Planta (1987) 171:11–18

Planta

Somatic instability of carotenoid biosynthesis in the tomato *ghost* mutant and its effect on plastid development

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Abstract. The tomato (*Lycopersicon esculentum* (L.) Mill.) *ghost* plant is a mutant of the San Marzano cultivar affected in carotenoid biosynthesis. *ghost* plants exhibit a variable pattern of pigment biosynthesis during development. Cotyledons are green but true leaves are white. Green sectors, which appear to be clonal in origin, are frequently observed in the white tissue. Because of the lack of photosynthesis *ghost* plants have a very low viability in soil. We have developed a strategy for propagating *ghost* plants that employs organ culture to generate variegated green-white plants which, supported by the photosynthetic green areas, develop in soil to almost wild-type size. These plants were used to analyze the pigment content of the different tissues observed during development and plastid ultrastructure. Cotyledons and green leaves contain both colored carotenoids and chlorophyll but only the colorless carotenoid phytoene accumulates in white leaves. The plastids in the white tissue of *ghost* leaves lack internal membrane structures but normal chloroplasts can be observed in the green areas. The chromoplasts of white fruits are also impaired in their ability to form thylakoid membranes.

Key words: Carotenoid biosynthesis – Chloroplast in tomato mutant – Chromoplast – *Lycopersicon* (*ghost* mutant) – Mutant (tomato) – Tissue culture (of mutant).

Introduction

The tomato *ghost* mutant, which was isolated by Rick et al. (1959), is coded by a single gene, *gh*. Plants homozygous recessive for *gh* show a green-white variegated phenotype. Cotyledons and first true leaves are generally green but mosaics of normal and chlorotic tissue are frequently observed. In the second-leaf stage the pattern of chlorophyll deficiency becomes more conspicuous and by the third set leaves are completely without chlorophyll. Thereafter most plants die off, but a few remain variegated, with the green tissue showing as islands in the leaves and as long streaks in stems and petioles. Although most *ghost* plants perish before flowering those with enough chlorophyll flower and set fruit after both self- and cross-pollination. Seeds collected from self-pollinated plants give rise to plants with the same pattern of expression, regardless of the phenotype of the branch from which they originated, indicating that the green areas formed in *ghost* plants are not the result of either nuclear or plastid mutation (Rick et al. 1959). Biochemical analyses indicate that the colorless carotenoid phytoene accumulates in the white tissue of *ghost* plants (Mackinney et al. 1956).

Phytoene is the first compound of the carotenoid biosynthetic pathway. Through a series of dehydrogenation reactions, it is converted into colored carotenoids (for a review, see Goodwin 1976). The main function of these pigments is to protect the cell against photooxidation that can occur as a byproduct of the light absorption by chlorophyll. In the absence of colored carotenoids the chlorophyll can be destroyed by photooxidation, resulting in an albino phenotype which in general has very low viability, at least in high light. In addition, carotenoids can function as accessory antenna pigments in both bacteria and plants (Goedheer 1969, 1972) and they are also responsible for the red and yellow colors of fruits, flowers and some endosperms. In these tissues they accumulate in organelles called chromoplasts.

As reported by Rick et al. (1959) *ghost* plants

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are hard to grow, making biochemical and molecular studies very difficult. We report a strategy for propagating *ghost* plants through tissue culture which allows the continuous production of plants that can reach sizes similar to their wild-type counterparts. These plants were used to study pigment biosynthesis and plastid structure during different stages of development.

Material and methods

Tissue culture. The medium for shoot culture (MSO) contained the salts of Murashige and Skoog (1962) supplemented with B5 vitamins (Gamborg 1982) and 30 g/l of sucrose. Seeds (kindly provided by Dr. C. Rick, University of California, Davis) were disinfected by sequential submersion in 10% commercial bleach (approx. 0.5% sodium hypochlorite) + 0.1% sodium dodecyl sulfate (SDS), 100% ethanol, and sterile distilled water, and germinated in flasks containing MSO medium and 1.2% agar. Shoots containing at least one leaf with green sectors were cut from the seedlings and transferred to sterile flasks containing the same medium. After new shoots developed the procedure was repeated, selecting for increasing amounts of green tissue until a mostly green plant was obtained. Well-rooted, mostly-green plants, with an average height of approx. 10 cm, were planted in soil in a covered container and grown in a growth chamber. The cover was removed after 2 d and in some cases the plants were transferred to the greenhouse. Shoot culture was also used to maintain stocks of the homozygous dominant and heterozygous plants. Shoots containing at least one leaf were transferred under sterile conditions to flasks containing MSO medium. Some of these plants were transferred to soil according to the conditions described for *ghost* plants. For regeneration from leaf disks, green and white leaves from plants grown in a growth chamber were surface-sterilized by the same procedure as used for seeds. Disks of 3 mm diameter were cut under sterile conditions and placed in Petri dishes containing medium T1 (MS salts supplemented with 2.5 mg/l N6-benzyladenine [BA], 1 mg/l indole-3-acetic acid [IAA], 30 g/l sucrose and B5 vitamins) and 1.2% agar. Regenerated shoots were rooted in MSO medium. Pollen viability was determined according to Burham (1984).

Light conditions. For high-light (120 μmol photons $\cdot m^{-2} \cdot s^{-1}$; photosynthetically active radiation) experiments the growth chambers were equipped with four fluorescent lamps (General Electric, Cleveland, O., USA; cool white, 72 W each) and nine incandescent lamps (100 W each). The fluorescent lamps were turned on from 06:00 to 22:00, the incandescent lamps from 06:30 to 21:00. Temperature was kept at 25° C and 22° C during the light and dark periods, respectively. Low-light (0.1 μmol photons $\cdot m^{-2} \cdot s^{-1}$) experiments were done from 05:30 to 21:00, with the same temperature settings.

Pigment characterization. One gram of leaf material from plants grown in the greenhouse was extracted repeatedly with 5 ml of acetone until no color was observed in the solvent. Between extractions the sample was centrifuged at 1500 $\cdot g$ for 15 min. An equal volume of petroleum ether (b.p. 30–60° C) was added to the supernatant and after a brief centrifugation the upper phase was removed and dried under a stream of nitrogen. The sample was resuspended in hexane and loaded into a magnesium-oxide column, 10 mm diameter, 80 mm long. Carotenoids were eluted sequentially with 2% acetone in hexane, 5% acetone in hexane and 20% acetone in hexane. The samples were dried, resuspended in hexane, and the absorption spectra determined in a 3480 Perkin Elmer (Elmwood Park, N.J., USA), spectrophotometer.

Electron microscopy. Leaf samples containing white and green sectors were taken from a tissue-culture-propagated *ghost* plant grown in a growth chamber. For the analysis of fruits a homozygous dominant (gh^+/gh^+) and a homozygous recessive (gh/gh) plant, grown in the greenhouse, were hand-pollinated on the same day. Only one fruit per inflorescence was allowed to develop since a number of very small fruits are otherwise set in each inflorescence. When the wild-type fruits reached the "turning" stage (as defined by the U.S. Department of Agriculture tomato-ripening chart) both wild-type and the *ghost* fruits were harvested, but some wild-type fruits were allowed to proceed to the red stage and then harvested. Both leaf and fruit samples were fixed in 4% glutaraldehyde in 0.1 M sodium-cacodylate buffer, pH 7.2, for 2 h and then rinsed four times, 15 min each, in the same buffer. Postfixation was carried out in 2% OsO_4 in 0.1 M sodium cacodylate, pH 7.2, for 2 h. The samples were dehydrated in a graded series of ethanol followed by 100% propylene oxide, and infiltrated and embedded in PolyBed 812 (Polysciences, Warrington, Pa., USA). Polymerization was carried out at 60° C for 64 h. Samples were sectioned with a diamond knife using a Sorvall MT5000 ultramicrotome (Sorvall Instruments, Wilmington, Del., USA). After post-staining with 2% uranyl acetate (15 min) and lead citrate (5 min) the samples were examined with a Phillips (Eindhoven, The Netherlands) 201 transmission electron microscope operated at 60 kV.

Results

Propagation of ghost plants by tissue culture. In many plants, shoot tips can be cultured under sterile conditions to generate a large number of vegetatively derived plants (Hu and Wang 1983). Typically, shoots that may contain one or more leaves are cut and placed in a sterile growth medium. With time, roots and new shoots develop and the plant can be either transferred to soil or used as a source of shoots for further propagation. We reasoned that this technique could be used to propagate *ghost* plants by selectively choosing shoots containing leaves with large green sectors. This selective propagation should result in plants that contain more photosynthetic tissue than the ones germinated directly from seed, and thus be more likely to develop normally when transferred to soil.

In order to test the usefulness of this approach we germinated, under sterile conditions, seeds from a heterozygous ($^+/gh$) plant which had been self pollinated, obtaining plants of both green and *ghost* phenotype. The *ghost* plants (gh/gh) can be easily identified by the differential pattern of chlorophyll biosynthesis during development. The hypocotyls lack chlorophyll. The cotyledons are generally green, but occasionally bleached at the margins. The first true leaves and the stem are white

Fig. 1. A three-months-old tomato *ghost* plant propagated through tissue culture and grown in the greenhouse. This plant was 1.2 m high

but green sectors are frequently observed. When subepidermal layers are examined under the microscope the sectors appear to originate from single cells. Shoots containing leaves with green sectors were removed, sterilized, and cultured. These explants developed new roots and shoots, giving rise to variegated white-green *ghost* plants. Successive rounds of propagation through shoot culture, always selecting for green tissue, resulted in *ghost* plants having an increased amount of green leaves, usually not attainable when the plants are raised from seed. When these plants were transferred to soil they reached a height of 1 m in an average of two to three months (Fig. 1). The same procedure was used to propagate heterozygous and homozygous dominant plants. Thus, we now have plants of all three genotypes in tissue culture. Our biochemical and structural work was conducted on homozygous recessive and dominant plants propagated by shoot culture; heterozygotes were not used.

Morphology of ghost plants. The morphology of the *ghost* plants has been illustrated in the paper by Rick et al. (1959) and we have made similar observations with the plants propagated through tissue culture. Roots, stems and petioles are largely unaffected by the *gh* mutation. Fully expanded white leaves, however, show a profoundly altered morphology. This is particularly evident when the plants are grown in high light, while at low light this effect is reduced and the white leaves are morphologically more similar to their green counterparts. Unopened leaves or leaves of plants grown in low light in tissue culture are similar to wild-type or green *ghost* leaves, indicating that the alteration is a consequence of photooxidative damage that occurs when the leaves are exposed to high light. Anthocyanin pigmentation is very conspicuous in the white portions of *ghost* plants, probably because the color of this pigment is not masked by chlorophyll and carotenoids, as in wild-type plants. However, this assumption needs to be confirmed by a quantitative analysis of the anthocyanin content.

ghost plants flower and set fruit normally in the green branches. The petals have an intense yellow pigmentation along the midrib but tend to lose color at the margins. The size of the fruits obtained is roughly proportional to the amount of green tissue in the corresponding branch, probably as a consequence of the degree of nutrient available. In white branches, flowers generally fail to open. However, analysis of pollen from these flower buds by vital staining indicated a high level of viability and pollen extracted from these flowers has been successfully used to pollinate wild-type plants.

Fruits in *ghost* plants are set only on green branches. A pronounced loss of chlorophyll is observed during the development of these fruits. Even fruits arising from mostly green branches tend to show an almost total loss of chlorophyll before reaching the mature stage. A yellow pigment can be observed in the skin of the fruits during subsequent stages. This probably corresponds to the alkali-soluble, non-carotenoid pigment described by Rick et al. (1959). At the same time softening of the fruit becomes evident.

Stability of the ghost green phenotype. The propagation strategy outlined above always results in variegated white-green *ghost* plants, even when shoots that appear to be all-green are used for the primary cutting. This could be a reflection of the occurrence of different phenotypes in different cell layers. It is also possible that the green tissue gives rise to white cells. An alternative approach to propagation of plants is to regenerate shoots from callus tissue. In the case of *ghost* this was an attractive possibility because we could regenerate plants

from tissues of defined phenotype. From a *ghost* plant we selected green and white leaves. Microscopic examination of some of the green leaves indicated that all the mesophyll layers contained well developed chloroplasts and that no evidence of white *ghost* cells could be found (not shown). Disks, 3 mm in diameter, were cut from these leaves, the lower and upper epidermis were peeled off, and the remaining tissue was placed in regeneration medium (see *Material and methods*). An average of ten shoots were obtained from each disk. These shoots were green and retained their phenotype when transferred to MSO medium for shoot culture. Twenty plants were allowed to develop in sterile flasks and then transferred to soil. In all cases pale-green and white sectors appeared at different stages during the development of the plants, indicating that the green phenotype is unstable and can give rise to white tissue. Microscopic examination (not shown) of some of the pale-green sectors showed that many mesophyll cells contained chloroplasts but the remaining layers had the typical structure of *ghost* white tissue (Rick et al. 1959). Thus, the pale-green phenotype consists of white and green layers of cells in the same leaf.

Plants were also regenerated from white leaf tissue. However, the efficiency of regeneration was markedly decreased, with a typical frequency of only one or two shoots arising from each 3-mm disk. On visual inspection the new shoots were either predominantly white or green. Two shoots of each class were transferred to flasks containing MSO medium and allowed to develop into plants. The plants originating from white shoots showed numerous green sectors while the ones from green shoots had pale-green and white sectors. This result strongly indicates that a bidirectional switch occurs between the green and white *ghost* phenotypes.

Pigment characterization. Having obtained a constant supply of *ghost* plants, we were able to examine the chlorophyll and carotenoid content of these plants. As mentioned above, cotyledons of homozygous recessive plants tend to green more than the hypocotyls or the first true leaves. A qualitative extraction of pigments in cotyledons (Fig. 2) indicates that both chlorophyll and colored carotenoids are present. The absorption spectrum of the pigments extracted from white leaf tissue (Fig. 2) indicates that a colorless carotenoid, later identified as phytoene, accumulates in this tissue, whereas chlorophyll is detected only in very small amounts. It is possible that this trace amount of chlorophyll originates in the small green sectors that contaminate the white tissue. Analysis of the green *ghost* tissue shows that, as in cotyledons and wild-type leaves, both chlorophyll and colored carotenoids are present.

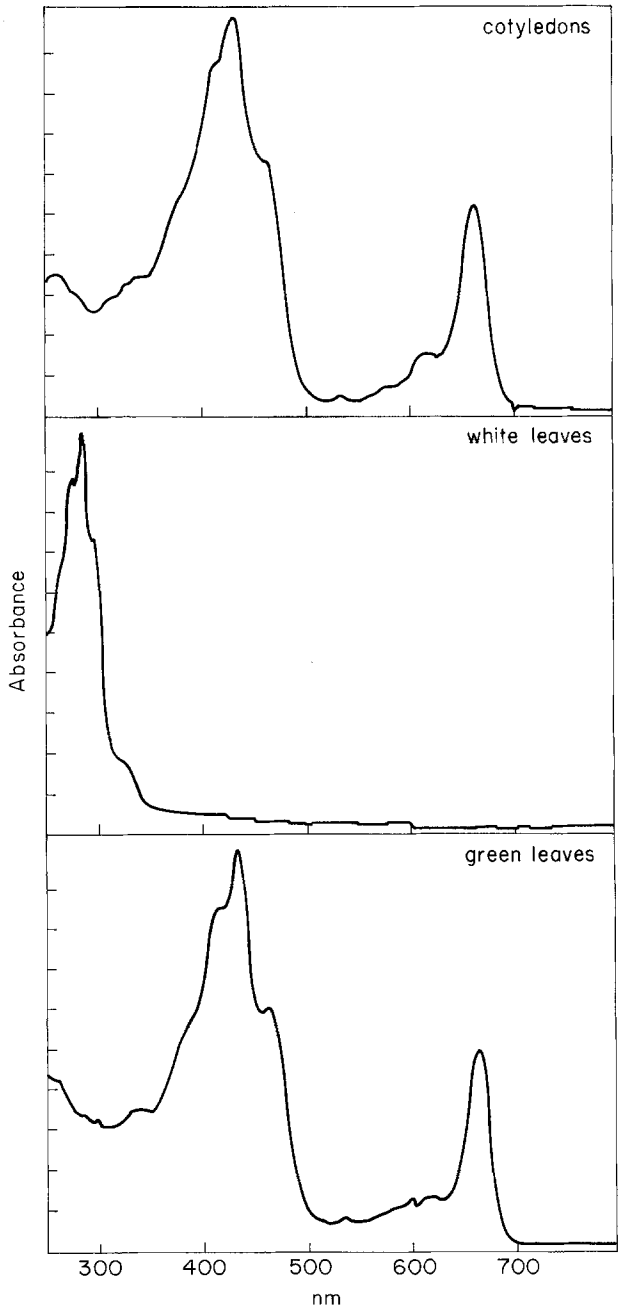

Fig. 2. Absorption spectra of carotenoids from different tissues of tomato *ghost* plants. Pigments were extracted according to *Material and methods* from cotyledons of *ghost* plants germinated under sterile conditions or from leaves of plants propagated through tissue culture. Wavelengths are indicated at the bottom. The three-peak spectra correspond to carotenoids. Colored carotenoids absorb between 350 and 500 nm. Phytoene absorbs below 300 nm. Monomeric chlorophyll absorbs at 680 nm

We also conducted a quantitative analysis of

Table 1. Chlorophyll and carotenoid content of different tissues of *ghost* plants. Average of two determinations. Values expressed in $\mu g \cdot (g\ FW)^{-1}$; – = below detectable levels

	Wild type (leaves)	*ghost*		
		Green leaves	White leaves	Cotyledons
Phytoene	–	–	49	–
β-carotene	42.3	55.5	–	22.6
chlorophyll	1819	1979	5	949

the chlorophyll, β-carotene and phytoene content of *ghost* cotyledons and of wild-type and *ghost* green and white leaves (Table 1). The results indicate that β-carotene, the main leaf carotenoid, is present in all three green tissues, and that the ratio of β-carotene to chlorophyll is similar in all three cases. Phytoene accumulates in *ghost* white leaves but not in green *ghost* tissue. Thus, the *ghost* green and the wild-type leaf tissues are phenotypically not distinguishable.

There is a third phenotype (yellow) that forms occasionally in *ghost* plants (Rick et al. 1959). Extraction of carotenoids from yellow leaves indicates that both phytoene and colored carotenoids accumulate (not shown). However, we were unable to stabilize this phenotype in tissue culture and conducted no further work on it.

Ultrastructural analysis. In the thylakoid membranes of normal, mature chloroplasts, chlorophyll and colored carotenoids bind specific proteins to form the antenna and reaction-center complexes.

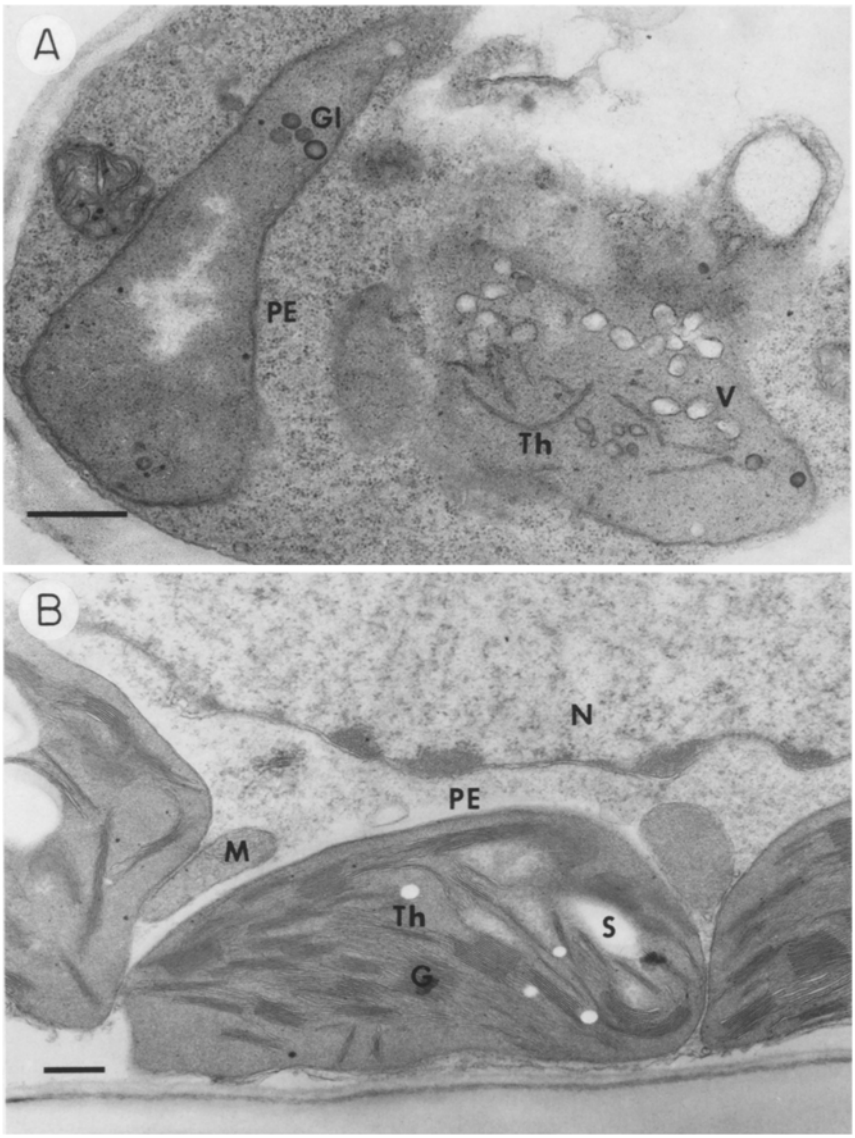

Fig. 3A, B. Plastids from leaves of tomato *ghost* plants grown in a growth chamber at high light, **A** from white sectors (×27000); **B** from green sectors. *Gl*, globuli; *Th*, thylakoids; *V*, vacuole; *PE*, plastid envelope; *M*, mitochondrion; *G*, grana; *S*, starch; *N*, nucleus. ×17000; bars = 0.5 μm

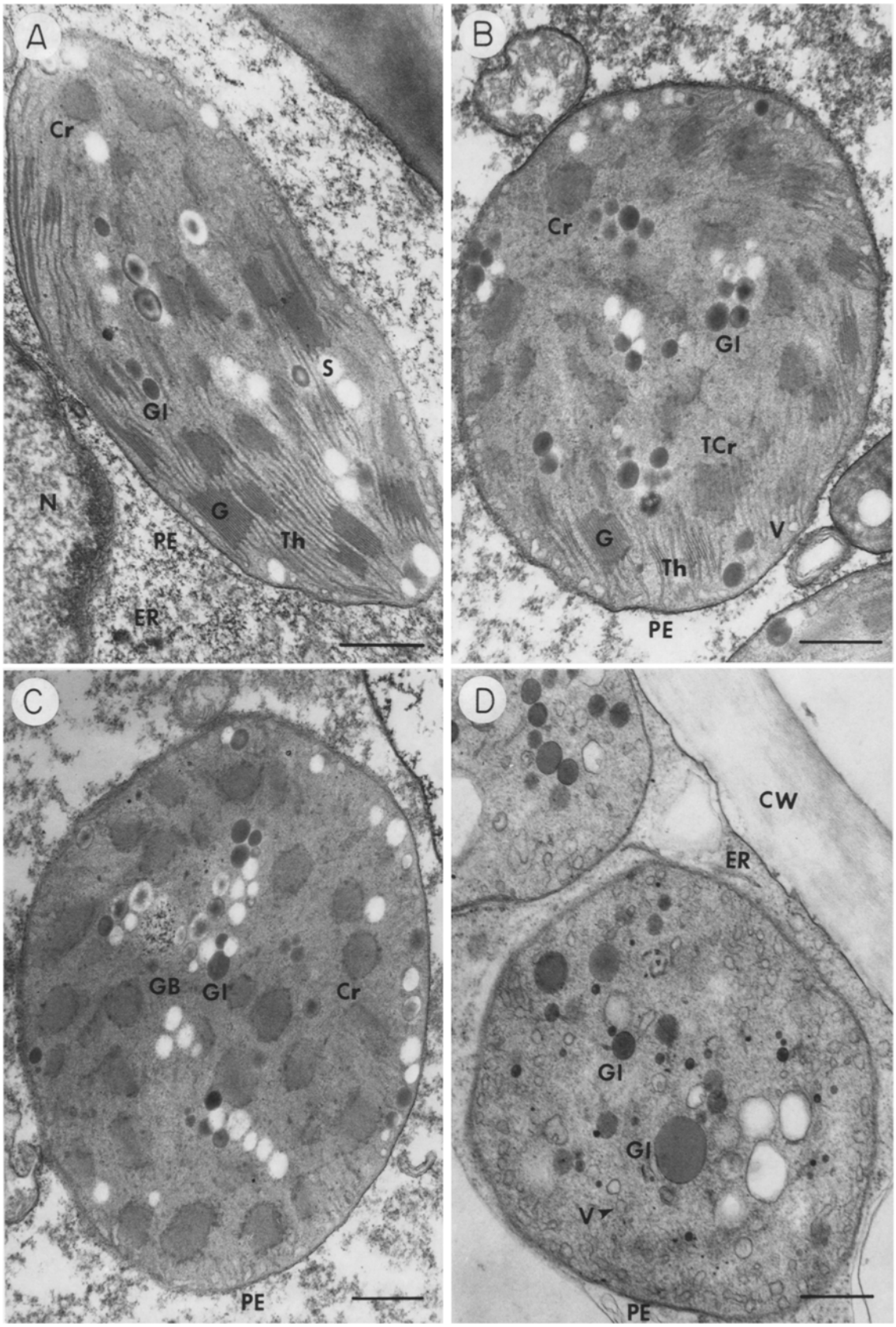

Fig. 4A–D. Plastids from wild-type and *ghost* fruits of tomato: **A**, **B**, **C** from wild-type fruits at the "turning" stage of ripening; **D**, from a *ghost* fruit. *Cr*, crystalloid; *ER*, endoplasmic reticulum; *CW*, cell wall; *GB*, granular body; *Tcr*, transition crystalloid; *S*, starch; *G*, grana; *Gl*, globuli; *Th*, thylakoid; *PE*, plastid envelope; *N*, nucleus; *V*, vacuole. **A**, ×28500; **B**, ×30000; **C**, **D**, ×24000; bars = 0.5 μm

We wanted to determine the kind of plastids formed in the white and green sectors of *ghost* leaves and fruits. Electron-microscopic examination of cells from white leaves shows that the plastids are of irregular shape with little or no thylakoid membranes (Fig. 3A), whereas normal-looking chloroplasts, containing stacked thylakoids, develop in green *ghost* leaves (Fig. 3B). Only one type of plastid was observed within a single cell, indicating that mixing of chloroplasts and undifferentiated plastids within single cells does not occur.

During the early stages of development of the wild-type tomato fruit, chloroplasts which resemble leaf plastids are formed (Harris and Spurr 1969a, b; Rosso 1968). At the onset of ripening there is unstacking and partial disappearance of the photosynthetic membranes, while chlorophyll and starch grains are slowly degraded. Next, colored carotenoids begin to accumulate in the form of membrane-bound "crystals". Representative micrographs of plastids formed in a San Marzano fruit at the "turning" stage are shown in Fig. 4A–C. In the green area of the fruit, chloroplasts are well developed, with extensive grana formation (Fig. 4A), while typical plastids are formed in the red area of the "turning" fruit (Fig. 4B, C). The chromoplast in Fig. 4B represents a transitional stage in the differentiation of chloroplasts into chromoplasts. Grana and thylakoids are still clearly distinguishable but membrane structures (crystalloids) that lack the stacking of the grana are also present. These structures resemble the carotenoid-accumulating "crystalloids" described in other lines of tomatoes (Harris and Spurr 1969a, b). In the structure labelled *TCr* (transition crystalloid) thylakoids are connected to a central body which seems to have a diffuse internal membrane structure. This could represent an intermediate stage in the process of transition between grana and crystalloids. Analysis of the plastid structure in a late red stage indicated that the long "crystals" observed by Harris and Spurr (1969b) in the red Pearson line also form in the San Marzano fruit (not shown). It is therefore likely that, as suggested by Rosso (1968), the thylakoid membranes, after the chlorophyll is degraded, participate in the process of formation of the carotenoid-containing "crystals", which may arise as a product of fusion of several "crystalloids". However, at the present time we lack direct evidence for this process.

For the analysis of *ghost* fruits, samples were taken from fruits formed on green branches. This material was harvested at the time when wild-type fruits which had been pollinated at the same time were at the "turning" stage. The yellow non-carotenoid pigment that forms in *ghost* fruits during ripening (Rick et al. 1959) was not observed in these samples. High levels of phytoene were detected in these fruits (not shown); this is consistent with the results of Mackinney et al. (1956). The plastids of *ghost* fruits (Fig. 4D) do not show thylakoids, grana or crystalloids but extensive formation of osmiophilic globules and vacuoles takes place. Thus, the lack of colored carotenoids evidently results in both fruit and leaves of *ghost* plants in the formation of plastids that fail to develop organized internal membrane systems.

Discussion

The *ghost* mutant offers interesting opportunities for the study of gene expression in plants. A major hurdle in these studies was the difficulty in growing mature *ghost* plants. This has now been overcome with the use of tissue culture, and the success of this approach underlines the potential of tissue culture for selectively propagating tissues with different phenotypes from variegated plants.

In the light of our results we can reexamine the nature of the *ghost* mutation. Rick et al. (1959) determined that chlorophyll accumulates in the green but not in the white *ghost* sectors, and they characterized *ghost* as a chlorophyll-unstable mutant. Mackinney et al. (1956) determined that phytoene accumulates in *ghost* white tissue, but they did not analyze the carotenoid content of the green *ghost* tissue. Our results indicate that the carotenoid composition of *ghost* green tissue is similar to wild type. Thus, it is likely that the *ghost* locus affects the conversion of phytoene into colored carotenoids. This locus may code for the enzyme phytoene dehydrogenase or for a regulatory protein that affects carotenoid biosynthesis. The loss of chlorophyll is probably a secondary event resulting from the lack of photoprotection in the white tissue. The *gh* locus, unlike the other known carotenoid mutants of tomato, affects both leaves and fruits. It has been postulated that there are two pathways for carotenoid biosynthesis in tomato, one affecting the leaves and the other the fruits (Raymundo et al. 1976). If the *gh* mutation is actually in the gene for phytoene dehydrogenase, the bifurcation of pathways must occur after this enzyme.

The development of normal chloroplasts correlates with the accumulation of colored carotenoids, which are necessary to protect chlorophyll against photooxidation. When only phytoene accumulates the resulting photooxidative process destroys the internal membranes of the chloroplast. This is con-

sistent with the observations of Robertson et al. (1978) on the plastids of maize carotenoid mutants. We also found that colored carotenoids are needed for normal chromoplast development. The chromoplasts of the white fruits seem to develop without going through the initial chloroplast stage. It is known that chloroplasts are not required for chromoplast formation (Smith and Smith 1931). However, the plastids in *ghost* fruits do not seem to form the crystaloids observed in wild-type fruits, an observation consistent with the idea that the thylakoid membranes are precursors of these structures (Harris and Spurr 1969b).

Seeds of self-pollinated *ghost* plants give rise only to plants with ghost phenotype, thus confirming the observation of Rick et al. (1959) about the epigenetic nature of the variegation in these plants. Another well-documented case of variegation based upon physiological rather than mutational events is the *im* mutant of *Arabidopsis thaliana* (Redei 1967). In homozygous *im* plants variegation results from the presence of both cells with undifferentiated plastids and cells with normal chloroplasts. As in the case of *ghost*, within a single cell plastids develop uniformly and the distribution of the two types of cells is not random. Also, the progeny of the two types of sectors is indistinguishable. Biochemical analysis indicates that *im* controls the synthesis of a ribonuclease.

At the present time we do not have information about the molecular basis for the variegation in *ghost*. Involvement of a diffusible substance can be excluded because the boundaries between sectors are very sharp. The fact that carotenoid biosynthesis proceeds normally in green *ghost* tissue indicates that the synthesis of enzymes for carotenoid biosynthesis is induced during the development of the plant, but this induction disappears before or during meiosis.

We would like to acknowledge the secretarial work of L. D'Alessandro and K. Otto, and the art work of J. Roberts and D. Greene. The excellent work of L. Corella (U. of Connecticut) on the first electron-microscope characterizations is also acknowledged.

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Received 2 September; accepted 31 December 1986