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MUTANTS OF BARLEY HEAT-SENSITIVE FOR CHLOROPLAST DEVELOPMENT

by

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Six nuclear gene mutants of barley, heat-sensitive for chloroplast development, are described. These conditional lethal mutants in six different genes can be grown as homozygous viable plants in the field, their mutant phenotype only being expressed with development at high temperature. Chlorophyll accumulation in the mutants but not in the wild type is inhibited by a growth temperature of 31°C. The degree of temperature sensitivity varies amongst the mutants. For example, partial inhibition of chlorophyll production is evident in the mutant *vir-zf*^{ts4} above 20° while inhibition is complete at a growth temperature of 29°. The mutant *vir-zi*^{ts49} remains green at 29° but is bleached at 31°. The reduction in chlorophyll content of the leaves at high growth temperatures is accompanied by the appearances of structural abnormalities in the chloroplasts and reduced photochemical activity per mg chlorophyll. The inhibitory effect of elevated temperatures is confined to some early stage of plastid development as the light-dependent conversion of etioplasts into chloroplasts in the mutants is not inhibited at 32°. Leaf elongation in the mutants compared with the wild type is not affected by high growth temperatures. When the mutants are grown at temperatures permitting normal chlorophyll accumulation, the chloroplast thylakoid membranes appear to be no less heat stable than in the wild type.

1. INTRODUCTION

Higher plants frequently become chlorotic following exposure to temperatures, either hot (4, 17) or cold (2, 9, 15, 17), which are on the verge of being injurious to growth of the plants. Barley seedlings become chlorotic at growth temperatures in excess of 32°C (5, 17).

This phenomenon in barley and in rye results in plants deficient in chlorophyll (5) and chloroplast thylakoid membranes (13), chloroplast ribosomes (5, 13) and ribulose-1,5-bisphosphate carboxylase (5, 6). Several enzymes of the photosynthetic CO_2 fixation cycle (6) and the small subunit of ribulose-1,5-bisphosphate carboxylase (3), which is made on cytoplasmic ribosomes, continue to be synthesized. Thus the high temperature effect appears to be a specific inhibition of chloroplast-localized protein synthetic systems. As such it provides an experimental system for studying chloroplast-cytoplasmic interactions involved in the synthesis of chloroplast proteins (3), as well as the differential control of plant metabolism by temperature.

The power of this experimental system would be enhanced by the availability of mutants in which the minimum growth temperature needed to produce the chloroplast deficiencies has been altered. Such temperature sensitive, conditional lethal mutants in higher plants would furthermore provide selection mechanisms for somatic cell hybrids produced by protoplast fusion in an analogous manner to the light sensitive mutants previously employed for this purpose (10, 11). In this paper we describe six barley mutants, heat-sensitive for chloroplast development at temperatures lower than those producing chlorosis in the wild type. The mutants are the result of mutations in six different nuclear genes.

2. MATERIALS AND METHODS

2.1. Screening for heat sensitive mutants

Resting kernels of Svalöv's Bonus barley were mutagenized in 1969 with ethyleneimine or with γ-rays from a ^{137}Cs source and the M_1 generation grown in the experimental fields of the Swedish Seed Association at Svalöv and of the Agricultural Research Department of the Danish Atomic Energy Commission's Research Establishment at Risø. The M_2 spikes were planted at Risø in a temperature controlled greenhouse with artificial lighting. The air temperature was set at 35°C and continuous illumination supplied. As soon as the chlorophyll mutants had reached a primary leaf height of 5 cm, *albina*, *xantha* and *viridis* seedlings were transferred to a constant temperature room kept at 15°C and with continuous illumination. Seedlings which greened and survived were grown to maturity and progeny tested. Of the 57 mutants isolated, 9 proved in the progeny tests to have wild type phenotype if grown at 15°C and mutant phenotype if grown above 30°C. Six mutants have been kept as homozygous stocks in the Copenhagen collection since 1970. For genetic analysis they have been crossed with each other and with all *xantha* and *viridis* mutants of the collection (19).

They are designated *viridis-y*^{ts2}, *viridis-zf*^{ts4}, *viridis-zg*^{ts9}, *viridis-zh*^{ts46}, *viridis-zi*^{ts49} and *viridis-zj*^{ts57}. In abbreviated form the mutants are referred to as ts2, ts4, ts9, ts46, ts49 and ts57. Mutants ts2 and ts9 were induced by a treatment of Bonus grains with a solution of 0.014% (v/v) ethyleneimine (pH 7.7) for 5 hours, whereas mutants ts4 and ts49 were isolated after a treatment with a concentration of 0.030% ethyleneimine. Mutants ts46 and ts57 originated in an M_2 material from grains treated with an acute dose of 12,000 rad γ-rays.

2.2. Growth conditions

Wild-type barley (Hordeum vulgare L. cv. Svalöv's Bonus) and the six mutants were grown in a growth chamber or where growth at several temperatures was to be compared, in glass tubes in water baths as described previously (17). In this second method seeds were germinated for 28 hours in running tap water before being planted in vermiculite contained in glass tubes (4 cm dia. × 30 cm). The tubes

Abbreviations: DCIP = 2,6 dichlorophenol indophenol; DCMU = 3-(3',4' dichlorophenyl)-1,1-dimethylurea.

were placed in water baths (± 0.1°C) for the times indicated in the text. Continuous overhead illumination of 4 500 lux at seed level was provided by white fluorescent lights.

2.3. Absorption and fluorescence measurements

Absorption spectra or fluorescence measurements were made on the area of the primary leaf 3 to 4 cm below the leaf tip. An Aminco DW-2 spectrophotometer in the split-beam mode was used to record the absorption spectra. The same spectrophotometer equipped with a total fluorescence accessory was used for recording the heat-induced rise in chlorophyll fluorescense (16). The temperature of the leaf section was increased by water circulated from a water bath and heater at a rate of 1°C per minute.

2.4. Chloroplast isolation and assays

Chloroplast thylakoids were isolated from primary leaves (less the first one cm of the tip) as previously described (12).

Chloroplast activities were assayed at 23°C using an Aminco DW-2 spectrophotometer operated in the dual-wavelength mode. Photoreduction of ferricyanide was measured at 420 nm minus 450 nm in a reaction mixture (1.5 ml) consisting of chloroplast thylakoids (4 μg chlorophyll·ml^{-1}), 0.05 M-Sørensen's phosphate buffer (pH 7.5), 0.05 M-NaCl, 0.05% (w/v) bovine serum albumin, and 0.34 mM $K_3Fe(CN)_6$. Red actinic light (11x 10^4 ergs cm^{-2}.sec^{-1}) was provided by light from a 150 W tungsten lamp filtered through heat filters (Calflex C and Corning 1-75) and a Corning 2-60 red cut-off filter. Photoreduction of NADP using reduced DCIP as the electron donor (photosystem 1 activity) was measured at 350 nm minus 370 nm using a reaction mixture (1.5 ml) containing chloroplast thylakoids (8 μg chlorophyll·ml^{-1}), 0.03 M-Sørensen's phosphate buffer (pH 7.5), 0.03 M-NaCl, 0.03% (w/v) bovine serum albumin, 0.67 mM-NADP, 2.5 mM-ascorbate, 63 μM-DCIP, 1.4 μM-ferredoxin from Anacystis nidulans, 6.7 μM-DCMU and gramicidin D (4 μg·ml^{-1}). The intensity of red actinic light was 3 × 10^4 ergs cm $^{-2}$·sec^{-1}. Chlorophyll concentrations in leaves and chloroplast suspensions were determined (1) after extraction with 80% (v/v) acetone.

2.5. Electron microscopy

The primary leaf tissue was cut between 3 to 4 cm below the tip into pieces 3 mm × 0.5 mm and fixed at 20°C in formaldehyde-glutaraldehyde (8) containing 7% sucrose. After washing in 0.1 M-phosphate buffer pH 7.2 containing 7% sucrose, the tissue was post-fixed for 2 hr in 1% OsO_4, stained in 2% aqueous uranyl acetate for 30 min, washed and dehydrated through a graded series of alcohol and embedded in Spurr's resin. Thin sections were stained with 2% uranyl acetate and with lead citrate and examined in a Siemens Elmiskop I electron microscope.

3. RESULTS AND DISCUSSION

3.1. Genetic analyses of the mutants

When tested under restrictive temperature conditions, the six mutants behaved in crosses as recessive nuclear gene mutants. All pairwise combinations of the six mutants yielded F_1 seedlings with wild-type phenotype showing them to be mutations in six different genes. Allele tests of five of the mutants with the 32 previously identified *viridis* genes and the 20 previously identified *xantha* genes (19) as well as with 23 other loci giving rise to seedling lethals revealed no allelism with any of these loci. The five mutants are therefore given the gene designations *vir-zf* ^{ts4}, *vir-zg* ^{ts9}, *vir-zh* ^{ts46}, *vir-zi* ^{ts49} and *vir-zj* ^{ts57}. The sixth mutant *vir-y* ^{ts2} was found to be allelic to the recessive lethal *viridis-y*^{59} (7). The F_1 of the cross +/*y*^{59} x ts^2/ts^2 grown at 35°C segregated 47 green to 33 white seedlings, i.e. approximately in a 1:1 ratio. In the gene *viridis-y* there is thus available now a lethal and a conditional lethal allele. The mutant *viridis-y*^{59} is white with a green tinge and frequently with a green tip when grown at temperatures ranging from 2° to 31°C. It has previously been shown (7) to contain very little chlorophyll and to accumulate photoconvertible protochlorophyllide and some protopor-

Table I

Chlorophyll accumulation in ts-mutants grown at 21°C and 32°C

Mutant	Growth temperature			
	21°C		32°C	
	Total chlorophyll μg (g fresh wt)$^{-1}$	Chlorophyll *a/b*	Total chlorophyll μg (g fresh wt)$^{-1}$	Chlorophyll *a/b*
ts2	1390	3.6	390	2.7
ts4	1620	3.6	230	2.7
ts9	1560	4.4	580	2.8
ts46	1240	3.9	250	2.9
ts49	1530	3.6	380	3.2
ts57	1140	3.4	290	2.3

Seedlings were grown in growth cabinets at 21°C ± 0.5°C under continuous white light (1700 lux) for 8 days or at 32°C ± 0.5°C also under continuous white light (2400 lux) for 7 days. Analyses were carried out on the top 15-cm segment og the primary leaf.

phyrin IX upon feeding with δ-aminolevulinate. As expected from its low chlorophyll content, oxygen evolution could not be detected with the mutant.

3.2. Chlorophyll inhibition in ts-mutants at 32°C

Growth of the ts-mutants at 32°C resulted in inhibition of chlorophyll production. In Table I, chlorophyll production is compared in mutants grown at 21°C and 32°C. In mutants grown at the higher temperature, chlorophyll production was confined to the tip of the primary leaf. These green areas had lower ratios of chlorophyll *a* to *b* than plants grown at 21°C, but were capable of oxygen evolution in the light as determined using a platinum electrode. Chlorophyll accumulation is not inhibited in wild-type barley grown at 32°C (17, see also below).

Table II

Greening of ts-mutants

Mutant	Growth conditions							
	Dark, 6 days: 23°C		32°C					
	+ Light, 24 hr: 21°C		32°C		21°C		32°C	
	Chl[1]	a/b[2]	Chl	a/b	Chl	a/b	Chl	a/b
ts2	1010	3.2	1190	3.1	160	27	260	5.8
ts4	1140	3.2	1510	2.9	68	30	220	11.2
ts9	1010	3.0	1870	2.7	68	30	170	12.4
ts46	1280	3.0	1760	3.1	97	330	300	5.8
ts49	1300	3.0	1850	3.0	66	30	230	5.9
ts57	980	2.8	1590	3.1	96	30	270	5.4

The mutants were grown in the dark at 23 ± 0.5°C or 32 ± 0.5°C and then illuminated with light (~ 3000 lux) at the temperatures shown.
1) μg chlorophyll (*a* + *b*) per g fresh weight primary leaf
2) ratio of chlorophyll *a* to chlorophyll *b*

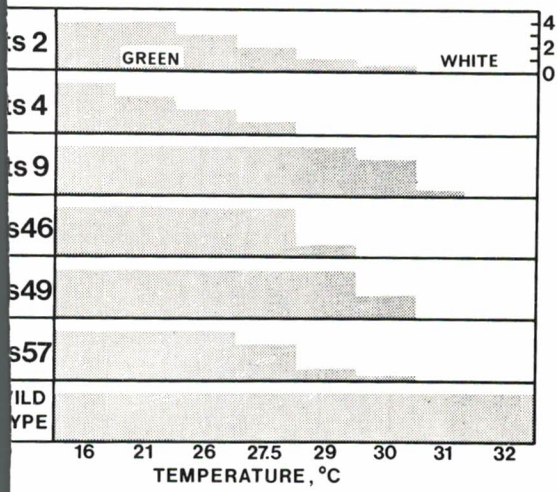


Figure 1a: Visual grading for chlorophyll in ts-mutants grown at different temperatures for 7 days. Grading was based on a scale of O (white) to 4 (wild type grown at 26°C) for the primary leaf (excluding the first 1 cm of the tip). Leaves showing a trace of chlorophyll were given a grading of 0.5.

3.3. Greening of the ts-mutants

The inhibitory effect of high temperature on chlorophyll accumulation shown in Table I could result either from inhibition of the light-dependent conversion of the etioplast into a chloroplast or from inhibition at some earlier developmental stage. It was possible to distinguish between these two possibilities by growing the mutants in the dark at either 23°C or 32°C, and then illuminating them and measuring chlorophyll production. The results are shown in Table II. Mutants grown in the dark at 23°C turned green when illuminated at either 21°C or 32°C. Thus a temperature of 32°C did not inhibit the conversion of etioplasts to chloroplasts. In contrast, chlorophyll synthesis was inhibited in mutants grown at 32°C in the dark, irrespective of the greening temperature. None of the growth conditions listed in Table II inhibited greening in the wild type.

3.4. Chlorophyll accumulation at different growth temperatures

Differential heat sensitivity between the various ts-mutants for chlorophyll production was demonstrated by growing the mutants at a variety of temperatures. Since the degree of temperature control needed for these experiments could not be obtained using the growth cabinets, the mutants were grown in glass cylinders immersed in water baths (see 2.2.). The effect of growth temperature on chlorophyll production was assessed by visual colour grading, extraction of leaf chlorophyll, or by recording the absorption spectrum of the intact leaf. Colour grading was developed as the most convenient and rapid procedure for surveying the effect of growth temperature on chlorophyll accumulation in mutants. Figure 1a shows results obtained with the six ts-mutants and the wild type grown at eight different temperatures and Figure 1b gives an impression of the colours for five of the mutants grown at different temperatures. As noted above, the tip of the primary leaf of the mutants was green, at high growth temperatures, presumably because this region contained cells which were formed during kernel development or during the 28-hour period of water imbibition. For this reason the

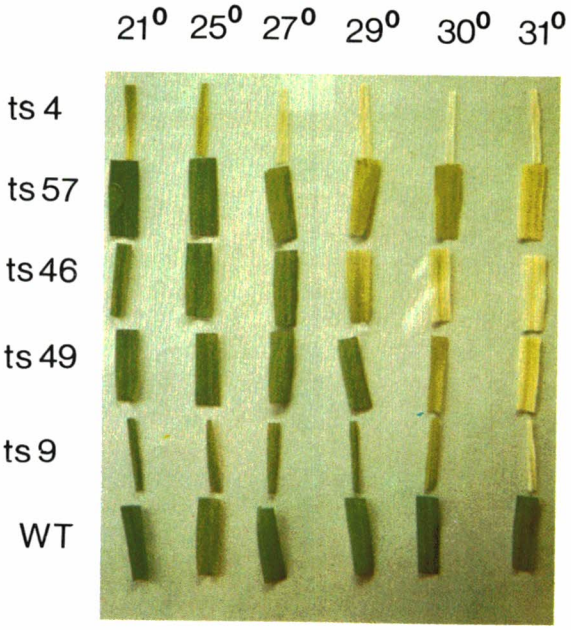


Figure 1b: Appearance of leaf segments from five ts mutants and wild type grown at a series of temperatures. The segments of ts4 and ts9 are from secondary leaves, those of the others from primary leaves.

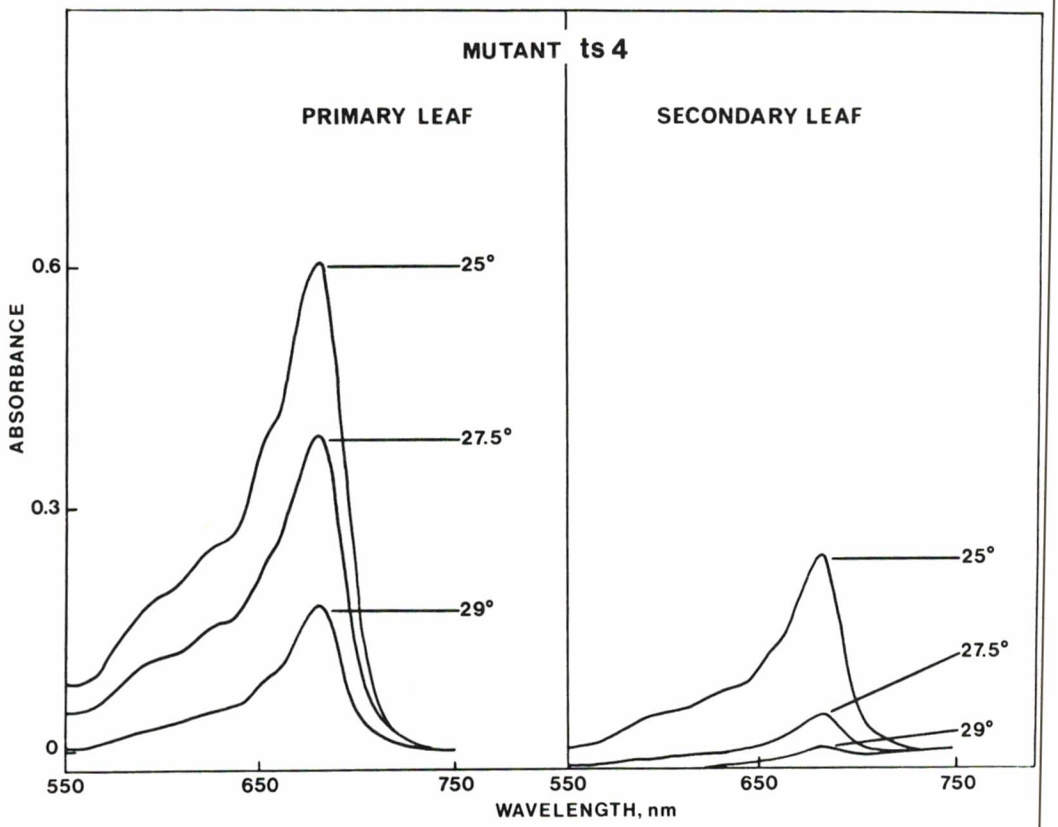


Figure 2: Absorption spectra of the primary and secondary leaves of the mutant ts4 grown at 25, 27.5 and 29°C.

first 1 cm-tip of the primary leaf was not used for any of the analyses which follow. Colour variations were not observed among seedlings of a given mutant grown at any one temperature.

The mutants ts2, ts9, ts49 and ts57 appeared white or contained traces of green colour when grown at 31°C and were white when grown at 32°C. The high temperature cut-off for chlorophyll production was sharp in ts9 and ts49; chlorophyll production was almost normal at 28 to 29°C but was completely inhibited by raising the temperature a further 2 degrees. The high temperature inhibition was more gradual in ts2, while ts57 was intermediate between ts2 and the other two mutans. Except for the green tip of the primary leaf, the colours of the primary and secondary leaves of these mutants were the same.

The most heat sensitive of the mutants were ts4 and ts46. In both of these mutants and in contrast to the other four, the central vein area of the primary leaf showed higher degrees of heat tolerance for chlorophyll production than the rest of the leaf. Thus in ts46 the central vein area was pale green at 30°C although chlorophyll synthesis in the rest of the leaf was arrested at 29°C.

The mutant ts4 also showed a considerable difference in colour gradation between the primary and secondary leaves, chlorophyll production in the latter leaves being even more heat-sensitive (Figure 1b) than was indicated for the primary leaf in Figure 1a. This difference is shown in Figure 2 in which the 'in vivo' absorption spectra of primary and secondary leaves of ts4 grown at different temperatures are compared.

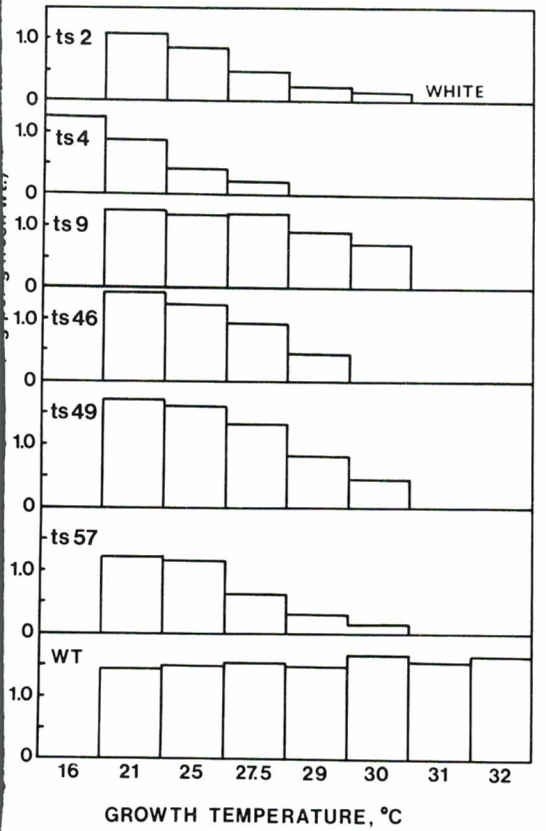


Figure 3: Chlorophyll content of primary leaves of ts-mutants and the wild type grown for 6 days at temperatures from 16°C to 32°C. The first 1 cm (tip) of each leaf was removed and discarded before the leaves were weighed and extracted for chlorophyll. Measurements were not made on leaves that were white or contained only traces of green.

Figure 3 shows estimations of the chlorophyll content of leaves of the ts-mutants and the wild type grown at different temperatures. The trends obtained are similar to those shown in Figure 1, except that the decreases in chlorophyll with increasing growth temperature are more gradual than it would appear from colour grading. This can be attributed to the difficulty in assessing degrees of colour at chlorophyll concentrations in excess of 50% of normal. In contrast to chlorophyll production, there were only small differences in growth rates between the mutants and wild type primary leaves (Figure 4). Thus the marked heat sensitivity of chloroplast development in the mutants was not paralleled by a similar effect of high temperature on leaf elongation.

3.5. Changes in chloroplast structure

Figure 5 shows the effect of elevated growth temperatures on the structure of chloroplasts in the mutant ts57. Grana were well developed in chloroplasts grown at 26°C and 28°C (Figures 5a and b) even though the chlorophyll content of the leaves was considerably reduced at the latter temperature (Figure 3). However, at 28°C the orientation of the grana was random with respect to each other in the majority of the plastids and the intergrana lamellae were poorly developed. Plants grown at 29°C contained a

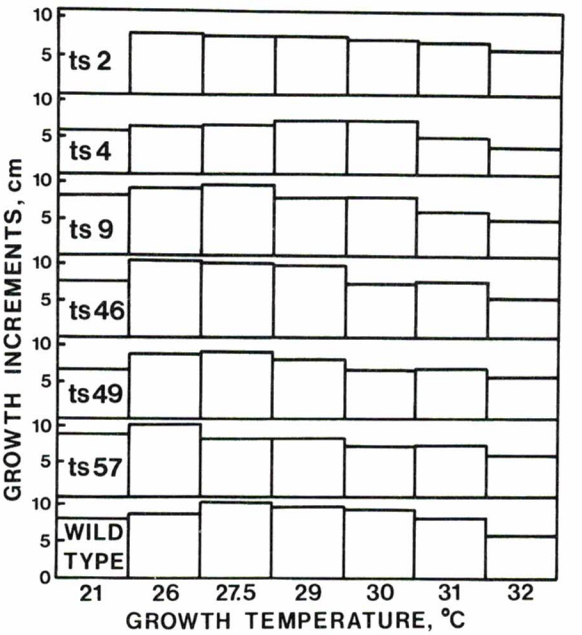


Figure 4: Elongation growth of ts-mutants and the wild type at different temperatures. Values shown are the average increase in height of 10 to 12 plants at each temperature between the 3rd and 5th day of growth.

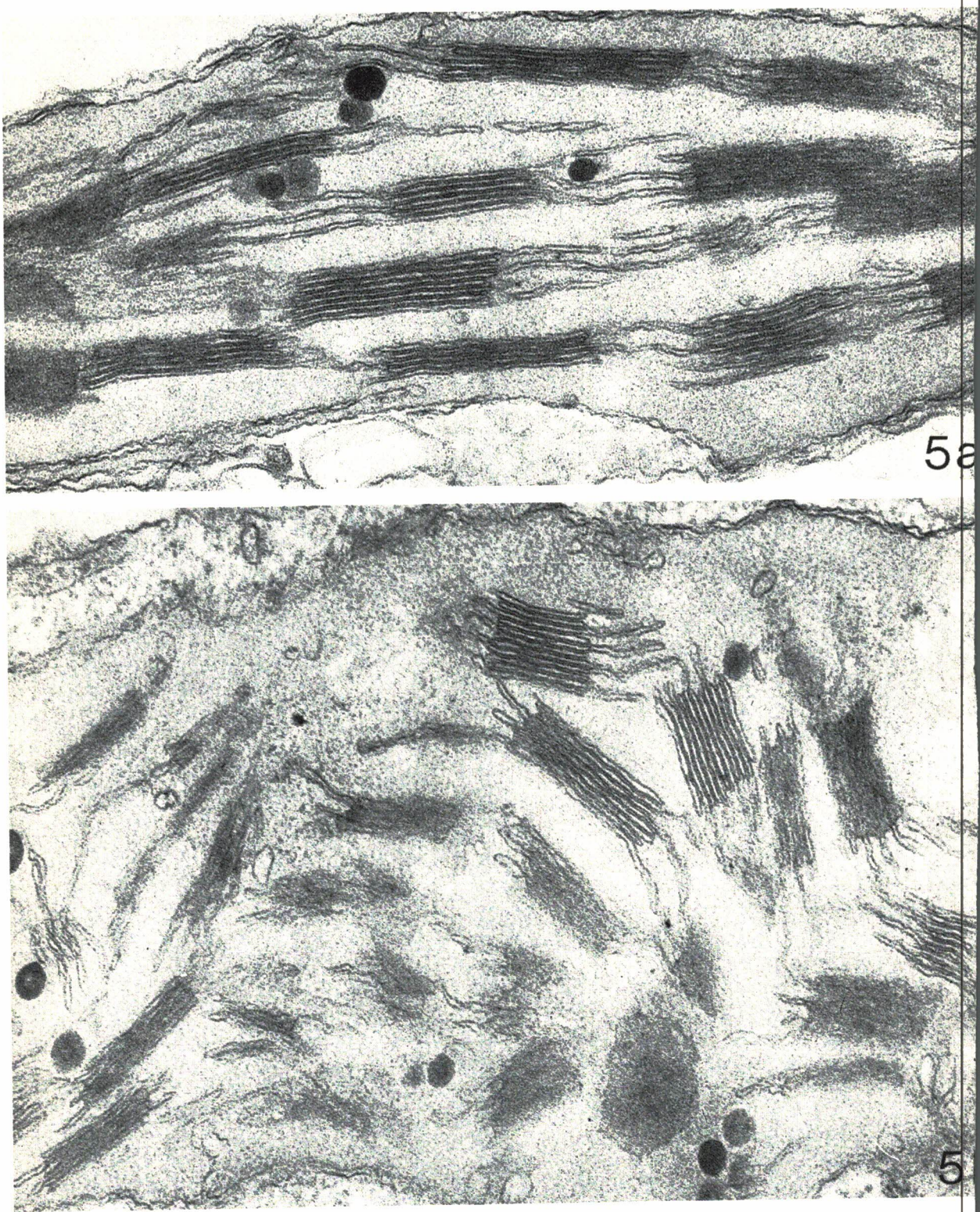
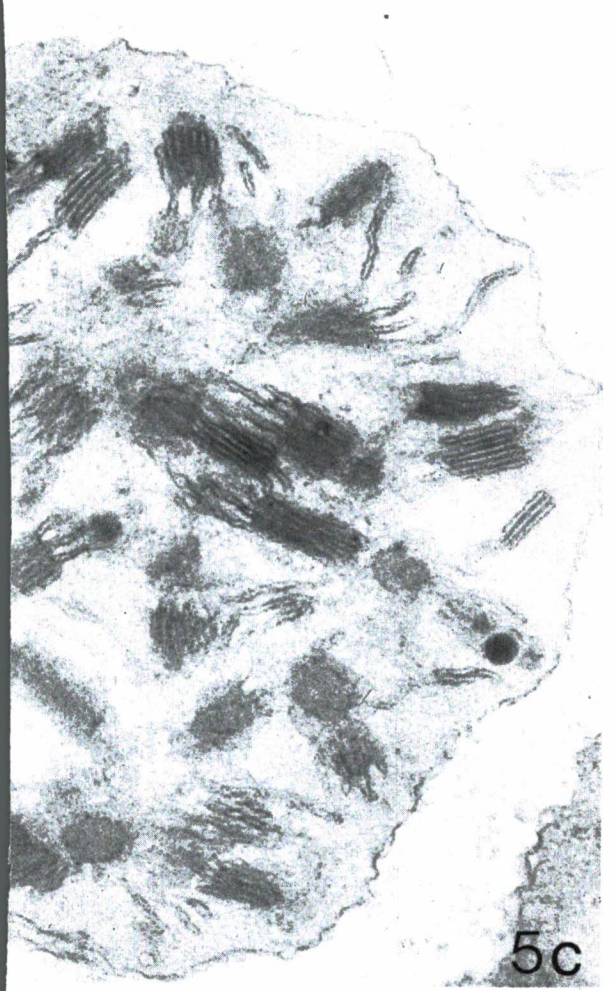
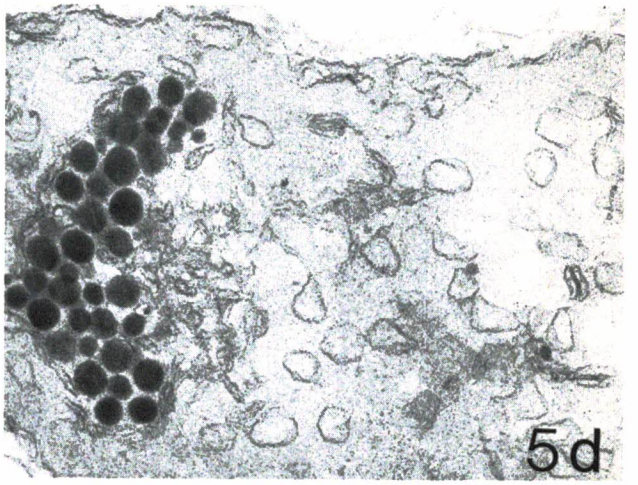


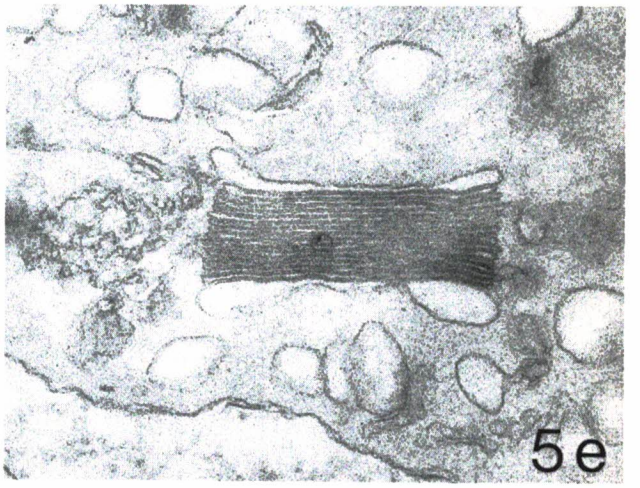
Figure 5: Ultrastructure of the plastids of mutant *viridis-zj* ^{ts57}.
a) grown at 26°C; 70,000 ×
b) grown at 28°C; 70,000 ×
c-e) grown at 29°C; 50,000 ×
f) grown at 30°C; 70,000 ×



5c



5d



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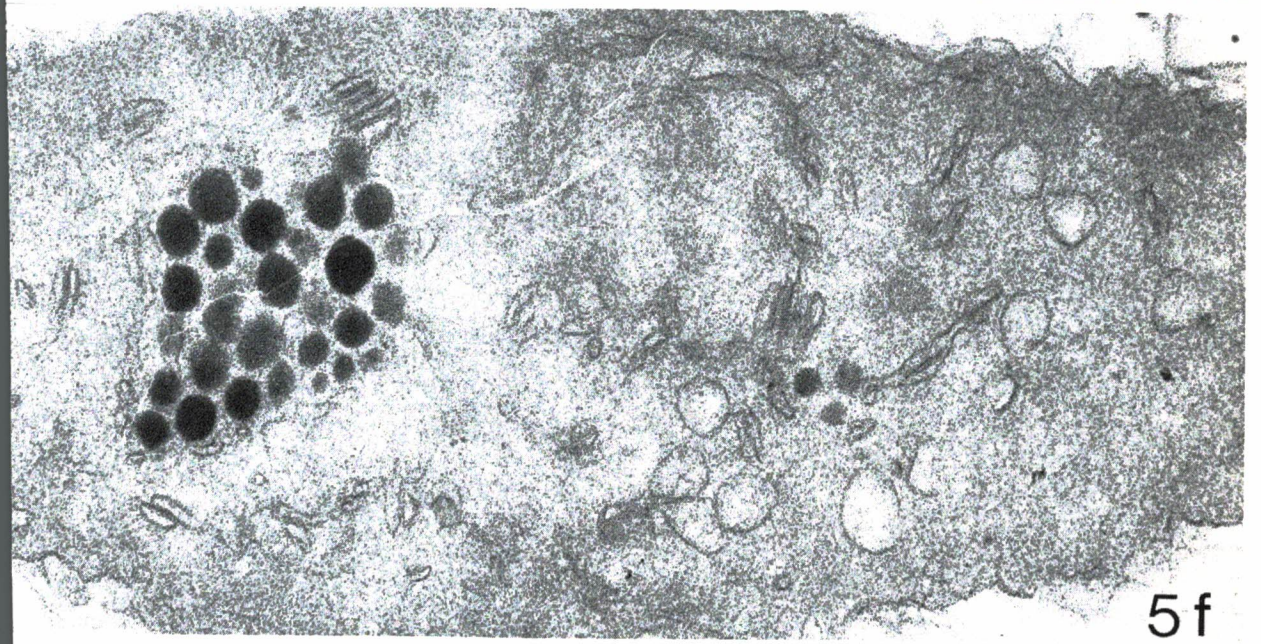

5f

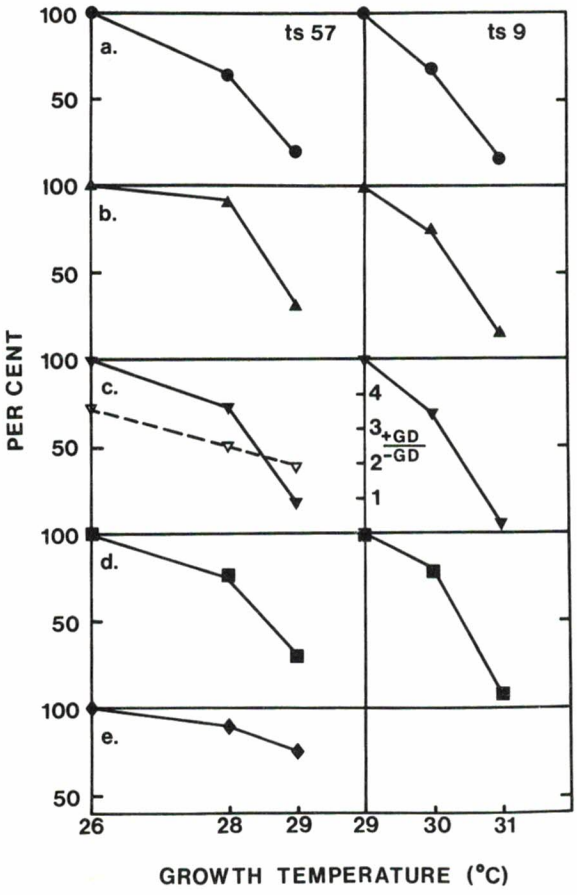

Figure 6: Photochemical activities of chloroplasts isolated from ts57 grown at 26°, 28° and 29° and ts9 grown at 29, 30 and 31°C. a, leaf chlorophyll; b, photoreduction of ferricyanide; c, photoreduction of ferricyanide in the presence of gramicidin D (4 μg·ml^{-1}). The dashed line in the case of ts57 shows the ratio of activities in the presence and absence of gramicidin D (GD); d, photoreduction of ferricyanide in the presence of 0.1 mM *p*-phenylenediamine; e, photosystem 1 activity (cf. 2.4). Results are expressed as a percentage of the values obtained at the lowest growth temperature used for each mutant. For ferricyanide photoreduction these were around 300 μmoles reduced (mg chlorophyll)$^{-1}$ hour^{-1} in the presence of either gramicidin D or *p*-phenylenediamine.

variety of chloroplasts with different internal organizations. A few plastids contained grana with random orientation (Figure 5c). In most plastids the internal membranes consisted of swollen thylakoids, small grana containing two discs and an occasional abnormally large granum (Figure 5d, e). Clusters of osmiophilic globuli was a common feature. In cells grown at 30°C the reduction and disorganization of the internal membranes was enhanced (Figure 5f). Similar structural changes in the chloroplast organization were observed when mutant ts9 was grown at temperatures from 29°-31°C.

Table III

The effect of intermittently lowering the growth temperature on chlorophyll production in mutant ts9

Growth temperatures	Av. height of seedlings (mm)	Chlorophyll content [μg (g fresh wt)$^{-1}$]
31°C, 1 hr per day at 28°C	110	89
31°C, 1 hr per day at 16°C	110	139
31°C, 2.5 hr per day at 28°C	110	119
31°C, 2.5 hr per day at 16°C	110	101
31°C, 6 hr per day at 28°C	120	321
31°C, 6 hr per day at 16°C	80	281
31°C, constant	90	48
28°C, constant	119	955
16°C, constant	84	581

Seeds were germinated for 26 hours in running tap water and then transferred to glass containers in water baths maintained at the temperatures given in the table. Analyses were carried out 5 days later.

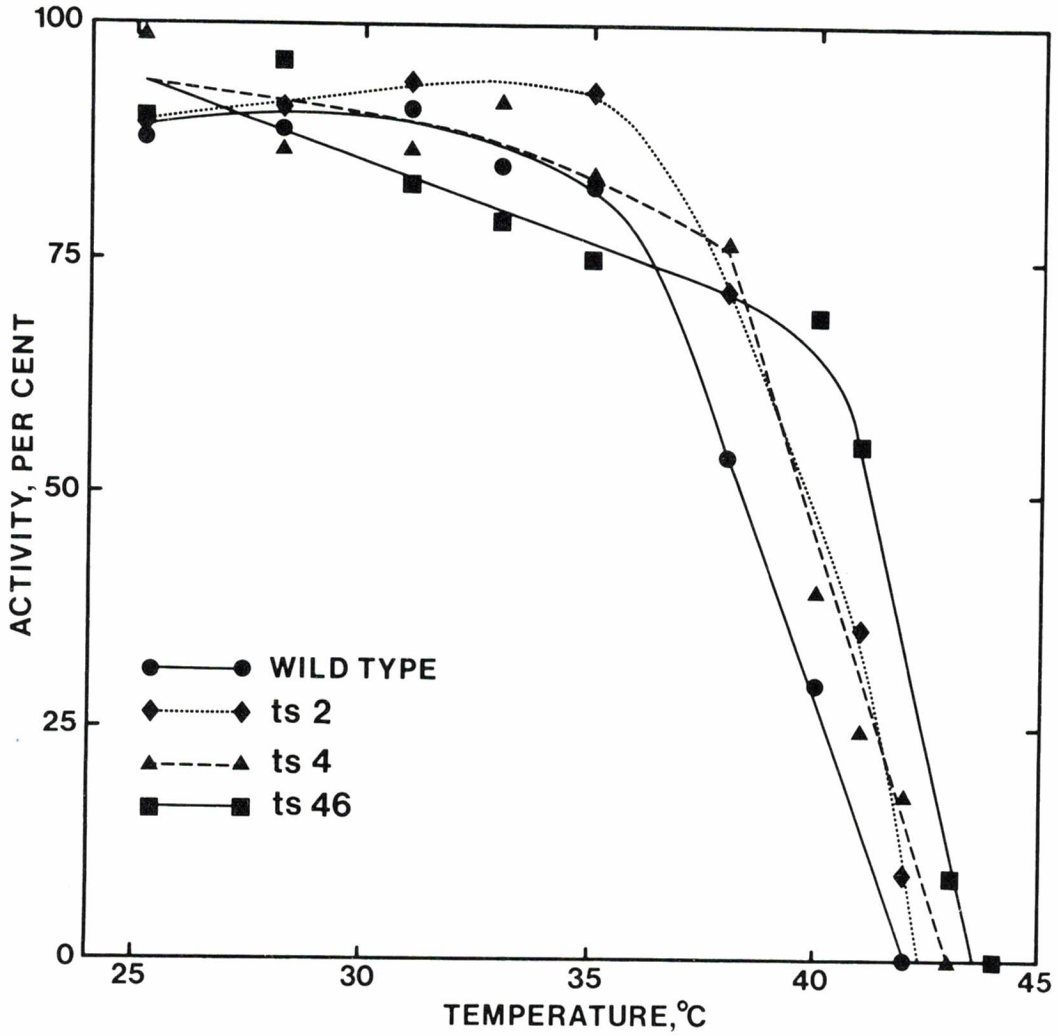

Figure 7: Heat lability of the photoreduction of ferricyanide in chloroplast thylakoids isolated from wild type and the mutants ts2, ts4, ts46. The isolated thylakoids were heated to various temperatures and then assayed for photoreduction of ferricyanide at 24°C as follows. Aliquots (20 µl) of thylakoid suspension (300 mg chlorophyll·ml^{-1}) were added to cuvettes immersed in a water bath with temperatures increasing successively from 25°C to 44°C (x-axis in the Figure). After 4 min, the cuvette was transferred to a water bath at 24°C and reaction mixture (1.5 ml at 24°C) was added. After a further 2 min for temperature equilibration, photoreductive activity was assayed at 24°C. The results are plotted as a percentage of the activity in control samples which were not heated.

3.6. Changes in chloroplast activity

Figure 6 compares photochemical activities of chloroplasts isolated from ts57 to ts9 grown at temperatures producing different degrees of inhibition of chloroplast development. The lowest temperature selected for each mutant allowed chlorophyll to accumulate to 80-100% of the maximum level. All of the preparations were active in reactions involving photosystems 1 and 2. However, the decrease in leaf chlorophyll (Figure 6a) was accompanied by a decrease in activity per mg chlorophyll indicating

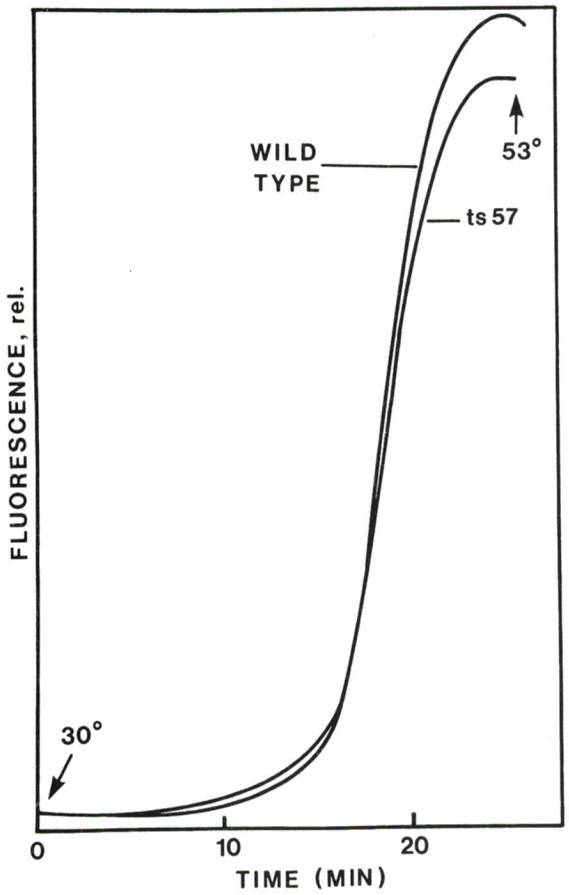

Figure 8: Increase in chlorophyll fluorescence in leaf sections of wild type and ts57 heated at 1°C per minute.

that chloroplasts which developed at high temperatures became more and more functionally incompetent as the growth temperature was raised. Activities of chloroplasts from ts57 involving both photosystems (Figure 6b, c) or photosystem 2 alone (Figure 6d) were more heat sensitive than photosystem 1 activity (Figure 6e).

3.7. The effect of intermittently lowered temperatures on heat bleaching

If high growth temperatures interfere with some regulatory mechanism controlling chloroplast development rather than by a direct inhibition of synthetic processes, then it might be possible to negate the effect of high temperature by interpolating relatively short periods of exposure to low temperature. This possibility was tested and shown to be unlikely in the mutant ts9. The results are shown in Table III. Decreasing the temperature to either 28°C or 16°C for 1 hour or 2.5 hours per day had only a slight effect in increasing the chlorophyll content of the leaves compared with control plants grown at a constant 31°C. Increasing the period of exposure to 28°C to 6 hours per day increased the chlorophyll content to 33% of the values found in plant grown a a constant 28°C. The comparable value for plants exposed to 16°C was 48%.

3.8. Thermostability of the chloroplast thylakiods of ts-mutants

Figure 7 compares the thermostability of Hill reaction activity in three of the mutants and the wild type. The plants were grown in a growth chamber at 17 ± 0.5°C. At this growth temperature, chloroplast development was not inhibited in the ts-mutants. It can be seen from the figure that the photoreductive activities of the isolated chloroplasts of ts2, ts4 and ts46 were at least as thermostable as the activity of chloroplasts isolated from the wild type.

The heat sensitivity of chloroplasts can also be monitored by an increase in fluorescence of chlorophyll (14). This heat-induced rise in chlorophyll fluorescence can be measured in intact leaves and provides a means of ranking plants according to their heat sensitivity (14, 18).

Figure 8 shows fluorescence-heating curves for ts57 and wild type. The curves were almost identical and similar measurements with ts9 and ts46 indicated that on the basis of the fluorescence changes, all three mutants were just as heat stable as the wild type.

4. CONCLUSIONS

The temperature sensitive reactions affected in the six investigated nuclear gene mutants are part of the early stages of plastid development. The phenotypes displayed by the mutants at

growth temperatures spanning the range from permissive to restrictive temperatures is similar with respect to chlorophyll production, development of internal chloroplast structures and photochemical activities. Phenocopies of the mutants can be obtained by growing the wild type at temparatures above 32°C (5, 17). Analogous phenotypes are obtained with oat, wheat and pea wild type seedlings grown under restrictive temperature conditions (5, 13). Since the etioplasts formed in the mutants at the permissive temperature can apparently develop into fully active chloroplasts at the restrictive temperature (32°C), the six genes must code for six products either not needed in the assembly of the photosynthetic membranes or preformed in the dark. The six temperature sensitive mutants are potentially useful as markers for experiments with somatic hybridization involving barley. Pairs of mutants could be grown at the restrictive temperature and protoplasts produced from them for fusion. Regenerated fusion products of the two mutants could be selected by their capacity to green at a temperature non-permissive for the mutants in question.

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