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Characterization of a Virescent Chloroplast Mutant of Tobacco[1]

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ABSTRACT

Virescent mutations produce plants in which young leaves have reduced chlorophyll levels but accumulate nearly normal amounts of chlorophyll as they age; they are predominantly nuclear mutations. We describe here a virescent mutation (designated Vir-c) found in a somatic hybrid line derived from *Nicotiana tabacum* L. and *Nicotiana suaveolens* Lehm. This mutation is inherited maternally. Young, half-expanded Vir-c leaves contained three to six times less chlorophyll than did control leaves, and reached maximum chlorophyll levels much later in development. Chlorophyll synthesis rates and chloroplast numbers per cell in Vir-c were similar to the control, and carotenoid content in Vir-c was sufficient to protect chlorophyll from photo-oxidation. Photosynthetic rates of Vir-c at low light intensities suggested a reduced ability to collect light. Electron micrographs of Vir-c chloroplasts from half-expanded leaves showed a significant reduction in thylakoids per granum. The decrease in granal thylakoids was strongly associated with low chlorophyll levels; mature Vir-c leaves with nearly normal chlorophyll content showed normal granal profiles. These results are discussed in relation to virescent mutants previously described.

Virescent mutants are a curious group of higher plants in which young leaves show much reduced Chl content, but recover more levels of Chl as they age. In contrast with many photosynthetic mutations, the virescent mutation is not lethal, and virescent plants survive to flower and produce seed.

Virescent mutations occur in a wide range of flowering plants, including tomato (22), maize (6), cotton (5), and peanut (1). Despite the number of virescent mutants that have been described, the primary effect of the mutation is unknown. The phenotype suggests a temporal aberration in some factor governing Chl content, such as the ability to synthesize, protect, or accumulate Chl.

The variety of ways Chl content can be influenced may explain why the virescent phenotype is produced by a number of distinct mutant loci within a given species. In maize, for example, 10 mutant loci on 8 different nuclear chromosomes each produce the virescent phenotype. In a few rare instances, virescent mutations have been reported which were maternally inherited. Presumably these were associated with the chloroplast genome (20, 26). The existence of both nuclear and cytoplasmic mutations producing the same phenotype supports the view that virescent mutants comprise a diverse group, in which each distinct mutant locus disturbs a specific factor regulating Chl content.

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We report here a new, maternally-inherited virescent mutation in tobacco. We analyze several possible influences on Chl content and show that the ability to synthesize and protect Chl is not affected in this mutant. Instead, the defect results in an inability to accumulate Chl at the appropriate stage in leaf development. This inability is correlated with a severe reduction in thylakoid membranes, the site where Chl is normally found. Recovery of more normal Chl levels in older leaves is associated with the appearance of normal thylakoid structure. These findings are discussed in relation to virescent mutations previously described.

MATERIALS AND METHODS

Plant Material. The virescent mutant, designated Vir-c, was found in a line of plants derived from fusion of a protoplast from a haploid *Nicotiana tabacum* L. var "Gatersleben" suspension culture with a protoplast from a male-sterile plant which had *N. tabacum* chromosomes and *Nicotiana suaveolens* Lehm. cytoplasm (14). Thus, the fusion product had a triploid complement of *N. tabacum* chromosomes and a mixed population of both *N. tabacum* and *N. suaveolens* organelles.

The plant regenerated from this fusion product was self-sterile. Viable seed was obtained by pollinating the regenerated plant with *N. tabacum* L. var "Turkish Samsun" ("Samsun") pollen. Cross-pollination with "Samsun" pollen was continued for three generations. This line of plants was shown to have *N. tabacum*-type chloroplasts by marker analysis (14). Seed from the third and fourth generation after fusion was used in the work discussed here.

Plant material used in experiments was grown under greenhouse conditions unless otherwise specified. Seeds were sown in vermiculite and seedlings were potted in soil approximately 4 weeks after germination. Sunlight was supplemented with a bank of lights containing Sylvania cool-white fluorescent lamps and Sylvania Spot-Gro 150 W incandescent lamps. The light bank was on for 16 h/d. For uniformity, half-expanded leaves were used in experiments requiring young leaf tissue. Half-expanded leaves were compared to mature leaves which had stopped expanding and were generally three to four nodes below the half-expanded leaf. Plants used in experiments had 10 to 12 nodes, counting from the half-expanded leaf toward the base.

Chl Analysis. Samples 1 cm in diameter were taken with a cork borer from intercostal regions in the middle of the leaf. The leaf pieces were weighed and then ground with 3 ml of 80% acetone. The O.D. of the clarified solutions was measured at 645 and 663 nm, and the quantities of Chl *a*, Chl *b*, and total Chl were calculated from the equations of Arnon (2) in μg/ml. Chl per chloroplast was estimated from isolated chloroplasts by counting the number of chloroplasts in a known volume, extracting the Chl with 80% acetone and calculating Chl amounts as described previously.

Electron Microscopy. Leaf pieces from Vir-c and "Samsun" plants were fixed in glutaraldehyde, post-fixed in osmium tetraoxide, and embedded in a mixture of Epon and Araldite.

Sections were stained and viewed in a Phillips 300 electron microscope.

Chloroplasts per Cell. Chloroplasts per cell were estimated by a modification of the method of Lamppa *et al.* (17). Protoplasts were prepared from leaves, washed free of debris, and fixed at least 1 h in a phosphate buffer solution containing 1.25% glutaraldehyde (v/v) and 0.2 M sorbitol. A drop of the protoplast suspension was placed on a glass slide and covered with a glass cover slip. Gentle pressure flattened the protoplasts and produced a monolayer of easily countable chloroplasts.

Cell Numbers per Unit Leaf Volume. Cells per unit volume and cells per unit fresh weight were estimated from 1 cm diameter leaf discs which were weighed on a Mettler type 15 balance and placed in 5% chromic acid (v/v). After 48 to 60 h cells had dissociated. Aspiration of the cell suspension with a syringe equipped with a 25 gauge needle dispersed any remaining cell clumps. Cells were counted in a hemocytometer. Samples taken from the same leaf for the estimation of dry weight were weighed on a Mettler microbalance, dried for 24 h at 80°C, and weighed again. Sample volumes were calculated using the formula for a cylinder; $\pi r^2 h$. Radius of the samples was 5 mm, and the average height (thickness) was estimated from measurement of free-hand longitudinal sections using an ocular micrometer.

Carotenoid Analysis. The extraction of carotenoids was based on the method of Davies (8). One g of half-expanded leaf tissue was ground in cold 100% acetone. The brei was filtered and the homogenized tissue washed five times with fresh acetone. The pigments were transferred to 100% diethyl ether in a separatory funnel and dried down with a rotary evaporator. The pigments were taken up in absolute ethanol and saponified overnight in 60% KOH (w/v). The pigments were transferred to ether, washed with water until the pH was 7 to 8, dried down, and taken up in hexane. The absorption spectrum for the hexane solution was determined on a Cary model 15 recording spectrophotometer. The relative amounts of carotenoids were compared using the optical density at 441 nm, the absorption maximum.

Pchlide Accumulation. Vigorous plants 10 to 15 nodes tall were cut back to four to five nodes and placed in the dark for 9 d. Axillary buds produced etiolated shoots, which were harvested, weighed, and incubated in 0.2 M sucrose solutions containing 0.01 M ALA[2] for 26 h. Control shoots were incubated in 0.2 M sucrose without ALA.

After incubation, shoots were quick-frozen in liquid N_2. The tissue was ground to a powder in a mortar and pestle and then extracted with 80% ice-cold acetone. The O.D. of the acetone extracts at 623 nm was determined with a Gilford-modified Beckman spectrophotometer, and the quantity of Pchlide measured as described by Troxler *et al.* (25), using the extinction coefficient determined by Falk (10).

Measurement of Photosynthetic Rates. A 1 cm^2 sample was cut into 1 mm wide strips with a razor blade. The strips were washed with a solution containing 0.4 M sucrose, 10 mM NaCl, and 20 mM Hepes-KOH (pH 7.4) and then incubated in fresh solution for 20 to 30 min at a light intensity of 1.4×10^{15} quanta/$cm^2 \cdot s$. The pieces, along with 4 ml of nitrogen-bubbled 20 mM $NaHCO_3$, were transferred to a water-jacketed glass chamber and allowed to equilibrate for 15 min in the dark at 25°C. Light was directed on the chamber using a Leitz slide projector equipped with an Osram Longlife 250 W lamp. O_2 evolution and consumption were measured with a Clark-type O_2 electrode.

Isolation of Chloroplasts. Three to 4 g of half-expanded leaf tissue was de-ribbed and plunged into ice water. The leaves were chopped in grinding buffer containing 2.0 mM NaEDTA, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$, 350 mM sorbitol, 0.5% BSA (w/v), 4.0 mM Na ascorbate, and 50 mM Hepes-KOH at pH 8.3 (11).

[2] Abbreviation: ALA, δ-aminolevulinic acid.

The brei was filtered through four layers of cheesecloth, the filtrate layered above 40% Percoll (Pharmacia Fine Chemicals, Sweden) in grinding buffer, and centrifuged for 2 min at 1,200*g*. The pellet was gently suspended in resuspension buffer containing 375 mM sorbitol, 2 mM Na EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.96 mM DTT, and 35 mM Hepes-KOH (pH 8.3). This procedure routinely produced 90 to 95% intact chloroplasts as judged by refractility with phase contrast microscopy.

RESULTS

Genetic Analysis. Reciprocal crosses were made between the mutant, Vir-c, and normally-pigmented *N. tabacum* L. var "Turkish Samsun" ("Samsun"). Seeds were sown in large flats and seedlings scored 10 d after germination. When the mutant was used as the female in the cross, all progeny were mutant (Table I). When the female was normally pigmented "Samsun," all progeny had normal pigmentation. Selfing of the F_1 and F_2 generations always produced progeny of the same pigment type as the original parental female.Thus, the mutation was maternally inherited and stable through subsequent generations.

Chl Content and Leaf Development. Chl content in Vir-c and "Samsun" was compared starting with immature leaves at the apex and progressing down the stem. For "Samsun," Figure 1 shows an increasing rate of Chl accumulation in the youngest leaves, followed by a linear rate, reaching peak Chl content in

Table I. *Results of Reciprocal Crosses of Vir-c with N. tabacum L. var "Turkish Samsun," with the Phenotypes of the F_2 and F_3 Generations*

Cross	Generation	No. of Virescent Seedlings	No. of Normal Seedlings
Vir-c × "Samsun"	F_1	422	0
F_1 self	F_2	915	0
F_2 self	F_3	760	0
"Samsun" × Vir-c	F_1	0	401
F_1 self	F_2	0	743
F_2 self	F_3	0	838

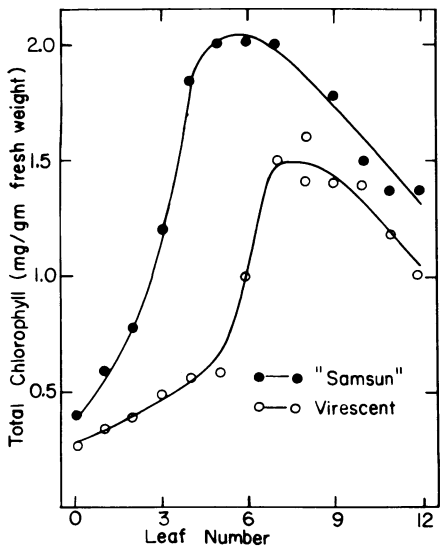

FIG. 1. Chl content of "Samsun" and Vir-c plants grown under identical conditions. Leaf numbers are from 1, the most apical leaf (less than 1 cm long), to the 12th leaf on each plant. The values at "leaf 0" represent Chl content in the apical bud above leaf 1. Leaves 2, 5, and 8 represent the three stages studied by EM. Leaf 5 is at the half-expanded stage and leaf 8 is fully expanded.

leaves which were half-expanded (leaf No. 5). In Vir-c, the initial rate of Chl accumulation was much slower until the half-expanded leaf stage, but was then followed by the same rapid rate of Chl accumulation as observed for the linear phase of "Samsun." The peak in Chl content for Vir-c occurred in the nearly fully-expanded leaf (leaf No. 7), two leaves later in development compared to "Samsun."

In the plants examined for Figure 1, the half-expanded "Samsun" leaf contained over three times the amount of Chl present in the half-expanded Vir-c leaf. Other experiments showed as much as six times more Chl in "Samsun" compared with the mutant. Comparison of the two at maximum Chl content for both showed a 1.3 to 1 difference between the "Samsun" and the Vir-c plants. In both plant types Chl content declined as leaves began to senesce.

Chl Synthesis. The difference in Chl content in young leaves of the virescent mutant and "Samsun" might result from an early depression in the rate of Chl biosynthesis in Vir-c. This hypothesis is difficult to test by simply measuring Chl content over time, since the presence of Chl depends not only on the pool of pathway intermediates, but also on the availability of Chl binding proteins (24). An alternative is to measure Pchlide, an intermediate in biosynthesis occurring near the end of the pathway. Conversion of Pchlide to the next intermediate requires light; therefore, the net rate of Pchlide synthesis can be determined by feeding young, dark-grown leaves with ALA, an intermediate in Chl biosynthesis occurring at the beginning of the pathway, and examining the amount of Pchlide accumulated in the dark in a given period.

Table II shows that endogenous levels of Pchlide were similar for Vir-c and "Samsun" (sucrose control). When fed with exogenous ALA, the amounts of Pchlide accumulated in 26 h were also very similar for Vir-c and "Samsun." Pchlide amounts accumulated in 5 and 12 h were close to endogenous levels in both plant types (data not shown). Thus, Chl synthesis in young leaves, measured in terms of Pchlide accumulation, was not affected by the mutation.

Carotenoid Content. Since the rate of Chl synthesis in Vir-c appeared similar to "Samsun," the carotenoid content was examined because of the role carotenoids play in protecting Chl from photooxidation (12). Reduced carotenoid pigment content could expose a like proportion of Chl molecules to destruction by light. Total carotenoids were extracted from half-expanded leaves of Vir-c and "Samsun" and compared on the basis of O.D. units at the absorption maximum. Table III presents the results from two experiments.

Total carotenoids per g fresh weight were three times lower in the mutant than in "Samsun," while mutant Chl content in these two experiments was about six times lower. Therefore, although total carotenoid levels in Vir-c were reduced, there was about two times more carotenoid pigment relative to Chl than in "Samsun." This suggests that while carotenoids were reduced in Vir-c, there was enough to protect twice as much Chl as was actually present. The reduction in carotenoid levels must be a consequence of the mutation, but not the cause of decreased Chl levels.

Table II. *Accumulation of Pchlide in Dark-Grown Leaves of Vir-c and "Samsun"*

Etiolated axillary shoots were placed in either 0.2 M sucrose (control) of in 0.2 M sucrose with 0.01 M ALA for 26 h. Pchlide in the leaves was then extracted and measured spectrophotometrically.

Experiment	0.2 M Sucrose		0.01 M ALA in 0.2 M Sucrose	
	Vir-c	"Samsun"	Vir-c	"Samsun"
	μg/g fresh weight			
1	4.95	5.35	19.13	14.94
2	8.49	4.25	13.37	14.37

Table III. *Carotenoid Content in Half-expanded Leaves of Vir-c and "Samsun" (in O.D. units)*

	Experiment	Carotenoids *per g fresh weight*	Carotenoids *per mg Chl*
"Samsun"	1	60.1	22.7
	2	44.6	22.5
Vir-c	1	20.5	63.3
	2	15.1	44.4

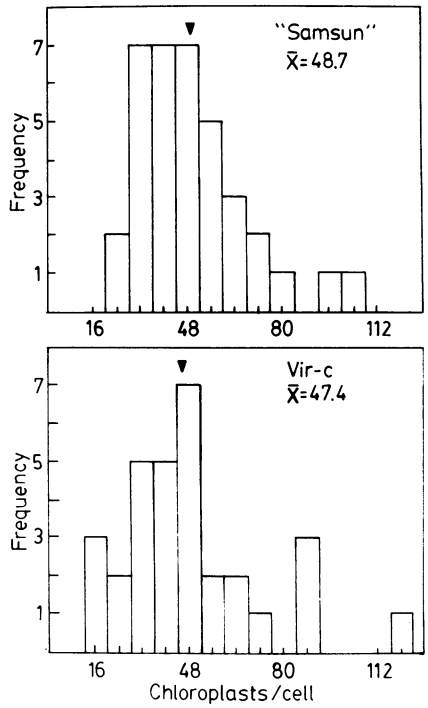

FIG. 2. Chloroplasts per cell in half-expanded leaves of "Samsun" and Vir-c. The data are from a frequency distribution. Abscissa divisions are the midpoints of the class intervals, with an interval width of 8. The arrows point to the mean values.

Chloroplasts per Cell. Chl content could also be affected by reducing the number of sites where Chl accumulates. On a simple level this could be achieved with slower chloroplast reproduction rates in the mutant, producing young leaves with fewer chloroplasts per cell. To account for recovery, chloroplasts would be predicted to continue reproducing until the wild-type number per cell was attained.

To compare chloroplast numbers on a per cell basis, cells must be of approximately the same size, since Reiss (21) and Frandsen (13) have shown that average chloroplast numbers per cell increase as cell volume increases. Average cell volumes of leaf cells from Vir-c and "Samsun" plants were sufficiently alike so that the comparison of chloroplasts per cell was valid (data not shown).

The histogram in Figure 2 shows the number of chloroplasts per cell in half-expanded leaves of Vir-c and "Samsun" were quite similar, and indicated that the difference in Chl content was not due to a decrease in chloroplast numbers per cell. In recovered leaves chloroplast numbers also were found to be similar for Vir-c and "Samsun" (data not shown).

When Chl content per chloroplast was examined, chloroplasts in half-expanded Vir-c leaves contained four to five times less

Chl than "Samsun" (Table IV). Chl content in chloroplasts of mature leaves of both plants was similar. Chl content per chloroplast thus reflected the same magnitude of deficiency observed in leaf tissue, and confirmed that reduction in Chl content was not due to fewer chloroplasts.

Thylakoid Ultrastructure. Within the chloroplast, Chl is accumulated in the thylakoid membranes. A reduction in thylakoid membranes then would also reduce Chl accumulation sites. Electron micrographs of chloroplasts from half-expanded leaves of Vir-c showed a distinct decrease in thylakoid membranes, predominantly in the granal stacks (Fig. 3). Granal profiles in chloroplasts from a half-expanded "Samsun" leaf averaged 10.1 thylakoids, compared to 5.7 thylakoids in Vir-c grana (Table V).

Micrographs of recovered Vir-c leaves (leaf No. 8, Fig. 1) and "Samsun" leaves of the same age showed similar numbers of thylakoids per granum, correlating more normal thylakoid profiles in Vir-c with recovery of more normal Chl levels. Micrographs of chloroplasts from very young leaves (leaf No. 2, Fig. 1) showed almost no differences in the size of granal profiles. Figure 1 also shows that at this stage there is less difference in Chl content, again linking thylakoid development with Chl levels.

Table IV. *Chlorophyll Content of Chloroplasts from Vir-c and "Samsun" Leaves*

Experiment	Half-Expanded Leaves		Mature Leaves	
	Vir-c	"Samsun"	Vir-c	"Samsun"
	ng Chl/10^5 chloroplasts			
1	11.5	61.2	49.9	59.4
2	11.0	49.2	46.9	42.5

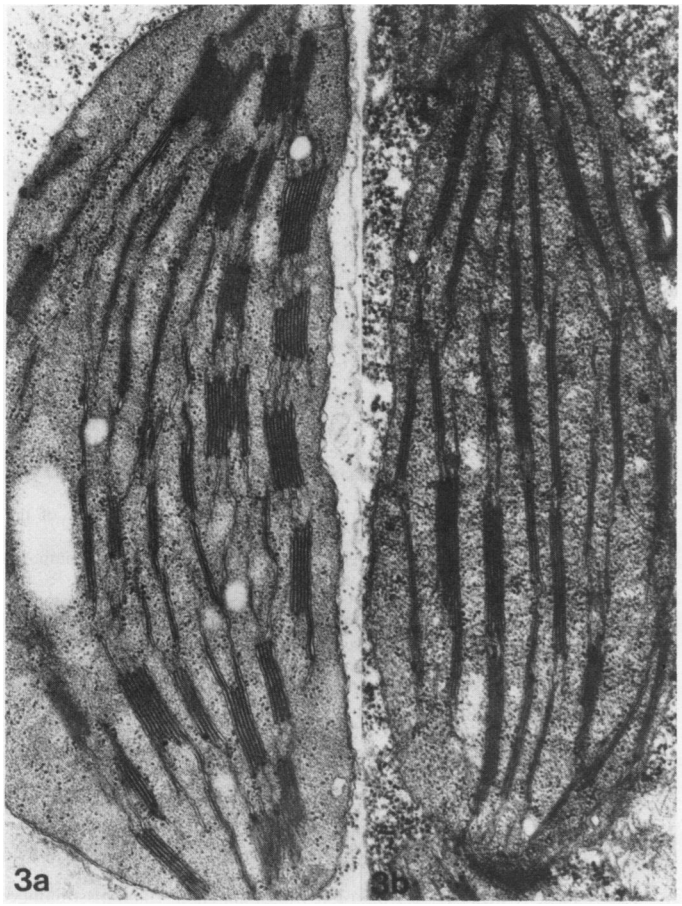

FIG. 3. Representative chloroplasts from leaf number 5 of a "Samsun" (a) and a Vir-c (b) plant. The only obvious difference in structure was in the degree of stacking of thylakoids, which was much more prevalent in the "Samsun" chloroplast than in the mutant. a, ×26,000; b, ×32,000.

Table V. *Thylakoid Profiles in Grana of Vir-c and "Samsun" Chloroplasts at Three Stages in Leaf Development*

Data are means of the number of thylakoid profiles per granum. The five largest grana were counted in each chloroplast section. Grana were counted in 6 to 10 different chloroplasts for each datum point. Data for half-expanded leaves are different at the 99% level of significance.

	Leaf Stage		
	Immature	Half expanded	Fully expanded
Vir-c	5.8	5.7	18.4
"Samsun"	5.6	10.1	16.3

Table VI. *Photosynthetic Rates for Half-expanded Leaves of Vir-c and "Samsun"*

Leaf strips were agitated in a 20 mM $NaHCO_2$ solution in a water-jacketed glass chamber at 25°C. O_2 evolution was measured with a Clark-type O_2 electrode.

	Saturating Light[a]	
	Rate/mg dry weight	Rate/cm^3 leaf tissue × 10^4
	μmol of O_2 evolved/h	
Vir-c	0.606 ± 0.110	2.259 ± 0.765
"Samsun"	0.477 ± 0.115	2.302 ± 0.273
	Low Light[b]	
	Rate/mg dry weight	Rate/cm^3 leaf tissue × 10^4
Vir-c	0.081 ± 0.074	0.457 ± 0.312
"Samsun"	0.231 ± 0.061	1.164 ± 0.714

[a] 2.5×10^{17} quanta/$cm^2 \cdot s$. [b] 7×10^{15} quanta/$cm^2 \cdot s$.

Photosynthesis. Since the thylakoid is the site of the light reactions of photosynthesis, the major reduction in thylakoids observed in Vir-c might be expected to affect the photosynthetic capability of the mutant. We determined that the photosynthetic rate in half-expanded Vir-c leaves saturated at a higher light intensity than in "Samsun" leaves. The half-saturation intensity for Vir-c was 2.7×10^{16} quanta/$cm^2 \cdot s$, compared to 0.94×10^{16} quanta/$cm^2 \cdot s$ for "Samsun." At a light intensity which saturated photosynthesis in both plants, the photosynthetic rates were very similar, when expressed on the basis of dry weight, or on the basis of unit leaf volume (Table VI). When expressed on the basis of Chl, the rate at saturation in Vir-c exceeded the "Samsun" rate, due to the approximately 5-fold difference in Chl content. The photosynthetic rates at low light intensity were noticeably different, with the mutant photosynthesizing at a much lower rate than "Samsun."

Coupled with the requirement for a higher light intensity to saturate the light reactions, the poorer photosynthetic rate at low intensities suggested that the light collecting ability of Vir-c was reduced.

Ratio of Chl *a* to Chl *b*. The Chl *a* to *b* ratio in half-expanded Vir-c leaves was 3.326 ± 1.28, compared to 3.158 ± 1.71 for half-expanded "Samsun" leaves. These ratios are very similar; moreover, no significant differences in Chl *a* to *b* ratios were detected at any of the developmental stages examined in Figure 1. Thus, mutant leaves did not sustain a major shift in the levels of Chl *a* relative to Chl *b*.

DISCUSSION

Whereas most virescent mutations are single-gene, recessive, nuclear mutations, the Vir-c mutation is maternally inherited. Chloroplasts and mitochondria are both maternally inherited in tobacco, but since mitochondria have never been shown to affect Chl accumulation, the most likely site of the mutation is the chloroplast genome.

The Vir-c mutation produced a pattern of Chl accumulation very similar to patterns found in nuclear virescent mutations. Machlachlan and Zalik (18) studied the nuclear virescent of barley and observed that maximum pigment levels were reached 2 to 3 d later than in the wild-type barley. In a virescent nuclear mutant of maize (16), peak Chl content also occurred about 2 d later than in the wild type. Once the rapid rise in Chl content began, the maize mutant, the barley mutant, and the tobacco mutant described here all demonstrated rates of accumulation similar to the wild type. In common to all these mutants was a lag in the rate of Chl accumulation which persisted to a significantly later stage in leaf development in comparison to wild-type plants.

The lag period in Vir-c was not due to a defect in the Chl biosynthetic pathway prior to synthesis of Pchlide. Similar results were obtained for another maternally inherited virescent mutation in soybean (20). These findings are not surprising, as most enzymes in the pathway have been shown to have Mendelian inheritance, although a chloroplast gene product could be necessary for normal pathway function.

Most nuclear virescent mutants also share with Vir-c the characteristic of decreased carotenoid levels in the young leaves. Typically, the carotenoid content in virescent leaves decreases about 2-fold, while Chl content decreases 3- to 5-fold (5, 6, 15, 23). Although the total amount of carotenoid pigments decreases, the ratio of carotenoids to Chl actually increases in virescent mutants and appears to be ample for Chl protection.

The bulk of carotenoid pigments in photosynthetic tissue is located in the thylakoid membranes (9). If carotenoids and Chl cannot accumulate when membrane binding sites are reduced, as suggested by Ogawa (19), then the reduced pigment levels in Vir-c may result from a delay in thylakoid development. Electron microscopic examination revealed significantly fewer thylakoids per granum in half-expanded leaves of Vir-c than in "Samsun." The reduction in thylakoid stacking was consistent with the reduction in Chl content shown by Vir-c. Thylakoid stacking in younger leaves showed no difference between Vir-c and "Samsun," and Chl levels were similar as well. A comparison of mature mutant leaves, which had recovered nearly normal Chl levels, with mature "Samsun" leaves showed that the degree of thylakoid stacking was indistinguishable. These observations linked the reduction in granal thylakoids specifically with the delay in Chl accumulation, and suggested that the mutation affected the timing of the thylakoid development.

Poor development of grana is the most frequently reported structural aberration in virescent mutants. In a nuclear virescent of cotton, the pale green leaves showed fewer thylakoids per granum than observed for the wild-type, while normal thylakoid stacking was found in mature, greened leaves (4). Other nuclear virescent mutants showing reduced thylakoid stacking per granum were peanut (3) and *Phaseolus* (7). Fewer thylakoids per granum could also be seen in electron micrographs of chloroplasts from the maternally inherited virescent of soybean (20). An interesting exception was the virescent corn of Chollet and Paolillo (6). Low Chl content in this mutant was associated with large grana and a poorly developed fretwork of stromal thylakoids. Normal thylakoid structure developed as Chl accumulated. Virescent mutations thus appear to affect thylakoid development, most by delaying development of granal stacks.

Fewer granal thylakoids in Vir-c in comparison to "Samsun" was associated with reduced light-gathering capability as shown by poorer photosynthetic rates at low light intensities, and by a higher light requirement for saturation. In contrast to Vir-c, the virescent corn mutant described by Chollet and Paolillo (6) required less light to saturate the light reactions, indicating photosynthetic units were larger, or more numerous, or both. Photosynthetic rates were also different in various virescent mutants. For example, the nuclear virescent mutant of cotton photosynthesized at rates very similar to the wild type, whether light intensity was low or high (5), while the virescent mutant of peanut had a lower photosynthetic rate (1, 15). Thus, virescent mutations, although similar in phenotype, have varied effects on photosynthesis. These effects may reflect alteration of a different photosynthetic component in each virescent mutant. Such a conclusion is supported by the Chl *a* to Chl *b* ratios reported in different mutants. Some mutants have ratios higher than the wild type (1), others have lower ratios (5), and some like Vir-c are unchanged.

If several different proteins are required for the normal sequence of thylakoid stacking, defects in any one of them could lead to a reduction in thylakoid stacking and an inability to accumulate photosynthetic pigments. Specific effects on photosynthetic activity would depend on the role of the defective protein. The virescent phenotype may result from mutations within the structural gene of such a protein, with recovery from the mutant phenotype awaiting synthesis of other gene products of similar function later in leaf development. Alternatively, virescent mutations may be regulatory mutations controlling the timing of gene expression and, as such, may provide a genetic tool to understand the regulation of chloroplast development.

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