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# Regreening of senescent *Nicotiana* leaves

# II. Redifferentiation of plastids

**H.A. Zavaleta-Mancera[1,2,3], B.J. Thomas[1], H. Thomas[1] and I.M. Scott[2,4]**

[1] *Institute of Grassland and Environmental Research, Aberystwyth SY23 3EB, UK*

[2] *Institute of Biological Sciences, University of Wales Aberystwyth, Aberystwyth SY23 3DA, UK*

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## Abstract

**Single senescent leaves attached to decapitated shoots of *Nicotiana rustica* L. regreened, especially when treated with cytokinin. Regreening caused an increase in leaf thickness, due to cell expansion. Senescent leaf plastids (gerontoplasts) were smaller than green chloroplasts, with degenerated membrane systems and stroma, and larger plastoglobuli. At advanced senescence, micrographs showed disintegrating gerontoplasts, reduced numbers of plastids were counted, and regreening became variable. The redevelopment of grana and stroma in regreening plastids was accelerated by cytokinin. All plastids in regreening leaves were identifiable as redifferentiating gerontoplasts because of their content of plastoglobuli and starch. Immunogold labelling showed significant association of POR with etioplasts in cotyledons, but with mature plastids in regreening leaves. No proplastids or dividing chloroplasts were observed in regreening leaves. Plastid numbers declined during senescence and did not increase again during regreening. It is concluded that the chloroplasts of regreening leaves arose by redifferentiation of gerontoplasts.**

Key words: Chloroplasts, cytokinin, Nicotiana, senescence, regreening.

## Introduction

It has already been shown that regreening could be induced in yellow leaves of *Nicotiana rustica* (Zavaleta-Mancera *et al.*, 1999). The reappearance of chlorophyll (Chl) correlated with the reappearance of the Chl biosynthesis enzyme, NADPH-protochlorophyllide oxidoreductase (POR). Development of functional thylakoid membranes during regreening was indicated by the reappearance of the thylakoid component, light-harvesting chlorophyll *a*/*b*-binding protein. The nature of the plastids in which these regreening events occur is explored in the present paper.

Senescent leaves contain partially dismantled chloroplasts that function to salvage or compartmentalize degraded material. These senescent plastids are sometimes called 'chromoplasts' because of their content of residual pigments (Tuquet and Newman, 1980; Thomson and Platt-Aloia, 1987). However, the term 'gerontoplast' has been advocated to designate the specific plastid development of senescing leaves (Matile, 1992; Thomas, 1997). Gerontoplasts and chromoplasts have ultrastructural features in common, such as a reduced thylakoid system and prominent plastoglobuli. However, chromoplasts that develop from proplastids, amyloplasts or young chloroplasts retain the capacity for division and biosynthesis, whereas gerontoplasts have been suggested to have lost these capacities. Reported characteristics of gerontoplasts include reductions in size, electron density and buoyant density, along with loss of ribosome and DNA contents (Matile, 1992).

The question of whether gerontoplasts can contribute to leaf regreening has therefore not been settled. Although early ultrastructural studies of *N. rustica* leaves suggested that plastids of senescent leaves could redifferentiate and even increase in numbers during regreening (Kursanov

[3] Present address: Centro de Botánica, Colegio de Postgraduados, 56230 Chapingo, Mexico.

[4] To whom correspondence should be addressed. Fax: +44 1970 622350. E-mail: ias@aber.ac.uk

Abbreviations: BAP, 6-benzylaminopurine; Chl, chlorophyll; EM, electron microscopy; FW, fresh weight; POR, NADPH-protochlorophyllide oxidoreductase.

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*et al.*, 1964; Sveshnikova *et al.*, 1966; Ljubesic, 1968; Butler and Simon, 1971), the concept of plastid dedifferentiation and redifferentiation remained a minority viewpoint (Whatley, 1978). Chloroplasts in regreened soybean cotyledons have been shown to originate both from a separate proplastid residue present in mature cells, and by redifferentiation of slightly senescent plastids (Tuquet and Newman, 1980). The present study monitored cellular changes in regreening *N. rustica* leaves to establish the origins of their plastid populations.

## Materials and methods

### Plant material

*Nicotiana rustica* L. plants were grown and single senescent leaves regreened on decapitated shoots as described previously (Zavaleta-Mancera *et al.*, 1999). At each stage of development, samples of tissue were taken from between the 2nd and 3rd lateral veins of the sixth leaves, avoiding vascular tissue. Green tissue was from 10-week-old plants. Progressively advancing stages of senescence were designated from S-1 (yellow leaf tissue from 14-week-old plants with inflorescence buds) to S-3 (yellow-white leaf tissue from 16-week-old plants with flowers in anthesis). Regreening was started from S-1 unless stated, and leaf tissues were sampled 6, 13 and 20 d after decapitation of the plant. Regreening leaves were treated with benzylaminopurine (BAP) or control spray and kept under low light (12 $\mu mol\ m^{-2}\ s^{-1}$) (Zavaleta-Mancera *et al.*, 1999). In a further experiment, plants regreened for 20 d were held for a further 6 weeks in increased light of 100 $\mu mol\ m^{-2}\ s^{-1}$.

Relative Chl contents of tissue samples for microscopy were measured with a Minolta SPAD-502 Chlorophyll Meter. When absolute Chl values are given, these were obtained by calibration of the SPAD-502 meter as described previously, except for tissues below the meter detection limits (e.g. S-3 leaves), when Chl extracts were measured (Zavaleta-Mancera *et al.*, 1999).

### Microscopy

Tissue pieces (approximately 1.5 $mm^2$) were infiltrated under mild vacuum for 15 min in fixative (67 mM sodium cacodylate buffer containing pH 7.4, 5% (w/v) glutaraldehyde, 2% (w/v) paraformaldehyde), then kept in fresh fixative at 4 °C for 16 h. After two 1 h washes in cacodylate buffer, the tissue was post-fixed in 1% osmium tetroxide in cacodylate buffer for 16 h at 4 °C. After two further 30 min washes in cacodylate buffer and one in double-distilled water, the tissue was dehydrated in a graded ethanol series and the procedure of Xu and Mendgen was used for infiltration with LR White resin (The London Resin Co., UK) (Xu and Mendgen, 1994). The samples were transferred to BEEM capsules and the resin was polymerized at 60 °C for 24 h under nitrogen.

Semi-thin (1 μm) leaf cross-sections, cut with glass knives on a Reichert–Jung ULTRACUT Ultramicrotome, were used for light microscopy or tissue identification prior to electron microscopy (EM). Light microscopy sections (five from each of four leaves) were stained with a 1 : 1 aqueous mixture of 1% methylene blue (in 1% sodium borate) and 1% azure II. Randomized determinations of tissue or cell dimensions, or plastid numbers per cell cross-section, were replicated 15 times for each leaf ($n=60$). Ultrathin sections (silver/gold) for EM were collected on copper grids with a support film of formvar and carbon, which were glow discharged to assist section recovery and adhesion. The sections were counter-stained with 2% (w/v) uranyl acetate for 15 min followed by lead citrate solution (Reynolds, 1963) for 3 min, and examined in a JEOL JEM 1010 electron microscope (JEOL, Tokyo) at 80 kV. Cross-sectional areas of organelles were calculated by weighing EM images on photographic paper of known weight per unit area.

### Immunocytochemical labelling

Tissue pieces (approximately 1.5 $mm^2$) were fixed for 16 h in cold (2 °C) 67 mM sodium cacodylate buffer (pH 7.4) containing 1% (w/v) glutaraldehyde and 4% (w/v) paraformaldehyde, then given 1 h washes in cold cacodylate buffer (twice) and double-distilled water. The samples were dehydrated in a graded ethanol series, while lowering the temperature to −27 °C, and infiltrated with LR White resin (The London Resin Co., UK) at −27 °C and then finally −20 °C (Xu and Mendgen, 1994). Following three 12 h incubations at −20 °C in resin containing 0.1% (w/v) benzoin ethyl ether (Aldrich, UK), the resin was polymerized by UV (10 cm from a 6 W 360 nm light tube) at −20 °C for 24 h and at 10 °C for a further 24 h. Ultrathin sections (silver/gold) were cut with a Reichert–Jung ULTRACUT ultramicrotome using a glass knife and collected on nickel grids with a support film of formvar and carbon. Grids were glow discharged to assist section recovery and adhesion.

For immunogold labelling, grids were moistened on SSC-T (0.15 M NaCl, 15 mM sodium citrate pH 7.2, 0.015% (v/v) Tween-20) for 30 min, transferred for 1 h to CTM-T blocking buffer, consisting of CTM buffer (Kenna *et al.*, 1985) with 0.015% (v/v) Tween-20, and then incubated overnight at 4 °C on primary antibody (Zavaleta-Mancera *et al.*, 1999) diluted 1 : 100 in CTM-T. The grids were washed with double-distilled water, re-incubated on CTM-T for 25 min, treated for 2 h with EM : GAR15 goat anti-rabbit immunoglobulin conjugated with 15 nm colloidal gold (British BioCell International, UK) diluted 1 : 50 in CTM-T, and then washed by floating on five drops of SSC-T for 3 min each, and then five drops of double-distilled water for 3 min each. Sections were counter-stained and examined by EM as described above. Controls to ensure the specificity of the primary antibody were as described previously (Gordon *et al.*, 1992). Numbers of gold particles per $\mu m^2$ were counted on weighed EM images of organelles on photographic paper of known weight per unit area.

### Statistical analysis

Data were assessed by analysis of variance and Duncan's Multiple Range Test (Duncan, 1955).

## Results

Senescent leaves and regreened leaves were, respectively, 15% and 48% thicker than green leaves, due to increased size, rather than numbers, of mesophyll cells (Table 1). Following senescence and regreening, the palisade mesophyll remained as a single layer of cells, which were 40% longer. While the numbers of cells crossed by transects through the spongy mesophyll remained unchanged (3–5), the thickness of this tissue was 32% and 62% greater in senescent leaves and regreened leaves, respectively (Table 1). All tissue sections were examined without success for new cell wall formations as evidence of cell division.

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**Table 1.** *Changes in tissue and cell dimensions following senescence and regreening for 20 d*

Values are means ± SE ($n = 60$, consisting of 15 replicated measurements on each of four leaves). Within each column, data with different letters are significantly different ($P < 0.05$).

| Leaf stage | Mean leaf thickness (μm) | Mean palisade cell length (μm) | Mean thickness of spongy mesophyll (μm) | No. of cells across spongy mesophyll |
|---|---|---|---|---|
| Green | 386 ± 7 a | 143 ± 9 a | 189 ± 0.7 a | 3–5 |
| Senescent | 445 ± 12 b | 156 ± 11 a | 250 ± 13 b | 3–5 |
| Regreened | 569 ± 11 c | 200 ± 8 b | 307 ± 10 c | 3–5 |

Pre-senescent green leaves, with a mean Chl content of 0.65 mg $g^{-1}$ FW, contained chloroplasts with organized grana and an electron-dense stroma (Fig. 1A). Plastids of yellow leaves (0.07 mg Chl $g^{-1}$ FW) at senescence stage S-1 were gerontoplasts with a rounder cross-section, with reduced and disorganized membrane systems forming sheets or vesicles, and a flocculent stroma (Fig. 1B). Gerontoplasts were smaller than the green chloroplasts (Table 2). Plastoglobuli, which in green chloroplasts had diameters of 150–450 nm, were generally larger (300–800 nm) in gerontoplasts. Consequently, although plastoglobuli decreased in number during senescence, their total cross-sectional area and the proportion of the plastid they occupied increased (Table 2). Starch grains were conspicuous in plastids from both the green and senescent leaves (Fig. 1A, B; Table 2). Nuclei of senescent leaf cells retained an intact double membrane, though their chromatin was less electron-dense than in the green leaf cells.

Micrographs of cells at advanced senescence stage S-3 (0.026 mg Chl $g^{-1}$ FW) commonly revealed disintegrating plastids, and released plastoglobuli forming clumps of osmiophilic material in the cytoplasm (Fig. 1C). The nuclear membrane in these cells was still continuous. Regreening occurred when plants with leaves at the S-1 stage of senescence were decapitated, treated with BAP, and kept in diffuse light (Zavaleta-Mancera *et al.*, 1999). After 6 d of this regime, mean leaf Chl content had recovered to 0.13 mg $g^{-1}$ FW, while the plastids increased in size (Table 2) and showed structural changes. The regreening leaf plastids had features characteristic of gerontoplasts, including a rounded shape and large plastoglobuli (Fig. 1D). They also contained large starch grains, as in green or senescent leaves. Other features, however, were indicative of redeveloping chloroplast activities, including a more electron-dense and granular stroma, and the appearance of new membranes, mostly single swollen thylakoids, though in some plastids grana were starting to form (Fig. 1D). As a result, the proportion of plastid area occupied by plastoglobuli decreased (Table 2). Plastid redifferentiation appeared to be more advanced in the spongy mesophyll than in the palisade cells.

After 13 d of regreening, mean leaf Chl content had increased to 0.28 mg $g^{-1}$ FW. Plastids had differentiated granal and intergranal membranes, but while some grana were compact, others appeared loose (Fig. 1E). In the more redifferentiated plastids, grana tended to be more parallel to the long axis of the plastid. At this stage, plastid development appeared similar in spongy mesophyll and palisade cells. The plastids in these regreening leaves still contained large and numerous plastoglobuli as seen in gerontoplasts.

After 20 d, the regreened leaves had a similar mean Chl content (0.66 mg $g^{-1}$ FW) to the pre-senescent green leaves, but their plastids were recognizably different (Fig. 1F). The regreened plastids were flattened and elongated, some having irregular shapes, while plastoglobuli remained conspicuous and as large (up to 850 nm diameter) as those of the senescent leaf gerontoplasts. In other respects the regreened plastids had typical chloroplast features: the stroma was granular and highly electron-dense, while the thylakoid membranes were oriented parallel to the plastid long axis, in compact, well-formed grana without the swelling seen at earlier stages of regreening. By this stage of the regreening process, there was a dramatic reduction in starch and the plastids had reduced cross-sectional areas (Table 2). When fully regreened plants were transferred into light of 100 μmol $m^{-2}$ $s^{-1}$ for 6 weeks, the leaf plastids re-accumulated starch, while the total cross-sectional area of plastoglobuli was reduced (Table 2).

The pattern of plastid differentiation in decapitated plants without BAP treatment was similar but slower, reflecting the slower recovery of Chl under this regime. After 20 d of regreening, the plastids of these plants had ultrastructural features similar to those of the BAP-treated plants after 13 d.

Immunogold labelling localized POR in etioplasts of cotyledons (Fig. 2A; Table 3). Levels of POR were lower in regreening leaves than etiolated cotyledons (Zavaleta-Mancera *et al.*, 1999), resulting in weaker immunogold labelling (Fig. 2B), but gold particle densities were significantly greater in starch-free areas of plastids than in cytoplasm or cell walls (Table 3).

No proplastids were observed in regreening leaves, nor was any evidence for chloroplast division observed. To confirm that all the chloroplasts of regreening leaves arose by redifferentiation of gerontoplasts, plastid numbers per cell cross-section were monitored during senescence and regreening. Any increase in plastid numbers would imply that division or *de novo* biogenesis was occurring. Instead, plastid numbers declined during senescence but did not increase again during subsequent regreening (Fig. 3). Leaf Chl content, in contrast, decreased during senescence and recovered during regreening (Fig. 3). It is therefore most

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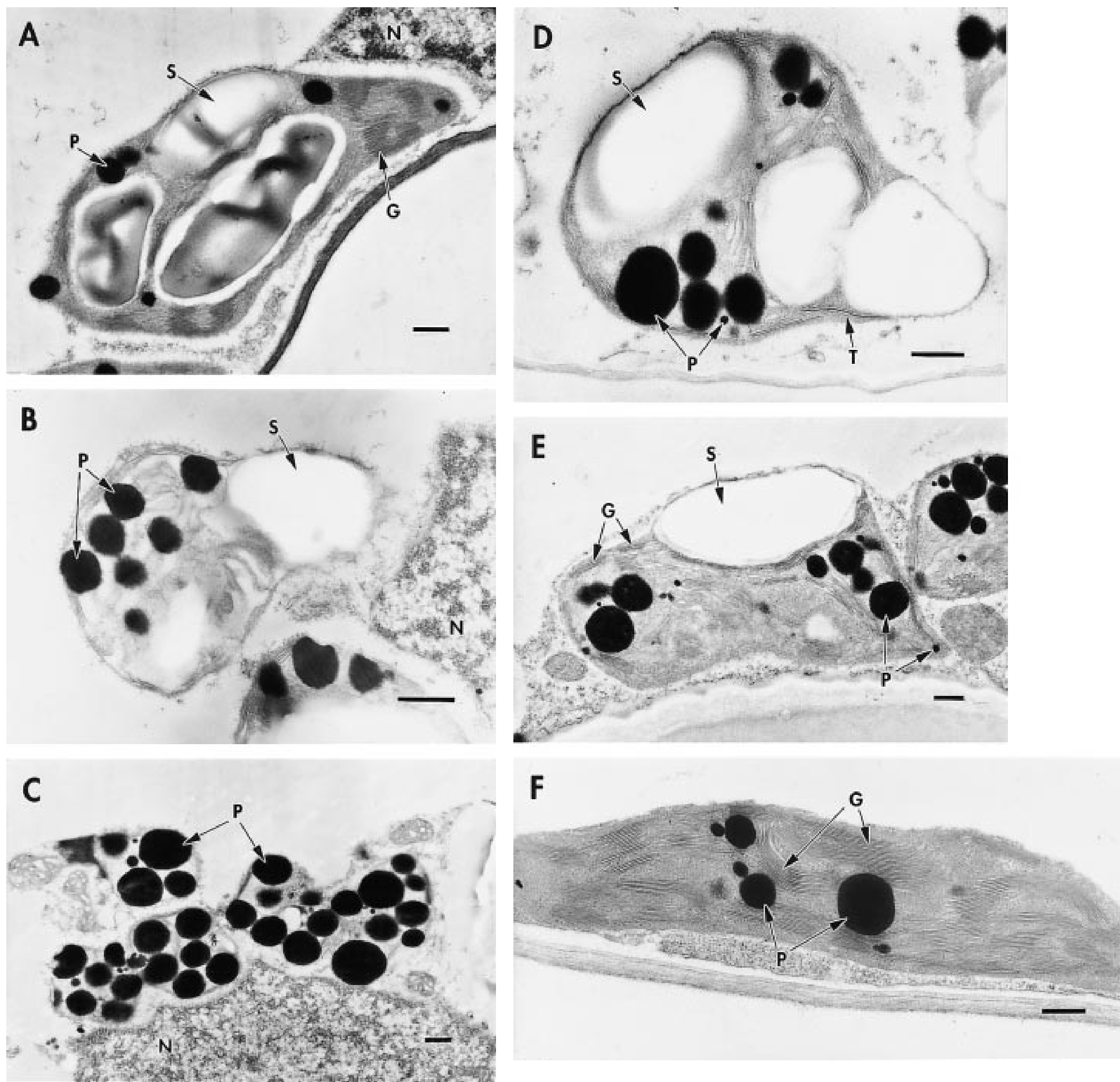


**Fig. 1.** Ultrastructure of plastids from leaves at different stages. (A) Pre-senescent green leaf, ×16 000. (B) Yellow senescent leaf (stage S-1), ×25 000. (C) Yellow-white leaf at advanced senescence (stage S-3), ×12 000. (D) Leaf regreened for 6 d, ×25 000. (E) Leaf regreened for 13 d, ×14 000. (F) Leaf regreened for 20 d, ×20 000. G, grana, T, thylakoid, P, plastoglobuli, S, starch, N, nucleus. Bars = 0.5 μm.

**Table 2.** *Changes in plastids and their constituents during senescence and regreening*

Values are means ± SE of 14–24 plastids. Within each column, data with different letters are significantly different ($P < 0.05$).

| Leaf stage | Mean plastid cross-sectional area ($\mu m^2$) | No. of plastoglobuli per plastid cross-section | Total area of plastoglobuli per plastid ($\mu m^2$) | % of starch-free plastid area as plastoglobuli | % of plastid area as starch |
|---|---|---|---|---|---|
| Green | 14.7 ± 1.3 a | 10.6 ± 1.1 a | 0.68 ± 0.1 d | 7.8 ± 0.5 e | 41.8 ± 2.6 c |
| Senescent | 7.1 ± 0.4 c | 7.4 ± 0.7 bc | 1.1 ± 0.08 bc | 42.9 ± 2.2 b | 59.9 ± 2.5 a |
| 6 d regreening | 9.8 ± 0.5 b | 7.1 ± 0.6 bc | 1.3 ± 0.1 b | 29.1 ± 2.0 c | 51.6 ± 2.7 ab |
| 13 d regreening | 11.1 ± 0.9 b | 7.4 ± 0.9 bc | 1.2 ± 0.2 b | 20.1 ± 2.3 d | 48.8 ± 1.6 bc |
| 20 d regreening | 7.2 ± 0.5 c | 5.9 ± 0.5 c | 1.0 ± 0.2 cd | 13.2 ± 1.6 e | 1.3 ± 0.5 d |
| 20 d regreening + 6 weeks in brighter light | 13.2 ± 1.0 a | 7.4 ± 0.9 bc | 0.7 ± 0.1 d | 10.1 ± 0.0 e | 40.7 ± 4.9 c |

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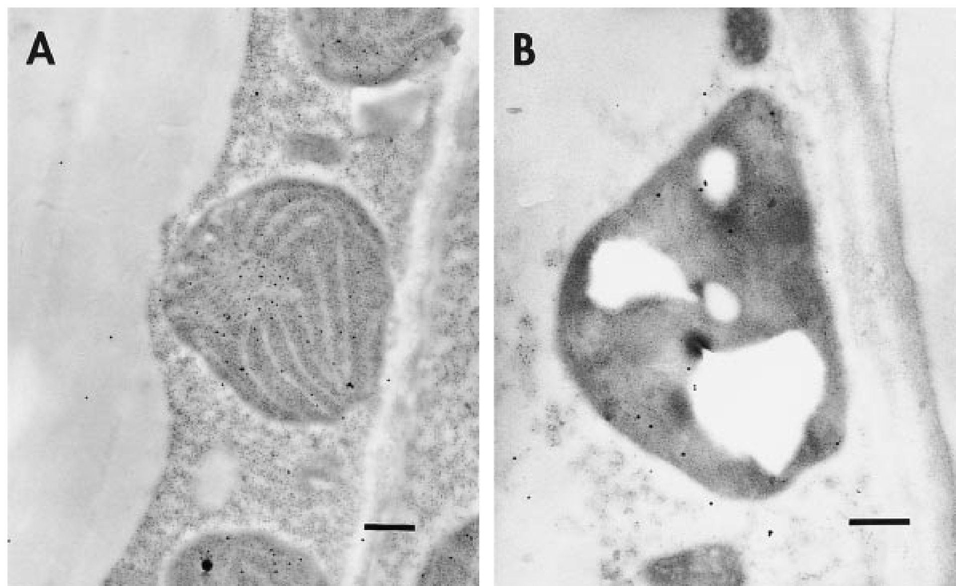


**Fig. 2.** Micrographs of *N. rustica* plastids in (A) etiolated cotyledons (×16 000; bar=0.5 μm), and (B) regreening leaves of plants 20 d after decapitation (×10 000; bar=1 μm), stained with antibody to POR and gold-labelled secondary antibody.

**Table 3.** *Immunogold labelling of POR in etiolated, green and regreening tissues of* Nicotiana rustica

Values are means ± SE ($n$=6–11).

| Material | Gold particle density per μm$^2$ | |
|---|---|---|
| | Pre-immune | Anti-POR |
| Etiolated cotyledons: | | |
| Etioplast | 0.07 ± 0.04 | 16.38 ± 1.06* |
| Cytoplasm + cell wall | 0.03 ± 0.02 | 1.94 ± 0.24 |
| Green leaves: | | |
| Plastid excluding starch | 0.23 ± 0.06 | 1.99 ± 0.42 |
| Cytoplasm + cell wall | 0.19 ± 0.08 | 1.79 ± 0.30 |
| Regreening leaves (18 d): | | |
| Plastid excluding starch | 0.20 ± 0.06 | 3.03 ± 0.48* |
| Cytoplasm + cell wall | 0.16 ± 0.08 | 1.42 ± 0.24 |

*Significant ($P$ < 0.05) association of label with plastid.

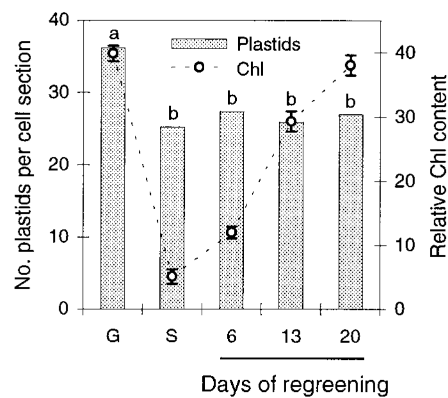


**Fig. 3.** Plastid numbers and relative Chl content in: G, green leaves; S, senescent leaves prior to regreening; and BAP-treated regreening leaves, at 6, 13 or 20 d after decapitation. Plastids were counted in 15 palisade cell cross-sections from each of four leaves at each stage. Bars with different letters differ significantly ($P$ < 0.05). Relative Chl contents are means ± SE ($n$=4).

likely that the same cohort of chloroplasts were losing and then regaining Chl.

In the preceding experiments, leaves were allowed to senesce to stage S-1, which was the maximum extent from which reproducible regreening could occur. At later stages of senescence, regreening was more variable (Fig. 4A) probably due to further loss of plastids as senescence advanced (Fig. 4B).

## Discussion

At the cellular level, leaf regreening could be explained by different hypotheses. Every mesophyll cell might have the capacity to rejuvenate, or regreening might require divisions in a stem-cell-like subpopulation. The first hypothesis appears to be correct in tobacco leaves. Although leaf thickness increased by nearly 50% during senescence and regreening, no mitoses or increases in cell numbers were observed. Instead, mesophyll cell enlargement accounted for increased leaf thickness. Similar conclusions were reached by Avery regarding the agronomic practice of 'topping' tobacco plants, in which removal of terminal flower stalks increases yield by enlargement of the upper leaves (Avery, 1934).

The tendency of leaves or cotyledons on decapitated plants to continue to grow has been observed in a wide range of plants (Leopold, 1961; Tuquet and Newman, 1980). Endogenous cytokinins accumulate in leaves of decapitated plants (Wang *et al.*, 1977), and cytokinins promote leaf expansion (Nielsen and Ulvskov, 1992). However, a significant 15% increase in thickness was also

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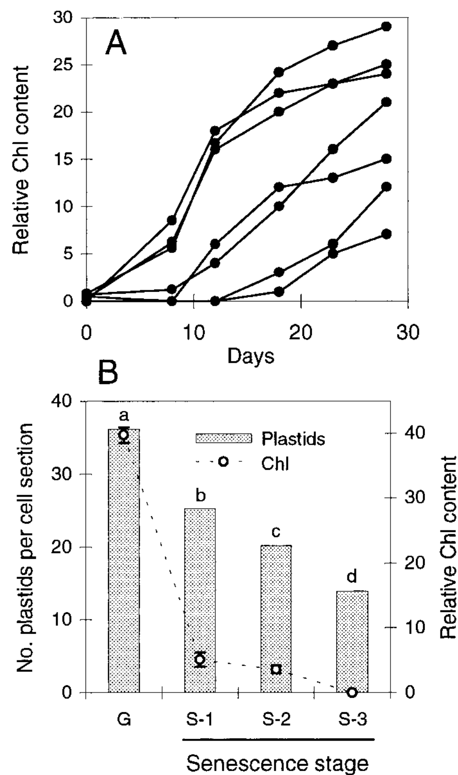


**Fig. 4.** (A) Variable regreening from advanced senescence in BAP-treated leaves on decapitated plants. Values are for individual leaves. Relative Chl contents prior to regreening were 0–0.8. (B) Decline in plastid numbers from green (G) leaves to progressively more advanced stages of senescence (S-1 to S-3). Plastids were counted in 15 palisade cell cross-sections from each of four leaves at each stage. Bars with different letters differ significantly ($P<0.05$). Relative Chl contents are means ± SE ($n=4$).

noted in senescent *N. rustica* leaves, in which endogenous cytokinin levels are reported to be lower (Singh *et al.*, 1992), so additional factors could be involved.

The chloroplasts of regreened *N. rustica* leaves were redifferentiated gerontoplasts. This was evident because, during the progressive recovery of the lamellar system and stroma, there was a persistence of gerontoplast features. These known characteristics of plastids in senescent cells included reduced size, rounded shape and larger plastoglobuli (Thomson and Platt-Aloia, 1987; Biswal and Biswal, 1988; Noodén, 1988). Proplastids were not observed at any stage of development from mature green to regreened leaves. In etiolated cotyledons, POR was immunolocalized in etioplasts, as has been well established (Forreiter *et al.*, 1990; Reinbothe *et al.*, 1996). In regreening leaves, however, POR was immunolocalized in mature plastids, providing further evidence that these organelles were the sites of Chl synthesis during reversal of senescence.

Trends in plastid numbers also supported the gerontoplast-redifferentiation route. Plastid numbers did not increase during regreening, and when too many plastids were lost during advanced senescence, regreening capacity deteriorated. One argument for the special definition of gerontoplasts is that chromoplasts of fruit and petals can divide while gerontoplasts have not been observed to do so (Matile, 1992). No evidence was found for division even of regreened gerontoplasts, either from plastid counts or from ultrastructural observations. The ability of tobacco leaves to regreen without an increase in chloroplast numbers is further evidence against new chloroplasts arising from proplastids. This contrasts with soybean cotyledons in which proplastids were observed during early senescence (Tuquet and Newman, 1980), while in regreening cotyledons a subpopulation of chloroplasts was found with no senescent characteristics, implying recent differentiation from proplastids.

It is believed that plastoglobuli in mature or senescent plastids represent residual hydrophobic components of degraded thylakoid membranes coalesced into lipid droplets (Biswal and Biswal, 1988; Matile, 1992). Consistent with this concept, plastids of mature green tobacco leaves contained plastoglobuli, which, during senescence and early regreening, decreased in number but increased in size, to give a substantial increase in total cross-sectional area per plastid. Conversely, plastoglobuli can diminish, as during thylakoid formation in greening etioplasts (Mackender, 1978), and it is interesting that in regreened leaves kept for a further 6 weeks in increased lighting, the total area of plastoglobuli per plastid decreased to a similar level to that of pre-senescent green leaves.

The integrity of the gerontoplast envelope was maintained at least until the most advanced stages of senescence, and even then apparently ruptured organelles could be artefacts of the increasing difficulties of fixation of senescent tissues (Thomson and Platt-Aloia, 1987; Matile, 1992). Regreening of senescent leaves is likely to be an adaptation to partial defoliation caused by herbivore grazing, so retention of plastid integrity as late as possible will maximize the plant's survival potential (Woolhouse, 1984). Loss of plastid DNA early in senescence has been reported in some species (Matile, 1992). The resilience of the plastid genome during senescence is therefore also worth investigation as a potential determinant of regreening capacity. Indeed, the recovery of biosynthetic capacity implied by the structural restoration of regreening gerontoplasts presents an additional dimension in the complex regulation of plastid gene expression, which may involve transcriptional and post-transcriptional mechanisms, in addition to interactions with the nucleus (Mayfield *et al.*, 1995).

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