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Regreening of senescent *Nicotiana* leaves

I. Reappearance of NADPH-protochlorophyllide oxidoreductase and light-harvesting chlorophyll *a*/*b*-binding protein

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Abstract

Decapitation of *Nicotiana rustica* L. plants above a single senescent leaf induced regreening, which was promoted by cytokinin treatment. Regreening required low light. The decline in leaf protein content and increase in protease activity seen during senescence were reversed on regreening. Western blotting showed that light-harvesting chlorophyll *a*/*b*-binding protein declined considerably during senescence, but on regreening it increased back to the levels seen in green leaves. NADPH-protochlorophyllide oxidoreductase (POR) was found by Western blotting at high levels in etiolated cotyledons, but at low levels in green leaves and not at all in senescent leaves. However, POR reappeared in regreening leaves, and cytokinin accelerated its increase.

Key words: Cytokinin, light-harvesting chlorophyll *a*/*b*-binding protein, protochlorophyllide oxidoreductase, *Nicotiana*, senescence.

Introduction

Greening and senescence normally occur at opposite ends of the life-span of a plant tissue. In the de-etiolation of seedling organs, biogenesis of chloroplasts involves development of thylakoids and expression of genes for assembly of the photosynthetic apparatus (Chory *et al*., 1994; Kusnetsov *et al*., 1994; Thomas, 1997). In yellowing leaves, disassembly of chloroplast molecules and structures is a programmed process (Smart, 1994; Buchanan-Wollaston, 1997; Thomas, 1997). It has been known for many years, however, that greening and senescence can occur in a different sequence. It has been shown that when all buds and leaves except one yellow leaf are removed from a *Nicotiana rustica* plant, then regreening of the leaf can occur (Mothes and Baudisch, 1958).

A common feature of these processes is the influence of cytokinins, which promote de-etiolation (Chory *et al*., 1994; Kusnetsov *et al*., 1994, 1998), while promotion of leaf regreening in *N. rustica* was one of the earliest discovered cytokinin effects (Kursanov *et al*., 1964). Today, cytokinin-transgene technology is available for senescence autoregulation (Gan and Amasino, 1995), so understanding the reversibility of senescence has renewed importance.

One question is how chlorophyll (Chl) biosynthesis is regulated when yellow leaves regreen. A non-constitutive step of Chl biosynthesis is the photoreduction of protochlorophyllide to chlorophyllide, catalysed by NADPH-protochlorophyllide oxidoreductase (POR) late in the pathway (Reinbothe *et al*., 1996; Thomas, 1997). This enzyme is the predominant protein in the paracrystalline

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Abbreviations: BAP, 6-benzylaminopurine; Chl, chlorophyll; EM, electron microscopy; FW, fresh weight; LHC, light-harvesting complex; LHCP, light-harvesting chlorophyll *a*/*b*-binding protein; POR, NADPH-protochlorophyllide oxidoreductase.

structure of the prolamellar body of etioplasts. Illumination initiates the rapid disappearance of POR, so that by the time Chl accumulation reaches its maximum rate, only trace amounts of the enzyme remain (Forreiter *et al*., 1990; Reinbothe *et al*., 1996).

The present paper therefore examines whether the renewed biosynthesis of Chl in regreening senescent leaves is associated with changes in POR levels. Also examined is the light-harvesting Chl *a*/*b*-binding protein (LHCP), whose accumulation during de-etiolation, and degradation during senescence, are believed to be dependent on its association with Chl (Thomas, 1997). Amounts of this protein component of the thylakoid light-harvesting complex (LHC) would therefore be expected to be co-ordinated with Chl levels during regreening.

Materials and methods

Plant material and treatments

Plants of *Nicotiana rustica* L., and *Nicotiana tabacum* L. cvs Samsun and Wisconsin 38, were grown in 13 cm diameter pots of John Innes No. 3 compost, in a glasshouse heated to a minimum of 18 °C, with light periods extended when necessary to 16 h by 400 W sodium lamps. No nutrient solutions were applied. When inflorescence buds appeared, plants were transferred to a controlled environment chamber at 24 °C under a 16 h light period of 12 or 100 μmol $m^{-2}s^{-1}$ (400–700 nm) obtained respectively by diffuse or direct fluorescent lighting. The 12 μmol $m^{-2} s^{-1}$ light level was used unless stated. 'Decapitation' involved cutting off the main shoot above a single leaf (the 6th, counted upwards, unless stated) with a low Relative Chl Content of 5–10 units measured with a Minolta SPAD-502 Chlorophyll Meter. Lateral shoots were removed as they appeared. Cytokinin-treated plants were sprayed daily for 7 d on the upper face of the single remaining leaf with a 10^{-4} M benzylaminopurine (BAP; Sigma, UK) solution containing 0.025% dimethylsulphoxide (Sigma), used to dissolve the BAP, and 0.02% Nonidet P40 (BDH Ltd, UK) as surfactant. Control sprays were similar but omitted BAP. Etiolated cotyledons were obtained from 8-d-old *N. rustica* seedlings grown in darkness in compost, and were harvested and extracted in dim green light.

Pigment measurements

To follow regreening non-destructively, Relative Chl Content was measured with the SPAD-502 meter, and then converted to absolute Chl amount using a calibration curve. To construct this curve, 70 regreening *N. rustica* leaves were sampled over 5 d for Chl estimations both with the SPAD-502 meter and following extraction in ethanol. The best fit by non-linear regression analysis with the GENSTAT 5 program was:

$$\text{Chl content (mg g}^{-1}\text{ FW)} = \exp[1.351 - 4.546(0.97575)^{\text{SPAD}}]$$

where SPAD = SPAD-502 meter reading. Chl contents of whole leaves were averaged from meter readings at eight different sites on each leaf. All Chl measurements presented were obtained from converted SPAD-502 readings, except those in Fig. 2, for which Chl *a*, Chl *b*, and carotenoids including xanthophylls, were measured in ethanol extracts as described previously (Lichtenthaler and Wellburn, 1983).

Gas exchange

Net CO_2 assimilation rates were measured by infra-red gas analysis using a CIRAS-1 portable photosynthesis system (PP Systems, Herts, UK) in atmospheric CO_2 levels and saturating light intensity (950 μmol $m^{-2} s^{-1}$). Three readings per leaf were averaged.

Analysis of leaf proteins

Leaf tissue was ground in liquid N_2, and then 5 vols of ice-cold lithium extraction buffer (Mae *et al*., 1993) and 0.5 vols of 20% (w/v) lithium dodecyl sulphate were added. Proteins were precipitated overnight at −20 °C following addition of equal volumes of 20% (v/v) trichloroacetic acid and 0.14% (v/v) 2-mercaptoethanol in acetone (TASH), and pelleted at 14 000 rpm, 4 °C for 5 min. The pellet was washed twice with an equal volume of cold TASH, dried for 1 h, then suspended overnight at 40 °C in 1 vol. of 50 mM SDS. Total protein was determined using bovine serum albumin as standard (Lowry *et al*., 1951). Proteins were separated by SDS-PAGE using a Bio-Rad Mini-Protean II cell, and visualized with Coomassie Blue and silver stain as described previously (Davies *et al*., 1990).

For Western blotting, proteins separated by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane using a Bio-Rad Mini Trans-Blot cell. Immunological detection of proteins was carried out by standard procedures. The antibody against *Festuca* LHCP was raised as described previously (Hilditch, 1986) and used at a dilution of 1 : 200. The antibody against barley POR was kindly provided by Dr WT Griffiths (Bristol, UK) and was used at a dilution of 1 : 1000. Peroxidase-conjugated swine anti-rabbit immunoglobulin (DAKO, UK) was used as secondary antibody at the same dilution as the primary antibody. Immunoreactive proteins were visualized by either chemical development for 5–15 min with a developer containing 4-chloro-1-naphthol and H_2O_2, or by enhanced chemiluminescence (ECL) following the kit manufacturer's procedure (Boehringer Mannheim, Germany).

Protease gel assay

Leaf tissue samples (0.1 g FW), crushed in 200 μl TRIS buffer (50 mM, pH 7.5), were absorbed onto discs of Whatman 3MM filter paper (6 mm diameter). The discs were then placed on a 2 mm deep gel (containing 0.4% (w/v) gelatin, 12 mM KH_2PO_4, 3 mM K_2HPO_4, 0.2 mM reduced glutathione, and 1.2% (w/v) agar) in a Petri dish sealed with parafilm, and incubated for 20 h at 30 °C. Following the incubation, the filter paper discs were removed and the gel surface was flooded for 2 min with 0.2% (w/v) amido black in 7% (v/v) acetic acid. After destaining with H_2O, protease activity was visualized as discs of white coloration against the black background of the stained gel.

Statistical analysis

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

Results

Treatments inducing leaf regreening

The original research on regreening used *N. rustica* (Mothes and Baudisch, 1958; Kursanov *et al*., 1964). In preliminary studies, *N. rustica* showed stronger regreening than *N. tabacum* cvs Samsun and Wisconsin 38, and was therefore selected as the experimental species. Senescence

of yellow leaves on intact *N. rustica* plants could be prevented with BAP (Fig. 1A). Leaves showing different degrees of yellowing regreened slowly when the plants were decapitated (Fig. 1B), but recovered comparable final Chl levels more rapidly when BAP was also applied (Fig. 1C). There were differential changes in Chl *a* and *b* and carotenoids during senescence and regreening (Fig. 2). The Chl *a*:*b* ratio of 2.68 in pre-senescent green leaves declined to 1.7 in senescent leaves, but recovered to 2.55 by 20 d of regreening. The ratio of carotenoids to Chl was 0.11 in green leaves, 0.4 in senescent leaves, and 0.12 in regreened leaves. Net CO_2 assimilation rates of green, senescent and regreening leaves were correlated with their Chl contents, indicating recovery of photosynthetic activity during regreening (Fig. 3).

Effects of light intensity on regreening

Mild lighting has been recognized as a precondition for regreening of senescent leaves (Mothes and Baudisch, 1958). All the experiments above used diffuse light

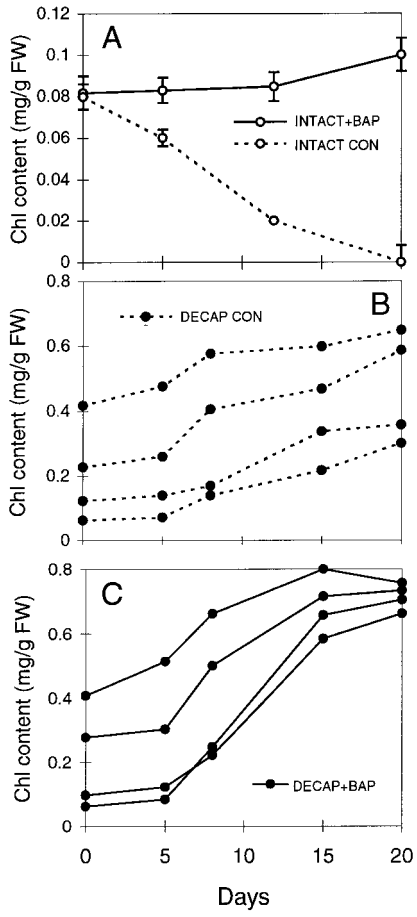

Fig. 1. Effects of decapitation and BAP on Chl content of leaves of *N. rustica*. (A) Intact plants with BAP or control treatments; (B) decapitated plants; (C) decapitated plants with BAP treatment. Values in (A) are means ± SE ($n = 10$). Curves in (B) and (C) represent individual leaves with different initial Chl contents.

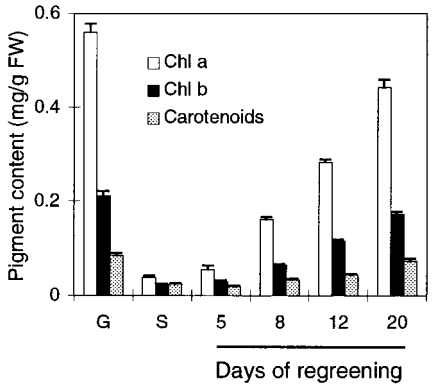

Fig. 2. Changes in leaf contents of Chl *a*, Chl *b* and carotenoids during senescence and regreening in BAP-treated decapitated *N. rustica* plants. G, pre-senescent green leaves; S, senescent leaves prior to regreening; and regreening BAP-treated leaves at intervals after decapitation. Values are means + SE ($n = 10$).

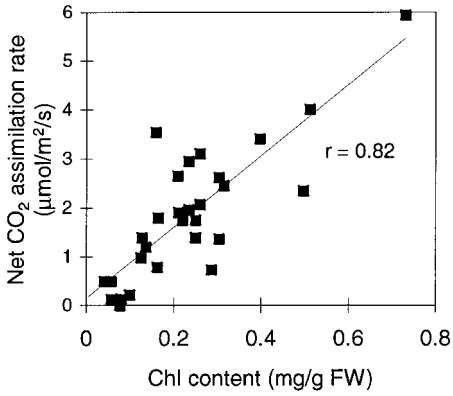

Fig. 3. Correlation of net CO_2 assimilation rates with Chl content of individual green, senescent and regreening *N. rustica* leaves.

(12 µmol m^{-2} s^{-1}); regreening was reduced in brighter light of 100 µmol m^{-2} s^{-1} (Fig. 4A), and was arrested when plants were transferred from 12 to 100 µmol m^{-2} s^{-1} (Fig. 4B).

Proteins in regreening leaves

Leaf proteins declined during senescence, but recovered during regreening, most strongly in BAP-treated leaves (Fig. 5A, B). Gel assays showed that the loss of proteins in senescent leaves was associated with a substantial increase in protease activity, which disappeared upon regreening (Fig. 5C). Western blotting revealed LHCP (25 kDa) and POR (36 kDa) in *N. rustica*. There was a dramatic decline in LHCP during senescence, but a strong recovery in regreening leaves (Fig. 6A). Protochlorophyllide oxidoreductase was immunodetected most strongly in Western blots of etiolated cotyledon extracts (Fig. 6B). In green leaves, a trace of POR was detectable with the sensitive ECL method, but not with peroxidase colour development. No POR was detected in senescent leaves using either method. However, POR was readily

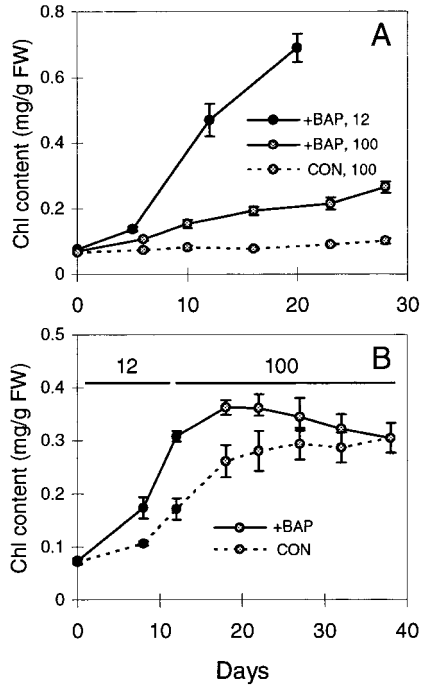

Fig. 4. Inhibition by increased light intensity of leaf regreening in decapitated *N. rustica* plants. (A) Plants in 12 or 100 μmol m^{-2} s^{-1} light; (B) plants transferred after 12 d from 12 to 100 μmol m^{-2} s^{-1} light. Values are means ± SE ($n = 10$).

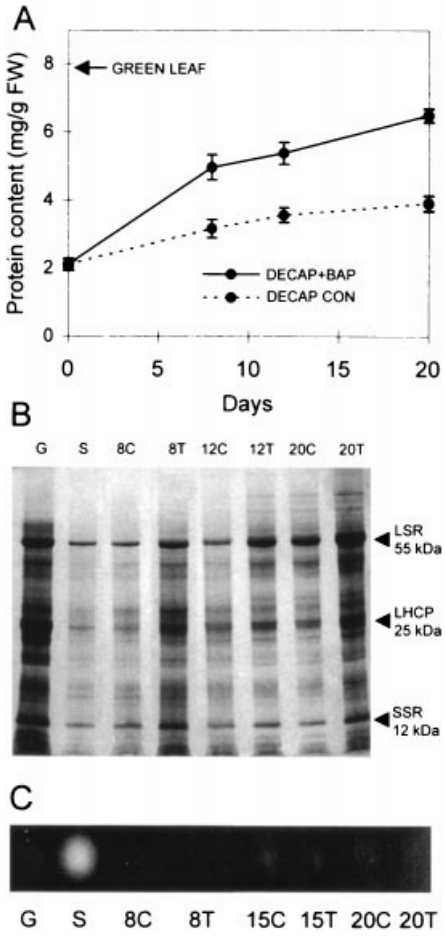

Fig. 5. (A) Total protein content of senescent (0.075 mg Chl mg^{-1} FW) leaves of *N. rustica* during regreening (to 0.69 mg Chl mg^{-1} FW) after decapitation on day 0. Protein content of pre-senescent leaves is marked 'green leaf' on the *y*-axis. Values are means ± SE ($n = 3$). (B) SDS-PAGE separations of total proteins from: G, pre-senescent green leaves; S, senescent leaves prior to regreening; and regreening control (C) or BAP-treated (T) leaves, numbers representing days after decapitation. Lanes were loaded on an equivalent FW basis. LSR, large subunit of Rubisco; SSR, small subunit of Rubisco. (C) Protease gel assays of equivalent-FW extracts of 4th leaves. Clear zones indicate protease activity (labels as in B).

detectable in regreening leaves (Fig. 6B). Analyses of the time-course of appearance of POR showed that BAP treatment enhanced expression in the early stages of regreening (Fig. 6C). In the first 2 weeks of regreening without BAP, low levels of POR were detected using ECL, but not using peroxidase colour development.

Discussion

The ecological significance of leaf regreening is probably the restoration of metabolic capacity following partial defoliation by herbivore browsing (Woolhouse, 1984). Recovery of photosynthetic capacity in *N. rustica* occurred during regreening, as shown previously in soybean cotyledons (Marek and Stewart, 1992). Decapitation and/or cytokinin can induce regreening in a range of plant species (Beever and Woolhouse, 1974; Venkatarayappa *et al.*, 1984; Marek and Stewart, 1992), although regreening capacity varies between species (Dyer and Osborne, 1971). Shoot decapitation increases cytokinin levels in xylem exudate (Beever and Woolhouse, 1974; Bangerth, 1994; Li *et al.*, 1995), and in still-attached leaves (Wang *et al.*, 1977). It has therefore been proposed that the senescence-delaying effects of this treatment may be due to increased availability of endogenous cytokinins rather than to changed source–sink relationships (Colbert and Beever, 1981). Cytokinin treatment in the present

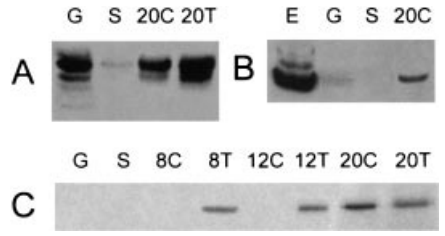

Fig. 6. Western blots of (A) LHCP and (B) POR in G, pre-senescent green leaves; S, senescent leaves prior to regreening; 20-d-regreening control (20C) or BAP-treated (20T) leaves; E, etiolated cotyledons. (C) Effect of BAP on the time-course of reappearance of POR (labels as above, numbers representing days after decapitation). SDS-PAGE lanes were loaded on an equivalent protein basis, and immunoreactive proteins were visualized by peroxidase colour reaction (A,C) or ECL (B).

study delayed or reversed senescence, particularly when applied in conjunction with decapitation.

As noted in the earliest studies (Mothes and Baudisch, 1958), ambient lighting needs to be of low intensity for regreening of yellow tobacco leaves. Without thylakoid LHC structures in an optimal state, leaves may have a poor ability to cope with high-light stress (Thomas, 1997). Effects on thylakoid structures of *N. rustica* leaves are implied by the observed changes in Chl *a*:*b* ratios during senescence and their reversal during regreening, but very few studies have looked at the association of different Chl types with the surviving complexes of the thylakoid in senescence. Changes in Chl *a*:*b* ratios during senescence have previously been observed, but not always in a consistent direction, even within a species. One reason seems to be that there is a cycle of interconversion between Chl *a* and *b* that is particularly significant in senescence, because only pigments with the '*a*' configuration on pyrrole group B are recognized by the Chl degradation pathway (Matile *et al*., 1999). Therefore, the relatively persistent Chl *b* in *N. rustica* may not be the same Chl *b* that was primarily localized in the antennae of pre-senescent leaves, but instead may be the product of unbalanced operation of the *a*-*b* interconversion cycle.

Chlorophyll and its binding-proteins are interdependent during the life-cycle of the LHC. Western blotting showed that LHCP disappeared during senescence in *N. rustica* leaves. During senescence, LHCP probably becomes vulnerable to proteases during Chl degradation; retention of pigment in *stay-green* senescence mutants is associated with immobilization of thylakoid proteins (Thomas and Smart, 1993; Bachmann *et al*., 1994). The decline in total protein content of senescent *N. rustica* leaves was shown to be associated with increased protease activity. Proteases are among the most readily detectable senescence-induced genes (Smart, 1994; Buchanan-Wollaston, 1997), and promoters of senescence-induced proteases are attenuated by cytokinin (Gan and Amasino, 1995).

During regreening, protease activity declined, total protein levels recovered, and LHCP reappeared. Cytokinin has been shown to enhance the expression of LHCP in cell cultures of *N. tabacum* cv. Wisconsin 38 and carnation (*Dianthus caryophyllus*), and in senescing barley leaves (Legocka *et al*., 1990; Abdelghani *et al*., 1991; Jackowski, 1996). The stoichiometry of Chl and LHCP in the LHC appears to be maintained by post-transcriptional regulation, such as proteolysis of uncomplexed LHCP, and both components may be necessary for stable integration into etioplast membranes (Flachmann, 1997; Kuttkat *et al*., 1997; Thomas, 1997). Similar mechanisms may apply in regreening leaves.

The resynthesis of Chl in regreening leaves coincided with renewed expression of POR, which occurred earlier when regreening was accelerated by treatment with cytokinin. It has recently been shown that faster greening of cytokinin-treated etiolated lupin cotyledons is accompanied by a higher level of POR (Kusnetsov *et al*., 1998). It therefore appears that cytokinin enhances expression of this enzyme when Chl biosynthesis occurs in the very different contexts of de-etiolation or reversal of senescence.

The identification of POR in *N. rustica* is supported by the immunolocalization of antigen in etioplasts (Zavaleta-Mancera *et al*., 1999), where POR is known to be abundant (Forreiter *et al*., 1990; Reinbothe *et al*., 1996). Two distinct forms of POR with different patterns of regulation by light have been found in green tissues of barley (Holtorf *et al*., 1995) and *Arabidopsis* (Runge *et al*., 1996), where some biosynthesis is needed to maintain Chl amounts. Further studies are needed to establish whether different PORs are expressed in de-etiolating, green or regreening tobacco tissues.

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