. In the dark the mutant accumulates lycopene and protochlorophyllide at 23 °C but almost none at 30 °C, while xanthophylls are deficient only at 23 °C. In the light, lycopene accumulation and β -carotene as well as xanthophyll deficiency is severe at 23 °C, whereas an improved content of β -carotene and xanthophyll is observed at 30 °C. An improved content can also be achieved by growing mutant seedlings in red light of wavelengths longer than 650nm at 23 °C.

Under conditions where lycopene accumulates in the dark the presence of abnormal membrane structures is correlated with a deficiency in certain membrane polypeptides, e.g. the α , β subunits of coupling factor.

Under conditions of severe β -carotene deficiency in light grown seedlings, photodynamic destruction of chloroplast ribosomes and preferential accumulation of light harvesting chlorophyll *a/b*-protein is observed. Eventually, the chloroplast structure disintegrates.

Under conditions of simultaneous lycopene accumulation and β -carotene as well as lutein deficiency the chlorophyll aggregates into a non-fluorescing form absorbing at 743nm. This form is part of a complex insoluble in 2% SDS and containing lycopene, chlorophyll *a* and protein.

Tigrina mutants at other loci have been characterized as to their temperature and light sensitivity.

Abbreviations: CF₁ = chloroplast coupling factor; Chl = chlorophyll; Chl-P = chlorophyll-protein; HPLC = high pressure liquid chromatography; kD = kilodalton; μ E = micro Einsteins; PAGE = polyacrylamide gel electrophoresis; SD = standard deviation; SDS = sodium dodecyl sulfate.

1. INTRODUCTION

The *tigrina* phenotype of barley, characterized by alternate transverse green and necrotic bands in the leaf (Figure 1), is only expressed when the *tigrina* mutants are grown under alternating periods of darkness and light (36). In continuous light the *tigrina* mutants are uniformly coloured and usually chlorophyll deficient while in the dark some are pale green due to the accumulation of protochlorophyllide in amounts exceeding that found in the wild-type (Figure 2) (28). The *tigrina* mutants are therefore considered to be defective in the regulation of chlorophyll biosynthesis (10, 21, 28, 37). In *tigrina-d*¹², *tigrina-b*²³ and *tigrina-o*³⁴ it was shown that the lesions are in control mechanisms operating normally in the dark to restrict δ -aminolevulinic acid formation and thereby prevent excessive protochlorophyllide accumulation (14). In *tigrina-b*²³ and *tigrina-o*³⁴ excessive protochlorophyllide

Figure 2. Phenotypes displayed by *tigrina-o*³⁴.

A. Wild-type and *tigrina-o*³⁴ grown in the dark at 23 °C. B. Wild-type and mutants mixed with heterozygotes grown in the dark at 30 °C. C. Wild-type and mutant grown in >600nm at 23 °C, D. >650 nm red light at 23 °C and E. in > 650nm red light at 30 °C.

accumulation in the dark was accompanied by partial blocks in the biosynthesis of carotenoids (29). In *tigrina-o*³⁴ lycopene accumulation was observed. This mutant is particularly interesting because under constant light at the low temperature (20-23 °C) deficiencies are observed of chlorophyll (28), chloroplast ribosomes and proteins synthesised thereon (17). These defects tend to normalise at the high temperature of 30 °C. At the high temperature other abnormalities are also reduced such as the amount of abnormal plastid membrane structures (17) and of chlorophyll *a*₇₄₃ (28).

Figure 1. Phenotypes shown by *tigrina* mutants grown under light-dark cycles.

A. The *tigrina* phenotype exemplified by *tigrina-d*¹² grown in the green-house. B. The zonata (arrow) and *tigrina* (double arrow) phenotypes shown by *tigrina-b*²⁴ grown in the growth chamber.

WT

tig-o³⁴

WT

WT
+
tig-o³⁴

WT

tig-o³⁴

WT

tig-o³⁴

WT

tig-o³⁴

This paper shows that in *tigrina-o³⁴* an increase in growth temperature coordinately affects the defect in carotenoid biosynthesis and the defective regulation of chlorophyll biosynthesis in the dark. In the light changes in pigment composition in *tigrina-o³⁴* grown at high and low temperature and under high and low intensity red lights are related to ultrastructural and molecular abnormalities in the plastids. The effect of temperature and light conditions on the phenotype of other *tigrina* mutants is also presented.

2. MATERIALS AND METHODS

2.1. Plant material and growth conditions

Seeds used were wild-type barley (*Hordeum vulgare* cv. Svalöfs Bonus), the mutant *tigrina-o³⁴* and its heterozygote in a 1:1.2 ratio (28), seeds of homozygous *tigrina-b²⁴*, *tigrina-c*, *tigrina-j²⁸*, seed material segregating for *tigrina* mutants at the *tigrina-a*, *tigrina-b*, *tigrina-d*, *tigrina-e*, *tigrina-f*, *tigrina-m*, *tigrina-n* loci and for albozonata-1. Seeds

were germinated in vermiculite moistened with tap-water. They were grown for seven days prior to analysis. Seedlings were grown either at 23 °C or 30 °C in complete darkness or under continuous illumination with white light or red light. Two different red lights were used; one with wave-lengths above 600nm and the other above 650nm. Seedlings were also grown in alternating periods of 8 hours dark and 16 hours light controlled by a time switch. For the discontinuous illumination experiments white light or red light with wave-lengths above 600nm or 650nm were used. Except when grown at 30 °C in the dark or in continuous red light at wave-lengths above 650nm, leaves of *tigrina-o³⁴* could be distinguished by their colour in white light from phenotypically wild-type heterozygotes. The leaves were frozen in liquid nitrogen in green safe light and sorted in white light at liquid nitrogen temperatures. Under these conditions protochlorophyllide was not photoconverted and pigments were protected against photooxidation by reason of the nitrogen atmosphere.

2.2. Light sources and energies

Two different white light sources were used. Four Osram Fluora 20W strip lights providing an intensity of $55\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ and two Philips TL, 40W striplights ($15\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) were used. Red light with wave-lengths above 600nm ($> 600\text{nm}$) was obtained from the stronger light source using a Rohm and Haas filter No. 502 (red). The light intensity was measured as $20\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$. Red 502 combined with blue 625 gave $> 600\text{nm}$ red light with an intensity of $2.5\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$. Red light with wave-lengths above 650nm ($> 650\text{nm}$) was obtained from the same light source using filter No. 602 (blue) and No. 502 (red) (Figure 3). The light intensity was $1.4\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$. The red filters formed the top of a large box ($100 \times 50 \times 30$ (depth)) cm^3 in which the plants were grown. Light intensities were measured using an LI-COR photometer model LI-185B and an LI-COR quantum sensor.

2.3. Extraction of pigments and preparation for their quantitative determination by high pressure liquid chromatography

Leaves were harvested by cutting them below the coleptile. One gram of plant material or sin-

Figure 3. Absorption spectra of filters used to obtain different red lights.

Blue 602 and red 502 were combined to produce red light of wave-lengths above 650nm. Red 502 was employed to give red light with wave-lengths above 600nm. When this filter was used alone an intensity of $20 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ was obtained. Red 502 combined with blue 625 gave $>600\text{nm}$ red light with an intensity of $2.5 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$.

gle plants were ground in a mortar with liquid nitrogen and respectively 200 or 10 mg NaHCO_3 . All operations were carried out in dim light or green safe light. For the larger amount of tissue the fine powder was transferred with acetone to a Buchner filter funnel and washed with 50ml of acetone. Ten ml of ethyl acetate was added to the filtrate and it was evaporated to final volume of 1 or 2 ml under a stream of nitrogen. If an aqueous layer formed it was dispersed by the addition of more acetone. The powdered single leaves were transferred with 5 ml acetone to a centrifuge tube and allowed to stand for some minutes in order to extract the pigments. After centrifugation one ml of ethyl acetate was added to the supernatant and the combined solution was evaporated as above to 50 or 100 μl . The pigments in the reduced extracts were then quantitatively determined by high pressure liquid chromatography (HPLC).

2.4. Determination of pigments by high pressure liquid chromatography

Chlorophylls and carotenoid pigments from plants grown in the light were injected into the separatory system by an automatic injection unit (WISP 710B) and separated on a C_{18} reverse phase contained in a plastic column (Radial Pack C18) and compressed in an RCM-100 unit. Elution was performed using a linear gradient of 90% methanol to 100% ethyl acetate over a period of 20 minutes followed by further isocratic elution with ethyl acetate for 5 minutes. The gradient was generated by two pumps controlled by a System Controller. The flow rate was 1 ml per minute. The eluting pigments were detected at 430nm at a sensitivity of 0.2 by a 450 Variable Wavelength Detector. The signal from the detector was integrated and the amounts of pigments calculated by a Data Module, programmed using factors obtained by linear regression of known

amounts of standard pigments and integrated peak area. All high pressure liquid chromatography equipment was manufactured by Waters. Protochlorophyllide and carotenoid pigments from plants grown in the dark were separated using the same system except for the introduction of 5 mM-tetrabutyl ammonium phosphate (Kodak) into the 90% methanol. In the presence of this anion-pairing reagent protochlorophyllide was retained for a longer time on the column.

2.5. Preparation of standard pigment solutions

All pigments were prepared in dim light or in green safe-light. Protochlorophyllide was prepared from dark grown barley shoots incubated overnight with their cut ends in 0.01 M- δ -aminolevulinate. The shoots were ground in liquid nitrogen, extracted with acetone: 0.3 M-NH₃, 80:20 v/v and filtered. The protochlorophyllide was transferred to diethyl ether by the addition of an equal volume of ether and saturated NaCl solution. The ether phase, containing protochlorophyllide and carotenoids, was washed free of acetone with water. Carotenoids were then separated from the protochlorophyllide. To 1 volume of ether was added 4 volumes of petroleum ether, 0.1 volume of a mixture (4:1 v/v) of methanol and 10 mM-NH₃. Protochlorophyllide was extracted into the ammoniaical methanol leaving all carotenoid pigments in the other phase. The protochlorophyllide solution was then washed with an equal volume of petroleum ether. The methanolic solution of protochlorophyllide was then diluted with saturated NaCl solution and the protochlorophyllide concentrated by absorption on a cartridge of C₁₈ reverse phase material (Waters Sep-Pak C₁₈). The Sep-Pak C₁₈ was pre-activated by passing 10 ml acetone and then 5 ml of H₂O. Protochlorophyllide was eluted with acetone and concentrated to a small volume under a stream of nitrogen. The concentration of protochlorophyllide was estimated in 80% acetone using $E_{m628} = 34.5$ (13). Standard solutions of chlorophyll *a* and *b*, lutein, violoxanthin, neoxanthin and β -carotene were prepared in the following manner. A solution enriched in leaf xanthophylls was obtained by grinding 50 g green leaves in liquid nitrogen and 1 g solid NaHCO₃ and extracting

the powder on a Buchner funnel with 50% acetone. The residue was set aside for extraction of chlorophylls and β -carotene. To the filtrate was added 0.2 volumes of chloroform. Acetone was then added until the two phases were dispersed. The mixture was then poured into 4 volumes of distilled water in a measuring cylinder. Pigment-containing droplets of the mixture fell through the water, were washed free of acetone and collected at the bottom as a relatively small volume of chloroform containing the pigments. The chloroform was washed several times with water removing each time the water and any solids that collected at the interphase after centrifugation. The chloroform solution of pigments was then dried over anhydrous NaSO₄ and the three xanthophyll pigments separated by reversed phase chromatography on thin layer plates (Whatman RC₁₈) using a solvent system of 90% methanol:ethyl acetate, 40:10 v/v. The separated xanthophylls were scraped off, eluted with acetone, concentrated under a stream of nitrogen and the concentration determined in ethanol using the extinction coefficients collected by DAVIS (6). The residue remaining from the extraction of xanthophylls was extracted with acetone until chlorophylls and β -carotene were removed and the acetone filtered. The chlorophylls were then separated from the β -carotene using an activated C₁₈ cartridge (7). The acetone was diluted with water and the pigments absorbed on the cartridge. Chlorophylls were then eluted with methanol and β -carotene with acetone. Pigments were separated on reversed phase C₁₈ thin layer plates in solvent systems of 90% methanol:ethyl acetate 50:50 v/v and 10:90 v/v consecutively. Pigments were eluted with acetone and the concentrations determined spectrophotometrically of the chlorophylls (25) and for β -carotene (6). Lycopene was purified from tomato paste (Italian dobbelconcentrato) as described in (19) and the concentration of all-trans-lycopene determined spectrophotometrically (6). After determination of the concentration of lycopene and the five chloroplast pigments the solutions were combined and concentrated to give a mixture of known composition. Different amounts of the mixture were injected into the high pressure liquid chromatograph in order to calibrate the response for each

pigment. Protochlorophyllide was calibrated by injecting it separately. Concentrations of the unknown epoxy-xanthophyll eluting between lutein and violoxanthin was determined using the same factor as determined for lutein.

2.6. Spectrophotometric and spectrofluorometric measurements

Absorption spectra were determined using an Aminco DW-2A. Low temperature fluorescence emission spectra were obtained as described by SIMPSON and VON WETTSTEIN (32).

2.7. Isolation of ribosomes, purification of thylakoids, and SDS-PAGE

Ribosomes were isolated as described earlier (17). In the preparations of ribosomes the ratio of A_{260} to A_{280} varied between 1.6 - 1.9 for crude ribosomes isolated from dark-grown plants and between 1.6 - 1.8 for those isolated from red light-grown plants. After separation of 70S and 80S ribosomes on a 10-30% sucrose gradient, the ribosomal proteins were analysed by SDS-PAGE. Internal plastid membranes were isolated and purified according to (18) and subjected to SDS-PAGE. The molecular weights of the separated polypeptides were determined by use of molecular weight markers. (Bovine serum albumin 68,000, ovalbumin 43,000, chymotrypsinogen A 25,000 and cytochrome c 12,400).

2.8. Electron microscopy

Tissue samples from dark-grown and red light-grown seedlings were collected and fixed under dim green light and red light, respectively. Specimens were sampled at different levels along the primary leaf, as illustrated in Figure 12. Tissue samples were prepared for electron microscopy by fixation in 3% glutaraldehyde and 10 mM- $MgCl_2$ in 0.05 M-cacodylate buffer, pH 6.9, and postfixed in 1% osmium tetroxide in the same buffer. Thereafter they were processed as described earlier (17).

2.9. Measurement of CO_2 fixation

The five cm top portion of the leaf was cut into 8 pieces and floated for 10 min in 0.4 ml of distilled H_2O containing $1\mu Ci$ of $NaH^{14}CO_3$ under $1000\mu E \cdot m^{-2} \cdot sec^{-1}$ of white light (Osram HQI lamp). After the incubation 1 ml of boiling meth-

anol was added, the leaf pieces ground in a mortar and an aliquot of the methanol extract counted.

3. RESULTS

3.1. The pigment composition of *tigrina-o³⁴* and the wild-type grown in the dark at 23 °C and 30 °C

In this section it is shown that the accumulation of excess protochlorophyllide in *tigrina-o³⁴* at the lower temperature was associated with a defect in carotenoid biosynthesis because both defects were reversed at the higher temperature. Figure 4A illustrates the separation of protochlorophyllide and carotenoids from mutants grown at the lower temperature and from the mixture of wild-type and mutants grown at the higher temperature. As can be seen large amounts of protochlorophyllide (peak 1) and lycopene (peak 8) were present in the mutant grown at the lower temperature. At the higher temperature the mutants could not be distinguished by eye from the phenotypic wild-type although they were known to be present in a ratio of about 1:1 (Figure 2B). However, pigments extracted from mixed leaves of these two genotypes contained low levels of protochlorophyllide and lycopene. In order to determine the pigment composition in the mutant at 30 °C analyses of extracts of single seedlings were carried out. Table I shows that the leaf identified as a mutant because of its small content of lycopene, had wild-type levels of β -carotene and the other pigments. Therefore, apart from the presence of a small amount of lycopene, the pigment composition of the mutant leaves grown in the dark at 30 °C is similar to that of the wild-type.

Both mutant and wild-type contained a pigment (Figure 4A, peak 4) which eluted between violoxanthin (peak 3) and lutein (peak 5) and had a spectrum very similar to lutein. The shift of the absorption maxima towards the blue caused by the addition of a drop of concentrated HCl and the magnitude of this shift (Figure 5) indicated that the pigment was a monoepoxy-carotenoid (6). The two neighbouring peaks were lutein (a dihydroxyxanthophyll) and violoxanthin (a diepoxydihydroxyxanthophyll). Therefore the elution position of the pigment suggests that it was a monoepoxydihydroxy-

Figure 4: Elution profiles of pigments from *tigrina-o³⁴* analyzed by HPLC.

The mutants were grown A. in the dark at either 30 °C or 23 °C, and B. in white light ($55 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) at either 23 °C or 30 °C. The peaks were identified as 1. protochlorophyllide, 2. neoxanthin, 3. violoxanthin, 4. monoepoxyxanthophyll, 5. lutein, 6. chlorophyll *b*, 7. chlorophyll *a*, 8. lycopene, 9. β -carotene.

xanthophyll. Lutein epoxide and antheraxanthin, two monoepoxydihydroxyxanthophylls which have been reported to be present in etiolated barley (24) were not detected in these experiments. The quantitative analysis of the pigment content (Table II) showed that at 23 °C, the mutant had more total carotenes (450% of wild-type levels) due to a large accumulation of lycopene, normal levels of β -carotene but decreased levels of the xanthophylls violaxanthin (16%), neoxanthin (50%), the unidentified epoxyxanthophyll (70%) and lutein (85%). The small amount of lycopene in the wild-type at 23 °C is due to a selecting error. Therefore, in the dark the *tigrina-o³⁴* mutation was expressed more strongly at the lower temperature i.e. was cold-sensitive. In the dark the mutant gene *tigrina-o³⁴* effected both an increased δ -aminolevulinate and a de-

creased xanthophyll formation. The latter was compensated by the accumulation of an equivalent amount of lycopene.

3.2. The pigment composition of *tigrina-o³⁴* and the wild-type grown under white light at 23 °C and 30 °C

As stated in the introduction, when *tigrina-o³⁴* was grown under constant light at the higher temperature of 30 °C plastid structure and components more closely resembled the wild-type and chlorophyll content was increased compared to the lower growth temperature of 23 °C. Quantitative data are now presented which show that the increased amount of chlorophyll at the higher temperature was accompanied by an increased ratio of β -carotene to chlorophylls thus indicating a greater pigment stability.

Figure 4B shows the separation by high pressure liquid chromatography of pigments from mutants seedlings grown under white light. The increased content of β -carotene (peak 9) at the high temperature is clearly seen. The mutant also contains at both temperatures a pigment (peak 4) (identified as an epoxyxanthophyll in

section 3.1) which is absent in the wild-type. The quantitative pigment compositions in *tigrina-o³⁴* and wild-type at 23 °C and 30 °C are given in Table III. At 23 °C compared to the wild-type the mutant had low levels of violaxanthin (13%), β -carotene (19%), total carotenes (61%)(of which accumulated lycopene represented 75%), neo-

Table I.

Pigment content of single leaves from seedlings of genotypes *tigrina-o³⁴/+* and *tigrina-o³⁴/tigrina-o³⁴* grown in the dark at 30 °C.

Pigment	Pigment content, nmoles per leaf		
	average of six <i>tig-o³⁴/+</i> leaves	S.D.	<i>tig-o³⁴</i> leaf
Protochlorophyllide	0.12	± 0.080	0.05
Neoxanthin	0.087	± 0.049	0.15
Violaxanthin	0.17	± 0.12	0.30
Epoxyxanthophyll	0.63	± 0.19	0.72
Lutein	1.9	± 0.52	1.8
Lycopene	0	0	0.1
β -carotene	0.16	± 0.054	0.15

Figure 5. Absorption spectra of pigment in peak 4.

The shift in absorption maxima upon the addition of HCl indicates that the pigment is a monoepoxy-carotenoid.

xanthin (23%) and lutein, chlorophyll *a* and chlorophyll *b* (all 28%). The unidentified epoxy-xanthophyll was also present in *tigrina-o³⁴* in amounts intermediate to those of neoxanthin

and violoxanthin. Thus in contrast to the carotenoid content of the mutant in the dark at 23 °C, in the light there was a deficiency of β -carotene and of total carotenes in addition to the xanthophyll deficiency. This suggested that photodestruction of both β -carotene and lycopene were occurring.

At 30 °C the levels of pigments in the mutant compared to the wild-type (beginning with the lowest levels) were violoxanthin (29%), chlorophyll *a* (46%), neoxanthin (50%), lutein (56%), β -carotene (56%), chlorophyll *b* (60%) and total carotenes (144%) of which lycopene represented 88%.

When the pigment composition of the mutant at 23 °C was compared with its composition at 30 °C the largest increase was seen for β -carotene (160%) followed by chlorophyll *b* (60%), lutein (50%), then chlorophyll *a* (35%), violoxanthin (30%) and the lowest in lycopene, the unidentified epoxyxanthophyll and neoxanthin (all 20-25%).

Therefore the pigment complement of the mutant resembled more closely the wild-type when grown at 30 °C than at 23 °C. The relatively large increase in β -carotene content can be attributed to a better conversion of lycopene to β -carotene at the higher temperature. The conversion of lycopene to lutein also increased and more xanthophylls were formed from β -carotene. Abnormalities still occurring at 30 °C in the mutant

Table II.

Pigment content of *tigrina-o³⁴* and wild-type (= *tig-o³⁴/+*) grown at 23 °C and 30 °C in the dark for 7 days.

Pigment	Content nmoles $\cdot$ g ⁻¹ fresh weight		
	<i>tigrina-o³⁴</i>	wild-type	1:1 mixture of <i>tig-o³⁴</i> and wild-type
	23 °C		30 °C
Protochlorophyllide	9.6	0.9	1.3
Neoxanthin	0.9	1.8	1.1
Violaxanthin	1.2	7.7	2.2
Epoxyxanthophyll	5.3	7.4	7.6
Lutein	22	26	25
Lycopene	16	1.7	0.2
β -carotene	2.4	2.3	2.2
Total xanthophylls	29	43	36
Total carotenes	18	4	2.2

Table III.

Pigment content of *tigrina-o³⁴* and wild-type (+/+) leaves grown in white light ($55 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) at 23 °C and 30 °C for 7 days.

Pigment	Pigment content, nmoles $\cdot$ g ⁻¹ fresh weight			
	<i>tigrina-o³⁴</i>		wild-type	
	23°C	30°C	23°C	30°C
Neoxanthin	9.6	12	41	24
Violaxanthin	4.3	5.5	32	19
Epoxyxanthophyll	6.5	7.8	0	0
Lutein	37	55	130	97
Chlorophyll <i>b</i>	62	99	219	165
Chlorophyll <i>a</i>	200	270	710	590
Lycopene	47	56	0	0
β -carotene	14	36	75	64
Total xanthophylls	57	80	200	140
Total carotenenes	61	92	75	64
Carotene/xanthophyll	1.1	1.1	0.38	0.46
β -carotene/chlorophyll	0.05	0.09	0.08	0.08

were the occurrence of the unidentified epoxy-xanthophyll, a low violoxanthin/chlorophyll ratio and a continued lycopene accumulation. At the higher temperature there was a partial reversal of the cold-sensitive lesion affecting β -carotene formation from lycopene. Consequently, the ratio of β -carotene to chlorophylls in *tigrina-o³⁴* increased from 0.05 at 23 °C to wild-type levels of 0.09 at 30 °C. The increased content of β -carotene and xanthophylls stabilised a larger amount of chlorophylls.

In white light the cold-sensitive carotenoid deficiency caused photoinstability and was associated with chlorophyll deficiency. It therefore might be expected that if *tigrina-o³⁴* was grown at the lower temperature but at light intensities and qualities where photodestruction would be lessened, an increase in chlorophyll content and plastid stability is to be seen.

3.3. The pigment composition of *tigrina-o³⁴* and wild-type leaves grown at 23 °C under >600nm and >650nm red light

To test the effect of light intensity and quality seedlings were grown under two light sources differing in intensity and quality. These were >600nm red light ($20 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) and >650nm red light ($1.4 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$). The lowest intensity light (>650nm red light) was of a

quality (Figure 3) such that any non-photoactive protochlorophyllide, with an absorption maximum at 635nm, that might accumulate, would not absorb the light and would not act as a photosensitiser. As shown in Table IV protochlorophyllide was detected in *tigrina-o³⁴* and the wild-type grown under the lower intensity >650nm red light in amounts similar to those found in dark grown wild-type (section 3.1). This suggests that the light intensity was low enough to limit photoconversion of protochlorophyllide and thus chlorophyll formation. The similarity of the chlorophyll content of the wild-type controls under the >600nm red light and white light (section 3.2) indicated that chlorophyll formation under >600nm red light was not limited by light intensity. Pigment levels in the mutant grown under >600 and >650 nm red light as a percentage of the comparable wild-type levels, were respectively for violoxanthin (18% and 12%), for β -carotene (14%, 31%), lutein (25%, 29%), neoxanthin (28%, 22%), chlorophyll *a* (26%, 54%) chlorophyll *b* (32%, 43%) total carotenenes (79%, 172%) of which lycopene represented 63% and 138%. Larger amounts of the unidentified epoxyxanthophyll eluting in peak 4 (see Figure 4) was present in the mutants grown under >650nm red light than under 600nm red light. It could be an unknown intermediate of

Table IV.

Pigment content of *tigrina-o³⁴* and wild-type grown for 7 days under >600nm (20 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) or >650nm (1.4 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) red light at 23 °C. ND, not determined.

Pigment	Content nmoles $\cdot$ g ⁻¹ fresh weight			
	<i>tigrina-o³⁴</i>		wild-type	
	Red >600nm	Red >650nm	Red >600nm	Red >650nm
Protochlorophyllide	ND	1.8	ND	1.1
Neoxanthin	8.2	4.4	29	20
Violaxanthin	4.1	2.8	23	23
Epoxyxanthophyll	5.9	7.9	0	0
Lutein	25	19	100	65
Chlorophyll <i>b</i>	28	40	87	94
Chlorophyll <i>a</i>	160	150	620	280
Lycopene	45	64	0	0
β -carotene	8.8	17	64	47
Total xanthophyll	43	34	150	111
Carotenes	54	81	68	47
Carotene/xanthophyll	1.3	2.4	0.43	0.42
β -carotene/chlorophyll	0.04	0.08	0.09	0.12

xanthophyll biosynthesis. In the wild-type grown under >650nm red light pigments eluted in peak 4. Their spectra, however, suggested that they could be a mixture of antheraxanthin and lutein epoxide. In the mutant, β -carotene to chlorophyll ratios under the >600nm red light (0.04) were appreciably decreased compared to the ratio (0.08) present in seedlings grown under the red light of lower intensity (>650nm) (Table IV).

In the light the phenotype of *tigrina-o³⁴* tended toward normality either when grown at 30 °C under white light or under >650nm red light at 23 °C. Could *tigrina-o³⁴* be completely normalised by growth at 30 °C under >650nm red light? Figure 2E shows that leaves from *tigrina-o³⁴* and wild-type grown under these conditions could not be distinguished by their pigment content. Lycopene and chlorophyll *a₇₄₃* were not detected in these plants. However, testing sixteen single leaves for their ability to fix CO₂ under high light intensity revealed two classes of leaves. Ten had a low activity of CO₂ fixation (50-100 $\times$ 10³ cpm leaf⁻¹) similar to wild-type in the dark (87 $\times$ 10³ cpm leaf⁻¹). The other six leaves showed high CO₂ fixation (120-240 $\times$ 10³ cpm leaf⁻¹), indicating the ability to photosynthesize. Since *tigrina-o³⁴* segregates 1:1 we consider the homozygous mutants unable to fix CO₂ even though

they appear normal. These results imply that although the pigment composition of *tigrina-o³⁴* resembled the wild-type when both were grown at 30 °C under >650nm red light the primary defect in this mutant remained and was not overcome by these conditions.

3.4. The accumulation of chlorophyll *a₇₄₃* in *tigrina-o³⁴* grown in the light

The defective formation of β -carotene from lycopene at lower temperatures in *tigrina-o³⁴* can also be related to another phenomenon observed in light grown mutants, i.e. the accumulation of chlorophyll *a₇₄₃*. In *tigrina-o³⁴* the accumulation of an abnormal form of chlorophyll *a*, having an absorption maximum at 743 nm in the leaf, has been shown to be much more pronounced at a lower growth temperature (20 °C) than at a higher one (30 °C) (28). Mutants grown at low light intensities such as in >650 nm red light studied in this paper also accumulated chlorophyll *a₇₄₃*. As shown in Figure 6 thylakoids isolated from the top half of the mutant leaf contained more chlorophyll *a₇₄₃* relative to chlorophyll *a₆₇₈* than thylakoids from the bottom half. Significantly the top half of the mutant leaf also contained the greatest amount of lycopene, representing the excess carotenes accumulated by *tigrina-o³⁴* compared to the wild-

Figure 6. Absorption spectra of purified thylakoids isolated from the bottom half (—), and the top half (---) of a *tigrina-o³⁴* leaf grown in continuous >650nm red light at 23 °C.

type control. As will be shown in section 3.7 a thylakoid membrane preparation of the mutant contained chlorophyll a_{743} in association with lycopene. The abnormal absorption maximum of the chlorophyll at 743nm must be due to an aberrant organisation of the chlorophyll molecules in the membranes.

Figure 7 illustrates that *tigrina-o³⁴* leaves grown in white light at 23 °C did not have the large wild-type fluorescence emission peak at 740nm. Instead a small fluorescence emission maximum at a wavelength slightly higher than 743nm was seen, but the size of this emission maximum was not proportional to the amount of chlorophyll a_{743} present in leaves of *tigrina-o³⁴* under these conditions (28). This provides evidence that chlorophyll a_{743} represents aggregates of chlorophyll which will be analysed in section 3.7.

3.5. Ribosome content of *tigrina-o³⁴* grown in the dark and in red light at 23 °C

It was previously suggested that the loss of 70S ribosomes in *tigrina-o³⁴* grown at 20 °C in the light could be explained by the abnormal carotenoid composition and consequent pho-

toinstability of these ribosomes (17). As shown below this explanation was supported by the finding of a normal 70S ribosome complement in mutants grown in the dark and under low in-

Figure 7. The fluorescence emission spectra in liquid nitrogen of wild-type (—) and *tigrina-o³⁴* (---) leaves grown in white light ($55 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) at 23 °C.

Figure 8. Separation of 70S and 80S ribosomes from wild-type and *tigrina-o³⁴* on 10-30% sucrose gradients.

Wild-type and the mutant were grown (A, B) in the dark at 23 °C, (C, D) in >650nm and (E) in >600nm red light. In each experiment 5 OD₂₆₀ units of crude ribosomes were applied. The gradients were tapped from the top and OD₂₆₀ (—) and OD₂₈₀ (---) were monitored continuously. The ribosomal peaks were identified by their position in the gradient and characteristic polypeptide patterns on SDS-PAGE.

tensity >650nm red light. Figure 8A and B shows the separation of 80 and 70S ribosomes from etiolated wild-type and *tigrina-o³⁴*, respectively. The 70S ribosomes from the etiolated plants are seen as a shoulder on the 80S peak. Chloroplast ribosomes are present in dark-grown mutant seedlings in amounts comparable to those of the wild-type. The photolability of the plastid ribosomes is demonstrated by their presence in the mutant grown in the dark at 23 °C and their deficiency in mutants grown at a similar temperature in white light (17).

As expected, at 23 °C, the chloroplast ribosomes were also more stable under the lower intensity >650nm red light than under the more intense >600nm red light. Figures 8C and D show the sucrose gradient separation of cytoplasmic (80S) and chloroplast ribosomes from wild-type and *tigrina-o³⁴* grown in >650nm red light. The amount of 70S ribosomes in the two cases are comparable showing that when grown under the low intensity >650nm red light the mutant is not deficient in chloroplast ribosomes. When the mutant is grown under >600nm red light the content of chloroplast ribosomes is decreased (Figure 8E). So, whereas the 70S chloroplast ribosomes were unstable when *tigrina-o³⁴* was grown under >600nm red light at 23 °C, they were stable under the lower intensity >650nm red light at the same temperature.

In order to determine whether the chloroplast ribosomes from mutants grown under >650nm red light had a normal polypeptide composition the isolated 70S ribosomes were subjected to SDS-PAGE analysis. Polypeptide patterns of 70S ribosomes from wild-type and *tigrina-o³⁴* grown in >650nm red light were identical, as judged by the limited resolution of one-dimensional SDS-PAGE (Figure 9).

3.6. The thylakoid polypeptide composition of *tigrina-o³⁴* grown at 23 °C under 650nm and 600nm red light and in the dark

The thylakoid polypeptide composition as determined by PAGE in SDS of the mutant grown at 23 °C cannot be simply reconciled with the observed stability or instability of the chloroplast ribosomes under the different growth conditions. As shown below, membrane polypeptides

Figure 9. The polypeptide pattern of 70S and 80S ribosomal proteins from *tigrina-o³⁴* and the wild-type grown in >650nm red light at 23 °C.

Figure 10. Thylakoid polypeptide pattern of wild-type and *tigrina-o³⁴* grown in the dark and in red light at 23 °C. The wild-type control was grown in white light (55 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$). In lanes 1-5 appr. 25 μg of chlorophyll was applied.

known to be synthesised in the plastid are deficient where the presence of normal amounts of 70S ribosomes would suggest that such polypeptides could be formed. It is also shown in this

section that the unusual chlorophyll a_{743} is present in the thylakoid membrane preparation from mutants but does not enter the gel.

In *tigrina-o³⁴*, grown under the lower intensity

>650nm red light, compared to the wild-type, the prominently reduced membrane polypeptides were chlorophyll *a* proteins 1, 2 and 3, the α - and β subunits of CF₁ and a 31 kD polypeptide (Figure 10). These are all synthesised on chloroplast ribosomes. A similar reduction was seen in mutants grown under the higher intensity >600nm red light where chloroplast ribosomes were deficient. Thus at 23 °C even when 70S ribosomes are present in *tigrina-o*³⁴, they are either not very active in the synthesis of thylakoid polypeptides or the polypeptides synthesised on these ribosomes are not inserted into the membranes. In contrast to the deficiencies of the membrane polypeptides known to be synthesised on chloroplast ribosomes, relatively large amounts of chlorophyll *a/b*-protein 2 synthesised on cytoplasmic ribosomes were found in the membranes of mutants grown under both red lights.

As has been shown, chloroplast ribosomes are present in normal amounts in dark grown *tigrina-o*³⁴ grown at 23 °C and the polypeptide composition of the isolated internal plastid membranes is qualitatively similar to that of the wild-type. However, just as in plastids grown under >650nm red light, plastid membranes from dark grown mutants contained fewer of the plastid synthesized polypeptides than the wild-type as especially noticable for the α and β subunits of coupling factor, CF₁. This deficiency has been previously noted (12) and may be due to assembly difficulties on the abnormal membranes. The prominent low molecular weight bands (Figure 10) in the pattern from etiolated mutant membranes were due to contamination with material of nuclear origin (18). This and the low yields of internal membranes from etiolated *tigrina-o*³⁴ seedlings may be attributed to the freezing in liquid nitrogen used for identification of the mutants.

Considerable differences between the polypeptide patterns of *tigrina-o*³⁴ and the wild-type were observed in dark and light grown seedlings. The 113 kD polypeptide, present in equal amounts in mutant and wild-type etioplast membranes, disappeared in the wild-type but not in the mutant as the light intensity was increased. The presence of this polypeptide in mutants grown in the light is one of the signs of

impaired development from etioplast to functional chloroplast. A 20.8 kD polypeptide was present in relatively minor amounts in wild-type etioplasts and almost absent in the mutant but was progressively increased in both in brighter light. The amount of this polypeptide was always less in the mutant. A 20.3 kD polypeptide present in equal amounts in wild-type and mutant etioplasts, was present in increased amounts under the lowest intensity >650nm red light but decreased in brighter light. In the light the mutant had greater amounts of this polypeptide than the wild-type. The significance of these observations is not apparent at the present time.

Chlorophyll *a*₇₄₃ could be identified on the gels after electrophoresis. In Figure 10 it can be seen that a relatively large amount of material fails to enter the gel in all tracks of mutant material. For light grown mutants this material was identified as the abnormal chlorophyll *a*₇₄₃ found in leaves of *tigrina-o*³⁴. This chlorophyll *a*₇₄₃ was present in the SDS treated mutant thylakoids applied to the gel but did not penetrate the gel during electrophoresis. The abnormal chlorophyll remained as an orange-green precipitate at the top of the gel during electrophoresis and could be stained with Coomassie Blue after electrophoresis indicating the presence of some protein. This portion of the gel and all the chlorophyll containing bands were cut out after electrophoresis and analysed in the spectrophotometer. Chlorophyll *a*₇₄₃ was found only in the precipitate that did not enter the gel. Pigments were extracted from the precipitate. The gel was ground in liquid nitrogen, extracted with acetone and pigments separated by chromatography revealing chlorophyll *a* and lycopene as the main pigments. Trace amounts of chlorophyll *b* were also present. Chlorophyll *a*₇₄₃ is therefore an aggregated form of chlorophyll *a* which is bound to lycopene together with some unidentified protein or proteins which under the circumstances are not soluble in SDS.

The relative amounts of membrane polypeptides in the top and bottom half of mutant leaves grown under >650nm red light was determined so that they could be compared with the distribution of chlorophyll *a*₇₄₃ and of structural features seen under the electron microscope which are

Figure 11. Polypeptide pattern of thylakoids isolated from the apical and the basal part of the mutant leaf grown in $>650\text{nm}$ red light. The wild-type was grown in white light ($55 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$).

reported in the next section. The polypeptide composition of thylakoids from the upper and lower parts of the primary leaf was determined as follows. Mutants were cut approximately at level 4 (Figure 12) and thylakoids were isolated, purified and analyzed by SDS-PAGE. Figure 11 shows that there are differences in the thylakoid polypeptide composition of plastids from the upper and lower parts of the mutant leaf. More chlorophyll *a/b*-protein 2, synthesised on cytoplasmic ribosomes was found in the upper part of the leaf, where chlorophyll *a*₇₄₃ is abundant. The chloroplast coded, α and β subunits of CF_1 , and the chlorophyll *a*-proteins 1, 2 and 3, were present in equal amounts in thylakoids isolated from the top and bottom parts of the leaf whereas there was a relative deficiency of a 31 kD polypeptide in the upper part of the leaf. This indicates that the chloroplastic ribosomes are more active in thylakoid polypeptide synthesis in younger than in older mutant plastids. The 20.8 kD and 42 kD polypeptides are absent in older plastids, whereas the amount of the 20.3 kD polypeptide is increased.

Altogether these results show that there is a relative deficiency of certain membrane polypeptides in plastid membranes from *tigrina-o*³⁴ grown at 23 °C. This deficiency is not due to photodestruction of chloroplast ribosomes.

3.7. Plastid membrane structure of *tigrina-o*³⁴ grown at 23 °C in the dark and in red light

In order to study the development of structural irregularities in *tigrina-o*³⁴ and relate them to the abnormal pigment content the etioplast and chloroplast structures were examined along the primary leaf from the youngest tissue (level 1) to the oldest (level 6), as illustrated in Figure 12.

In the dark grown *tigrina-o*³⁴ plastid, at level 1, the proplastids contain few internal membranes (Figure 13). At levels 2 and 3 where the plastids are more mature there is an increased but similar amount of membranes (Figures 14 and 15). The membranes were in places arranged into prolamellar bodies without paracrystalline structure and ribosomes were visible in these plastids. A further increase in the amount of internal membranes was found in the plastids of level 4 and regions of appressed primary lamellar layers were seen (Figure 16). Typical prolamellar bodies

with paracrystalline structures were visible in the etioplasts at level 5 (Figure 17) and 6 (Figure 18). In these older plastids few membranes radiating from the prolamellar bodies were seen and most of the membranes were appressed into some very electron-dense structures resembling tightly packed grana (e.g. Figure 17, arrow). Undulating osmiophilic sheets were also seen (Figure 18, arrows). All of these electron dense structures can be attributed to the excess amounts of carotene represented by lycopene and the deficiency of xanthophylls, seen in dark grown *tigrina-o³⁴* (section 3.1).

In plastids of the mutant grown in white light at the same temperature ribosomes were not detected above level 2 (17) but plastid ribosomes were clearly visible at all levels of the primary

leaf of *tigrina-o³⁴* grown at 23 °C in the dark. This indicated that the absence of plastid ribosomes in *tigrina-o³⁴* in white light at 23 °C was due to photodestruction.

In order to relate the deficiency of the pigment composition and 70S ribosomes in *tigrina-o³⁴* with changes occurring in the plastid ultrastructure, plants grown under >600nm and >650 nm red light were studied by electron microscopy. The ultrastructure of the plastids in the wild-type leaf at levels representing the different ages are presented first and then compared with the structures in the mutant leaf. In wild-type seedlings grown under >650nm red light (where the mutant plastid is more stable) very young plastids at level 1 contained few internal membranes (Figure 19). Both prolamellar bodies and small membrane stacks were present in these plastids. An increase in the amount of internal membranes were seen in plastids at level 2 (Figure 20). Large paracrystalline prolamellar bodies were usually interconnected with a well-developed thylakoid system. This contemporary presence of prolamellar bodies, grana and intergrana membranes in plants grown under low light intensity has previously been described (16). In plants grown under >650nm red light this system of membranes was found throughout the primary leaf. However, towards the tip of the leaf, the prolamellar bodies accounted for smaller and smaller amounts of the total membranes. At level 6 most of the plastid internal membranes were arranged into grana and intergrana lamellae, although some prolamellar bodies were still present (Figure 21).

In the mutant the development of the plastids under the >650nm red light was comparable to the wild-type up to level 4 (Figures 22, 23 and 24). Both normal and abnormal plastids were observed at level 4 (Figure 25). Prolamellar bodies were still present. In the aberrant plastids prolamellar bodies seemed to account for most of the normal membranes which remained (Figure 26). Very electron-dense large membrane stacks were visible, sometimes interconnected with the prolamellar bodies (Figure 26, arrow). Undulating osmiophilic sheets were seen in the aberrant plastids (Figure 26, double arrow). At level 5 all plastids were abnormal (Figure 27). Their internal membranes were arranged either

Figure 12. Positions along the primary leaf where samples for electron microscopy were collected.

◀ Figures 13 to 16. The *tigrina-o³⁴* mutant grown in the dark at 23 °C.

Figures 13, 14, 15 and 16 show plastids of increasing age from levels 1, 2, 3 and 4, respectively, and the corresponding increase in amount of internal membranes. Ribosomes are clearly visible inside all the plastids. (Bar = 0.5 µm).

as very electron-dense stacks (Figure 27, arrows) or as prolamellar bodies. Ribosomes were present inside these plastids as well as inside the plastids of level 6. At this level the prolamellar bodies were very small and the plastid stroma showed some vacuolation (Figure 28) indicating plastid destruction. The electron-dense stacks and the undulating osmiophilic membranes (Figures 27 and 28) may be derived, respectively, by the self-condensation of grana and of intergranal membranes (Figure 24).

The electron-dense stacks found in *tigrina-o³⁴* have been freeze-fractured and found to contain,

on the PFs face, hexagonally packed particles with a 100Å repeat (34). When purified light-harvesting chlorophyll *a/b*-protein containing mainly chlorophyll *a/b*-protein 2 was freeze-fractured, the fracture face was found to contain hexagonally arrayed particles with an average centre-to-centre distance of 122Å. Similar size particles were absent from the PFs face of the mutant *chlorina-f2* which lacks the chlorophyll *a/b*-protein 2 (31). This strongly indicates that the large amounts of chlorophyll *a/b*-protein 2 found

Figures 17 & 18. The *tigrina-o³⁴* mutant grown in the dark at 23 °C.

Figures 17 and 18 show plastids characteristic for levels 5 and 6, respectively. At both levels paracrystalline prolamellar bodies are seen. Very electron-dense membrane stacks (arrows) and undulating osmiophilic sheets, typical for lycopene accumulations (Figure 18, double arrow) are seen. Plastid ribosomes are clearly recognizable (Bar = 0.5 µm). ▼

◀ Figures 19 to 21. Wild-type grown in >650nm red light.

A few membranes arranged into a thylakoid system with small prolamellar bodies are visible in the young plastids at level 1 (Figure 19). Starch granules (S) are also present in these plastids. An increase in the amount of membranes is found in plastids of level 2 (Figure 20), showing a simultaneous presence of prominent grana and paracrystalline prolamellar bodies. Prolamellar bodies are still found in some mature plastids at level 6 (Figure 21, arrows). (Bar = 0.5 μ m).

in *tigrina-o*³⁴ grown under restrictive conditions is located in the electron-dense stacks. These abnormal grana may - as previously suggested (17) - also be the site of the aberrant chlorophyll *a*₇₄₃ since only the top part of the mutant leaf contains chlorophyll *a*₇₄₃ and the electron dense stacks. As reported above (section 3.6) chlorophyll *a*₇₄₃ was found associated with lycopene and protein.

In wild-type leaves grown under >600nm red light, very young plastids at level 1 had both very small prolamellar bodies and grana (Figure 29). At level 2 the plastids thylakoid system appeared to be fairly well developed and arranged into grana and inter-grana lamellae (Figure 30). When present, prolamellar bodies were very small. Towards the leaf tip development of the thylakoid system proceeds and finally, at the leaf tip (level 6) mature chloroplasts with well-developed thylakoid systems were usually found (Figure 31).

In mutants grown under the >600nm red light aberrant structures were seen in plastids of all ages. At level 1, close to the intercalary meristem, in the young plastids of the mutant leaf some undulating osmiophilic sheets were present (Figure 33). At level 2 most of the plastids were very aberrant with electron dense grana whereas some plastids contained exceptionally long grana (Figure 32). Ribosomes were still visible in the plastids of sections up to level 3. At this level plastids contained large undulating osmiophilic sheets as well as organised thylakoid membranes (Figure 34). The plastids at level 4 had an abnormal membrane structure with large electron-dense stacks and prolamellar bodies were frequent (Figure 35). As was the case in >650nm red light the large electron-dense stacks seen in Figure 35

must originate by self-condensation of the grana seen in Figure 34. Ribosomes were present in all plastids at this level, whereas at level 5 they were only present in some plastids (Figure 36). At this level vacuolation indicating plastid destruction was seen. Where the stroma appears disorganized ribosomes were no longer recognizable (Figures 37 and 38). In plastids at level 6 all membrane structures were abnormal and the stroma was completely disorganized. Thus, the decrease in the amount of 70S ribosomes in the mutant grown in >600nm red light was confirmed by the electron microscopical results. Ribosome destruction occurred only in the higher intensity red light and was always preceded by the appearance of aberrant membranes. When the ultrastructure of plastids in mutants grown under >600nm red light was compared with those grown under >650nm red light, plastid disorganisation and destruction was seen at earlier stages of plastid development (lower leaf levels). The greater stability of mutant plastids in the >650nm red light is correlated with the more balanced pigment content reported in section 3.3.

The study of the plastid ultrastructure revealed that plastid ribosomes were present in *tigrina-o*³⁴ grown in the dark at 23 °C but were destroyed by >600nm red light. Abnormal membrane structures were formed by condensation of membranes of relatively normal appearance. In sections of light grown plants the amount of electron dense stacks was correlated with the content of chlorophyll *a*₇₄₃.

3.8. The phenotype exhibited by *tigrina* and *albozonata* mutants under different growth conditions

The sensitivity of the phenotype of *tigrina-o*³⁴ to temperature and light intensity suggested that it would be of interest to see if the phenotype of other *tigrina* mutants was also affected by these parameters.

In Figures 1A and 1B examples were shown of two *tigrina* mutants which illustrate the *tigrina* and *zonata* phenotypes. These phenotypes were only exhibited when *tigrina* and *albozonata* mutants were grown under alternating periods of light and dark (36) and in addition depended on the intensity and quality of the light and the temperature (Table V).

For mutants grown at 23 °C using the higher intensity white light ($55 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) in the light period a *tigrina* phenotype characterised all *tigrina-a* and *tigrina-b* mutants and *tigrina-d*¹², *tigrina-e*²¹ and *tigrina-o*³⁴. A zonata phenotype was shown by *tigrina-n* mutants whereas the mutant *tigrina-f*¹¹ showed a zonata phenotype with a tendency for necrosis. Alboviridis was the phenotype exhibited by *tigrina-j*²⁸ and albozonata-l, while *tigrina-m*³¹ was viridis and is known to develop a zonata phenotype under less favourable conditions (36).

When the white light intensity of the light period was decreased to $15 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ some mutants showed a less pronounced phenotype. In *tigrina-d*¹², *tigrina-e*²¹, *tigrina-f*¹¹ and *tigrina-o*³⁴ the phenotype changes from *tigrina* to zonata; in *tigrina-n*³² from zonata to viridis and *tigrina-n*³³ from zonata to viridoxantha. Therefore in these mutants, the higher light intensity resulted in a more severe phenotype attributed to photodestruction.

Figures 22 to 24. The *tigrina-o*³⁴ mutant grown in >650nm red light.

Plastids of levels 1 (Figure 22), 2 (Figure 23) and 3 (Figure 24) are shown. As in the wild-type, in all these cases there is a simultaneous presence of prolamellar bodies and well developed grana. Plastid membranes have a normal appearance and ribosomes are visible in all the plastids. (Bar = 0.5 μm).

When grown at 30 °C compared to 23 °C under light periods of identical intensity ($15 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$) some *tigrina* mutants were positively affected by the increase in temperature and became wild-type in appearance or tended to do so. These were *tigrina-a*³, and *tigrina-e*²¹ which were almost completely wild-type in appearance, *tigrina-b*²⁴ where a wild-type phenotype with a suspicion of zonata was seen, while *tigrina-j*²⁸ became viridis. The phenotype of *tigrina-o*³⁴ was viridis with faint banding.

The increase of temperature from 23 °C to 30 °C adversely affected the phenotype of four

Table V.

The effect of growth conditions on the expression of the *tigrina* and albozonata phenotypes. The phenotype was determined on the seventh day after sowing using photoperiods of 8 hours dark and 16 hours light. Light intensity is given as $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$. Mutants *tig-b*²⁴, -c²⁰, -c²⁵ and *tig-j*²⁸ are homozygous viable. 1) With curled tips; ND = not determined.

	White light			Red light	
	23 °C 55 μE	23 °C 15 μE	30 °C 15 μE	>600nm 23 °C 2.5 μE	>650nm 23 °C 1.4 μE
<i>tig-a</i> ¹ , -a ⁶ , -a ¹³	<i>tigrina</i>	<i>tigrina</i>	<i>tigrina</i>	<i>tigrina</i>	normal
-a ¹⁷ , -a ¹⁸ , -a ²²	<i>tigrina</i>	<i>tigrina</i>	<i>tigrina</i>	<i>tigrina</i>	normal
<i>tig-a</i> ³	<i>tigrina</i>	<i>tigrina</i>	normal 1)	<i>tigrina</i>	normal
<i>tig-b</i> ⁷ , -b ¹⁹ , -b ²³	<i>tigrina</i>	<i>tigrina</i>	<i>tigrina</i>	<i>tigrina</i>	normal
-b ²⁴	<i>tigrina</i>	<i>tigrina</i>	zonata	normal	normal
<i>tig-c</i> ²⁰ , -c ²⁵	normal	normal	normal	normal	normal
<i>tig-d</i> ¹²	<i>tigrina</i>	zonata	<i>tigrina</i>	<i>tigrina</i>	normal
<i>tig-e</i> ²¹	<i>tigrina</i>	zonata	normal 1)	pale	normal
<i>tig-f</i> ¹¹	<i>tigrina</i> / zonata	zonata	<i>tigrina</i>	<i>tigrina</i>	normal
<i>tig-j</i> ²⁸	alboviridis	zonata	viridis	viridis	viridis
<i>tig-m</i> ³¹	viridis	viridis	xantha	viridis	viridis
<i>tig-n</i> ³²	zonata	viridis	viridis	viridis	viridis
<i>tig-n</i> ³³	zonata	virido/ xantha	xantha	ND	viridis
<i>tig-o</i> ³⁴	<i>tigrina</i>	zonata	virido/ zonata	<i>tigrina</i>	viridis
albozonata-l	alboviridis	viridis	albina	ND	viridis

25

26

27

28

- ◀ Figures 25 to 28. The *tigrina-o*³⁴ mutant grown in >650nm red light.

At level 4 (Figures 25 & 26) both normal and abnormal plastids occur. Prolamellar bodies are present and in the abnormal plastids very electron-dense membrane stacks (Figure 26, arrow) as well as structures characteristic for lycopene accumulation (Figure 26, double arrow). Plastids typical of levels 5 and 6 are shown in Figures 27 and 28, respectively. All plastids are abnormal with electron-dense membrane stacks (arrow) and undulating osmiophilic sheets (double arrow). Plastid ribosomes are present in all of them. (Bar = 0.5 μ m).

mutants. These were *tigrina-d*¹² and *tigrina-f*¹¹ which developed a clear *tigrina* phenotype at the higher temperature. The mutants *tigrina-m*³¹ and *tigrina-n*³³ developed xantha phenotypes and albozonata-1 showed an albina phenotype at 30 °C. The mutants discussed in this paragraph and the one above are therefore either cold-sensitive or sensitive to higher temperatures.

In order to see if the phenotype of the mutants could be positively affected by a further decrease of the light intensity and/or by changing its quality plants were grown using red light periods at 23 °C. Red lights used were of similar low intensity but different qualities: >600nm red light (2.5 μ E · m⁻² · sec⁻¹) and >650nm red light (1.4 μ E · m⁻² · sec⁻¹) light.

Under both low intensity lights protochlorophyllide photoconversion would limit chlorophyll synthesis (section 3.3) but any accumulation of photoinactive protochlorophyllide absorbing at 630nm would not act as a photosensitiser in >650nm red light. Mutants which accumulate excess amounts of photoinactive protochlorophyllide in the dark period would not be photobleached when exposed to >650nm red light. The >600nm red light periods positively affected the phenotype of three *tigrina* mutants compared to the higher intensity 15 μ E · m⁻² · sec⁻¹ white light at 23 °C. Under >600nm red light *tigrina-b*²⁴ had a wild-type phenotype, *tigrina-e*²¹ resembled the wild-type but had a paler green leaf base and *tigrina-j*²⁸ was viridis. The latter two mutants had also been noted to be sensitive to high intensity in the white light experiment.

Three mutants were adversely affected by growth under the >600nm red light compared to

the white light of 15 μ E · m⁻² · sec⁻¹ at 23 °C. These were: *tigrina-d*¹², *tigrina-f*¹¹ and *tigrina-o*³⁴ where the phenotype changed from zonata to *tigrina*. As these three mutants had previously been noted to be positively affected by a decrease in white light intensity they must be specifically sensitive to >600nm red light. This light is more intense in the >600nm region than the white light of 15 μ E · m⁻² · sec⁻¹.

The phenotype exhibited by some mutants clearly depended on the quality of the low intensity red light. When >650nm red light of approximately the same low intensity was used in the light periods instead of >600nm red light, many mutants changed phenotype from *tigrina* to wild-type. These were all *tigrina-a* mutants, *tigrina-b*⁷, *-b*¹⁹ and *-b*²³, *tigrina-d*¹², and *tigrina-f*¹¹; mutant *tigrina-o*³⁴ changed from *tigrina* to viridis and *tigrina-e*²¹ from wild-type with paler green leaf base to wild-type. These mutants are therefore specifically sensitive to >600nm red light.

It has been suggested that the bleached or necrotic bands observed in *tigrina* mutants are due to damage caused by a photosensitising pigment which accumulates in the dark but not in the light during extension growth (14). Nonphotoactive protochlorophyllide levels exceeding those of the wild-type are accumulated in *tigrina-d*¹², the *tigrina-b* mutants, *tigrina-o*³⁴ and the *tigrina-n* mutants grown in complete darkness. This protochlorophyllide could account for their *tigrina* phenotype. The non-photoactive protochlorophyllide has an in vivo absorption maximum at 630nm. Accordingly, the above mentioned mutants should develop the *tigrina* phenotype when grown under light-dark cycles with >600nm and not with >650nm red light. The expected results were obtained with *tigrina-d*¹², *tigrina-b*⁷, *-b*¹⁹, *-b*²³ and *tigrina-o*³⁴. The mutants *tigrina-b*²⁴, *tigrina-n*³² and *-n*³³ showed uniform leaf colour under both red lights. These three *tigrina* mutants are the ones which accumulate only twice as much or less protochlorophyllide in the dark (28) compared to the wild-type. The *tigrina-n* mutants accumulate excess amounts of abnormal carotenoids (29) and this could provide extra protection against a photosensitiser.

All *tigrina-a* mutants and *tigrina-f*¹¹ behaved just as the majority of the protochlorophyllide

◀ Figures 29 to 31. Wild-type grown in >600nm red light.

At level 1 the young plastids contain prolamellar bodies (Figure 29, arrows) as well as grana and starch granules (S). The thylakoid system is more developed at level 2 (Figure 30). The internal membranes of the plastids are arranged into grana and inter-grana lamellae. Mature chloroplasts are found at level 6 (Figure 31). (Bar = 0.5 μ m).

accumulators and exhibited a *tigrina* phenotype under >600nm but not under >650nm red light-dark regime. These mutants do not accumulate protochlorophyllide when grown continuously in the dark (28). However, in *tigrina-a*, spectra of leaves indicate that protochlorophyllide accumulates in excess of wild-type only after it has been induced by light. The mutant *tigrina-f*¹¹ is carotenoid defective in the light. It may be predicted that this mutant will accumulate excess protochlorophyllide in the dark compared to the wild-type especially at higher temperatures since it showed a more severe phenotype at 30 °C.

The mutants *tigrina-j*²⁸ and *tigrina-n*³², which are sensitive to light intensity and *tigrina-m*³¹ which is temperature sensitive showed no differential response to the two red lights.

The *tigrina-c* mutants showed a wild-type phenotype under all conditions tested and must require a lower temperature to exhibit a *tigrina* phenotype.

After seven days of growth under the >650nm red light/dark periods the plants were exposed to 55 μ E · m⁻² · sec⁻¹ white light for five minutes and returned to the previous growth conditions. Twenty-four hours later transverse bands which were paler green, yellowish or brownish-red compared to the rest of the leaf were seen in *tigrina-b*⁷, *-b*¹⁹, *-b*²³ and *tigrina-o*³⁴. Thus in *tigrina-b*⁷ three yellow bands were seen corresponding to the three dark periods experienced by the leaf from its emergence on the fourth day until the seventh day. In *tigrina-d*¹² a single band was seen. Photodestruction causing these bands must be due to the accumulation in the dark period of a photosensitising pigment, most probably protochlorophyllide. When grown in complete darkness at 23 °C *tigrina-b*⁷, *-b*¹⁹, *-b*²³, *tigrina-d*¹² and *tigrina-o*³⁴ appear pale green due to ac-

cumulation of excess protochlorophyllide (28). In *tigrina-d*¹² the accumulated pigment is dissipated with time. In the carotenoid deficient *tigrina-o*³⁴ and *tigrina-b* mutants the appearance of chlorophyll deficient bands implies that the deficient regulation of the chlorophyll biosynthetic pathway in the dark has more severe effects than in the light. In continuous darkness or under continuous illumination all *tigrina* mutants exhibited a uniform leaf colour (28).

4. DISCUSSION

This discussion relates the defective carotenoid biosynthesis in *tigrina-o*³⁴ and other *tigrina* mutants to: the regulation of chlorophyll biosynthesis, the formation of chlorophyll *a*₇₄₃ in the light, the formation of abnormal membranes and the defective synthesis in the plastid of membrane polypeptides.

It has been shown that both the regulation of δ -aminolevulinate biosynthesis in the dark and the defective carotenoid biosynthesis in *tigrina-o*³⁴ are cold-sensitive. Other *tigrina* mutants with defects in carotenoid biosynthesis which are associated with faulty regulation of δ -aminolevulinate formation in the dark are *tigrina-b*²³ and mutants at the *tigrina-n* locus where accumulation of excess protochlorophyllide indicates defective regulation of δ -aminolevulinate formation (28,29).

Therefore, the defective carotenoid biosynthesis and regulation of δ -aminolevulinate formation may have a common cause. The δ -aminolevulinate synthesising enzyme is strongly inhibited by heme (14,15). Genetic evidence suggests that regulation of the δ -aminolevulinate synthesising enzyme by heme in *tigrina-b* mutants and *tigrina-o*³⁴ is defective (14,21). Double mutants of these *tigrinas* with *xantha-f*¹⁰ accumulate the immediate heme precursor protoporphyrin, although *xantha-f*¹⁰ alone forms only minute amounts of protoporphyrin. Heme but not protoporphyrin inhibits the δ -aminolevulinate synthesising enzyme of the wild-type and thus a defective regulation by heme in these two carotenoid deficient *tigrina* mutants is deduced. As heme containing enzymes are most probably required in the desaturation processes in carotenoid biosynthesis, a heme deficiency could explain the phenotypes

◀ Figures 32 to 34. The *tigrina-o*³⁴ mutant grown in >600nm red light.

Lycopene accumulations can already be seen in plastids at level 1 (Figure 33, arrow) but thylakoid membranes have a normal appearance. Normal plastids with starch granules (S) are found at level 2 (Figure 32). Large lycopene accumulations (Figure 34, asterisks) are present together with normal grana and inter-grana membranes. Plastid ribosomes are clearly visible in all these figures. (Bar = 0.5 μ m).

of these carotenoid deficient *tigrina* mutants. When *tigrina-o*³⁴ was grown at 30 °C under >650nm red light the chlorophyll and carotenoid content was indistinguishable from the wild-type.

The relationship of deficient carotenoid biosynthesis in *tigrina* mutants to the accumulation of chlorophyll *a*₇₄₃ has been investigated in this paper. The aberrant chlorophyll is also observed in *tigrina-b* mutants, *tigrina-f*¹¹, *tigrina-m*³¹, *tigrina-n* mutants and *tigrina-o*³⁴ which are all now known to have partial blocks in carotene biosynthesis. No other *tigrina* mutants described have chlorophyll *a*₇₄₃ (28) or are known to have defects in carotenoid biosynthesis. The formation of chlorophyll *a*₇₄₃ has also been reported in wheat leaves treated with amitriole (20) which is an inhibitor of carotene biosynthesis (4). The observation that very little fluorescent emission attributable to chlorophyll *a*₇₄₃ could be observed in light grown *tigrina-o*³⁴ leaves suggested that it is an aggregated form of chlorophyll. Dimers or aggregates of chlorophyll have been shown to exhibit a decreased fluorescence (30). It has been suggested that aggregated chlorophyll having an absorption at 740nm is arranged as a stack of sheets of (-chlorophyll-H₂O-chlorophyll-H₂O)_n (22). In *tigrina-o*³⁴ chlorophyll *a*₇₄₃ is associated with lycopene and some unidentified protein. Aggregation of chlorophyll *a* giving a form with an absorption maximum at 740nm has been observed in non-polar solvents, is reversed by the xanthophyll, lutein, and by increases in temperature (2). This suggests that chlorophyll aggregation may also be caused by the lycopene accumulation (acting as a non-polar solvent) and lutein deficiency seen in light grown *tigrina-o*³⁴ and most probably in the other *tigrina* mutants which have partial blocks in the carotenoid path-

way prior to xanthophyll formation. In *tigrina-o*³⁴ grown at 30 °C in white light less chlorophyll *a*₇₄₃ was seen than at the lower growth temperatures. This decrease may be related to the increase in lutein and the increased content of the normal β -carotene relative to the abnormal lycopene. As shown in (2) increased temperature may also lead to deaggregation of the chlorophyll. If this is so, it is expected that carotenoid deficient *tigrina* mutants not affected by temperature such as *tigrina-b*⁷, *-b*¹⁹ and *-b*²³ should have less chlorophyll *a*₇₄₃ at 30 °C.

The photoinstability of pigments in *tigrina-o*³⁴ which can be attributed to the deficient carotenoid content leads to an instability of plastid ribosomes and plastid ultrastructure in general. In the light *tigrina-o*³⁴ is particularly deficient in β -carotene. In carotenoid deficient plants light absorbed by chlorophyll may result in the formation of singlet oxygen and cause photodamage (1, 9, 23).

The defective β -carotene biosynthesis at the lower growth temperature resulted in photoinstability of *tigrina-o*³⁴ plastids leading to destruction of plastid ribosomes. Previously it was shown that in white light at 30 °C *tigrina-o*³⁴ seedlings contained ribosomes in plastids throughout the entire leaf. However, in the light at 20 °C chloroplast ribosomes were only seen in the very young plastids close to the intercalary meristem (17). Consequently in white light at 20 °C chloroplast ribosomes are first formed and subsequently destroyed during plastid maturation due to carotenoid, primarily β -carotene, deficiency. At 30 °C the increased level of β -carotene in the mutant protects 70S ribosomes from photodamage. The photodestruction of plastid ribosomes has previously been demonstrated in β -carotene deficient mutants of sunflower (35) as well as in wheat and rye where carotenoid deficiency has been induced by herbicides (3, 8).

In *tigrina-o*³⁴ the destruction of chloroplast ribosomes is a light mediated process. The red light experiments showed that the intensity and the wave-length of light also influenced the stability of the ribosomes in plants grown at 23 °C. The >600 nm red light was more damaging to *tigrina-o*³⁴ than the >650nm red light. The more severe damage to pigments, ribosomes and generally to plastid ultrastructure seen under the

- ◀ Figures 35 to 38. The *tigrina-o³⁴* mutant grown in >600nm red light.

All plastids are aberrant at level 4 with very electron-dense large stacks formed by membranes radiating from a prolamellar body (Figure 35). Plastids with normal stroma and ribosomes (Figure 36) are present together with plastids containing disorganised stroma and no clearly recognizable ribosomes (Figure 37) at level 5. At level 6 no ribosomes can be seen and the stroma is completely disorganised (Figure 38). (Bar = 0.5 μ m).

>600nm red light than under the >650nm light can be attributed to the greater light intensity which results in an increased action of both chlorophyll *a* and *b* as photosensitisers. Successive increase in chlorophyll concentration during plastid maturation leads to the increase in photodamage seen from the bottom to the tip of the leaf.

The aberrant membrane structures seen in some of the *tigrina* mutants can be related to disturbances in carotenoid biosynthesis.

The accumulation of lycopene and/or other more saturated carotenes is always associated with the occurrence of crystalloids, osmiophilic granules, and undulating osmiophilic sheets (28). Investigations on the ultrastructure and pigment composition of mutant plastids from plants grown in the dark or under low intensity >650nm red light compared to the higher intensity 600nm red light confirmed that abnormal membrane structures were not due to photo-destruction but to the defective carotenoid biosynthesis.

The undulating membranes seen in *tigrina-o³⁴* plastids may be explained by the lycopene accumulation. Lycopene is believed to form crystalloids in the plastids and when these crystalloids dissolve during dehydration with ethanol after fixation the surrounding membranes are left behind as the undulating osmiophilic membranes (33). The electron dense stacks seen in *tigrina-o³⁴* are interpreted in the present paper as result of condensation of membranes due to the presence of lycopene and deficiency of β -carotene and xanthophylls.

The presence of membrane polypeptides known to be synthesised on chloroplast ribosomes in plastid membranes from *tigrina-o³⁴* was dependent only on the growth temperature and

not on the presence of chloroplast ribosomes in the plastids. Although these ribosomes were present in mutants grown in the dark or under >650nm red light they apparently functioned more effectively at 30 °C than at 23 °C. Evidence suggested that the protein composition of 70S ribosomes was normal at both temperatures. Deficiency of these membrane polypeptides must be related to the carotenoid deficiency in *tigrina-o³⁴* which has been shown to lead to abnormal membranes in the plastid. It has been shown that polysomes are bound to chloroplast membranes, and such membrane bound polysomes have been found to be active in protein synthesis in vitro, when supplemented with soluble cofactors (5, 11, 26). In *Chlamydomonas* unfinished products of such protein synthesis have been shown to be bound to the thylakoid membranes (11, 26, 27). The abnormal membranes could hinder insertion of membrane polypeptides either by inhibiting their synthesis or by preventing attachment of plastid ribosomes. It will therefore be of interest to see if the missing membrane polypeptides accumulate in the plastid stroma.

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