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Altered patterns of senescence and ripening in *gf*, a stay-green mutant of tomato (*Lycopersicon esculentum* Mill.)

Muhammad Shaheen Akhtar[1], Eliezer E. Goldschmidt[2], Isaac John[3], Simona Rodoni[4], Philippe Matile[4] and Don Grierson[1,5]

[1] *Plant Science Division, School of Biological Sciences, The University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK*

[2] *The Kennedy-Leigh Centre for Horticultural Research, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel*

[3] *Department of Biology, The University of Michigan, Ann Arbor, MI 48109–1048, USA*

[4] *Department of Plant Biology, University of Zurich, Switzerland*

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Abstract

The *gf* tomato mutant, which retains chlorophyll during ripening, has been found to be affected in leaf senescence. The leaves of the *gf* mutant show an absolute stay-green phenotype. As leaf senescence and fruit ripening proceed, there is a marked difference in chlorophyll content between wild-type and *gf*. In both attached and detached leaf studies, or after treatment with ethylene, the leaves withered and abscised in *gf* with only slight loss of chlorophyll and carotenoids. Total protein content declined and free amino acids increased during leaf senescence in wild-type and *gf*, but Western analysis showed that LHCII polypeptides were retained at higher levels in *gf*. Expression of senescence-related mRNAs increased normally in *gf* whereas those for *cab*, *rbcS* and *rbcL* declined in both mutant and wild-type. The mutant possesses enzyme activity for chlorophyllase, the formation of phaeophorbide a by the action of Mg-dechelatase and the oxygenolytic opening of the porphyrin macrocycle. Analysis of chlorophyll breakdown products in fruit indicated that *gf*, like other stay-green mutants, accumulates chlorophyllides *a* and *b*, but phaeophorbide *a* does not accumulate *in vivo*. This may indicate that, in the mutant, *in vivo* the action of phaeophorbide *a*-oxygenase is somehow prevented, either by altered accessibility or transport of components required for thylakoid disassembly or the absence of another factor.

Key words: Carotenoids, chlorophyll, ripening, senescence, tomato.

Introduction

The leaf senescence syndrome involves a large number of biochemical processes, many of which relate to the disintegration of the photosynthetic apparatus. As with other developmental phenomena, natural as well as genetically engineered mutants have become increasingly important for the analysis of leaf senescence and its regulation by environmental and hormonal stimuli (Canfield *et al.*, 1995; Gan and Amasino, 1995; Grbic and Bleecker, 1995; John *et al.*, 1995). 'Stay-green' mutants, in particular, have been highlighted as valuable tools for dissection of senescence and distinguishing between the degradation of different thylakoid constituents (Guiamet *et al.*, 1991; Thomas and Smart, 1993).

While tomato has been used as a major plant system for the study of fruit ripening, it has not been used extensively in early studies of leaf senescence (McGlasson *et al.*, 1975; Mizrahi *et al.*, 1975). The molecular characterization of tomato leaf senescence has been approached more recently (Davies and Grierson, 1989; John *et al.*, 1995, 1997; Drake *et al.*, 1996). The *green flesh* (*gf*) mutant of tomato was described long ago (Kerr, 1956), and shown to be located on chromosome 8 (Kerr, 1957). Only the altered fruit-ripening characteristics were described, however (Ramirez and Tomes, 1964; Grierson *et al.*, 1987; Cheung *et al.*, 1993), which include retention

[5] To whom correspondence should be addressed. Fax: +44 115 951 6334. E-mail: donald.grierson@nottingham.ac.uk

of chlorophyll, thylakoids, and some thylakoid proteins as the chloroplasts accumulate lycopene and are converted to chromoplasts. In the present study, the leaf senescence pattern of *gf* plants and the degradation of chlorophyll in both leaves and fruit were examined. The leaves of the *gf* mutant appear to contain all the enzyme activities required for chlorophyll catabolism yet exhibit an absolute, stay-green phenotype. General proteolysis of leaf proteins continues in *gf* during senescence, but the LHCII polypeptide was preferentially retained.

Materials and methods

Plant material

Wild-type and green flesh plants were grown in 7 cm pots in John Innes M2 compost in a growth room under controlled white fluorescent light (100 μmol m^{-2} s^{-1}) with a day/night period and temperature of 16/8 h, and 22/18 °C, respectively. The plants were given tap water every day and grown till they showed all the stages of leaf senescence (mature green, onset, mid and advanced) at about 6–7 weeks. Leaves at different stages of senescence were harvested as described previously by John *et al.* (1995) and immediately frozen in liquid nitrogen, and stored at −70 °C.

Protein extraction and Western analysis

Protein samples were extracted from tomato leaves and fruits according to the protocol of Meyer (1988). For Western blot analysis 50 μg of each protein sample was resolved on 14% SDS/PAGE and blotted onto nitro-cellulose membranes (Bio-Rad) according to the manufacturer's instructions. Antibodies against LHCPII, Rubisco (SSU and LSU) were obtained from Drs Kate Griffith and P Scott, respectively. The membranes were probed with antibodies as described by Cornelius *et al.* (Cornelius *et al.*, 1996).

RNA extraction and Northern analysis

Total RNA was extracted from leaves at different leaf senescence stages by the method of Wadsworth *et al.* (Wadsworth *et al.*, 1988). For RNA gels 5 μg of total RNA for each sample was loaded for electrophoresis. After electrophoresis the samples were capillary blotted onto GeneScreen membrane (Du Pont) and fixed using a Stratalinker UV-crosslinker 2400 (Stratagene) according to the manufacturer's instructions. DNA probes were radiolabelled using random primers by the method of Feinberg and Vogelstein (Feinberg and Vogelstein, 1983).

Extraction and quantitation of leaf and fruit chlorophyll and carotenoids

The method of Tomes (1963) was modified for carotenoids extraction. One gram of leaf tissue was ground with 5 ml of hexane:acetone (60 : 40, v/v) and a small amount of acid-washed sand. The aqueous phase was extracted many times until it became colourless. The total volume of the organic phase was measured and 1 ml was taken for scanning from 350–700 nm in a cuvette with a 1 cm path length. The amount of carotenoids (Davies, 1976) in 1 ml of sample was calculated by the following equation: μg of carotenoids = $A_{450} \times 4$. Carotenoids were finally expressed as μg $g^{-1}$of fresh weight (FW) of leaves.

Detached leaf senescence studies

Whole leaves were placed on three layers of wet Whatmann No. 1 filter paper in 14 cm Petri dishes (Fig. 2). The dishes, wrapped in parafilm and aluminium foil and incubated in the dark at 23 °C were opened for inspection and sampling every 2–3 d. The effect of ethylene was tested by placing the Petri dishes in a glass desiccator with 20 μl l^{-1} ethylene.

Determination of chlorophyll, catabolites, and catabolic enzymes

Chlorophyll content was determined spectrophotometrically in *N*,*N*-dimethylformamide extracts of leaf discs (Moran, 1982). Chlorophyllase activity was determined from fresh leaf extracts according to Trebitsh-Sitrit *et al.* (Trebitsh-Sitrit *et al.*, 1993) in 0.05 M phosphate buffer pH 7.4, 0.01% Triton X-100. Phaeophorbide *a* oxygenase activity was assayed as outlined in Hortensteiner *et al.* (Hortensteiner *et al.*, 1995). Dephytylated chlorophyll derivatives estimation was conducted by a phase separation assay as previously described (Amir-Shapira *et al.*, 1987), or by HPLC (Moser and Matile, 1997).

Determination of protein content, free amino acids and proteolytic activity

Total protein content was determined in leaf disc extracts in 0.05 M phosphate buffer pH 7.4, 0.01% Triton X-100 according to Bradford (Bradford, 1976). Free amino acids were determined in leaf disc extracts in 80% ethanol by the ninhydrin reagent Solution of Sigma (Moore, 1968). Proteolytic activity of leaf disc extracts was determined in 0.05 M phosphate buffer pH 7.4, 0.01% Triton X-100 by the release of acid-soluble products from radioactively labelled casein (α-casein, cold and ^{14}C-methylated from Sigma) as given by Katayama-Fujimura *et al.* (Katayama-Fujimura *et al.*, 1987).

Pigment measurement in senescent leaves after treatment with 8-hydroxyquinoline

Mature leaves were cut in half along the midrib and cut surfaces were placed on solutions in tap water of 1 mM ACC with or without addition of 8-hydroxy quinoline (1 mM) to block phaeophorbide *a* oxygenase. The tissue were allowed to senesce for 3.5 d in permanent darkness and then analysed for pigments.

Results

*Phenotype of green flesh (*gf*)*

The early development of plants and their general morphology did not appear to be altered by the *gf* mutation and the etiolation and de-etiolation behaviour of seedlings was similar. However, examination of mature *gf* plants (Fig. 1A) revealed striking differences in leaf senescence patterns. Unlike the wild-type, leaves on the lowermost nodes of *gf* plants did not lose their chlorophyll. The very old leaves eventually withered and abscised without any visible loss of chlorophyll. The *gf* fruit retained substantial amounts of chlorophyll during ripening (Fig. 1B), but other ripening changes, such as ethylene synthesis (data not shown) and lycopene accumulation (Table 1) were normal. Leaves of *gf* also retained chlorophyll when detached and treated with ethylene, or kept in the dark (Fig. 1C, D). Detached leaves of *gf* lost 5% chlorophyll

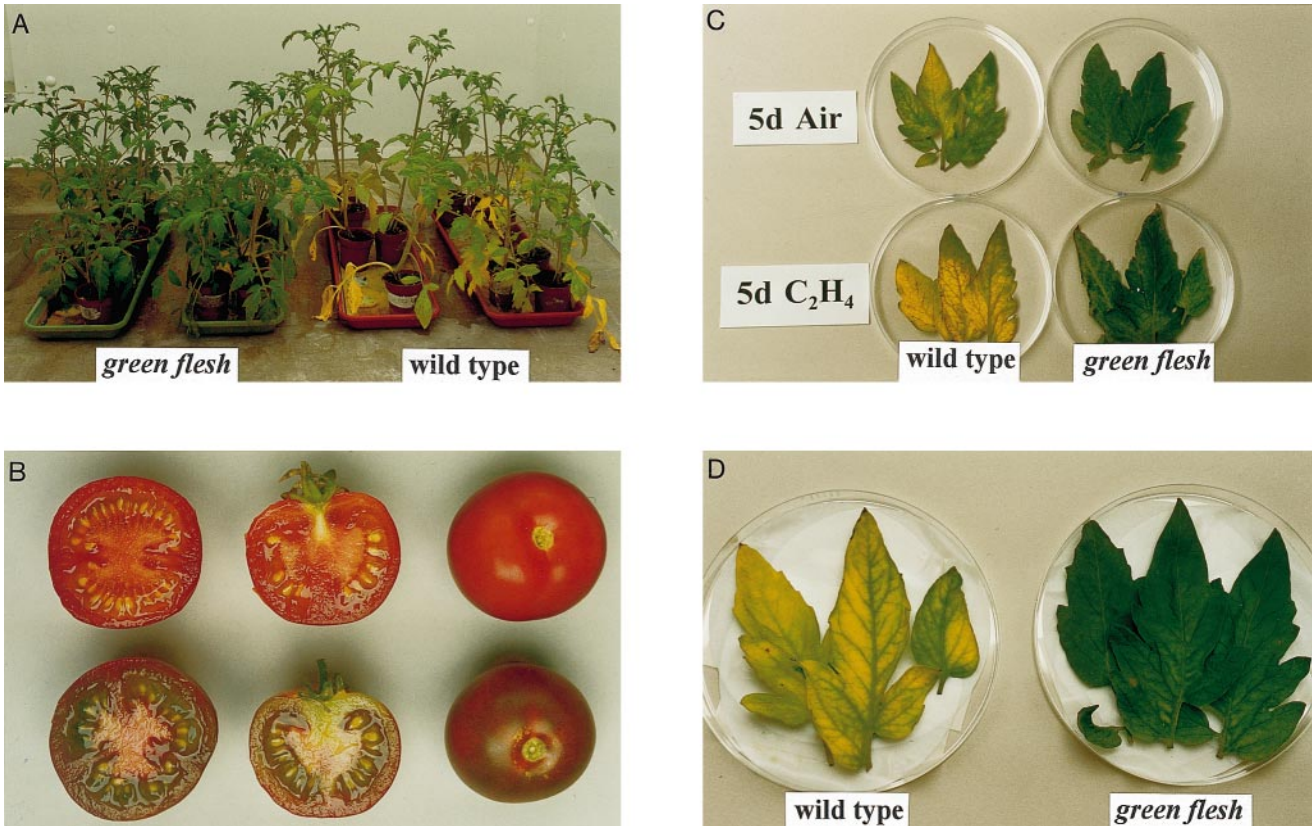

Fig. 1. Leaf senescence and ripening in wild-type and *gf* plants. (A). Five-week-old wild-type and *green flesh* plants grown in similar conditions of light and temperature. The wild-type plants show typical yellowing of leaves, while green flesh leaves wither and abscise without any sign of yellowing, giving it a stay green character. (B). Ripe wild-type (upper) and *green flesh* (lower) fruit. Retention of chlorophyll in the ripening fruit gives it a rusty red/dirty red phenotype (Clayberg *et al*., 1960). (C). Detached wild-type and *green flesh* leaves exposed to air or ethylene. (D). Detached wild-type and *green flesh* leaves kept in the dark for 9 d.

Table 1. *Chlorophyll and carotenoids in leaves and fruits of intact wild-type and* green flesh *plants*

In wild-type plants either fully expanded green leaves (MG) or those of the advanced senescence (yellow) stage (AD) were harvested. In *gf* leaves of a similar age and from a similar position to the wild type were taken for comparison, although there was little change in leaf colour. Fruits were harvested at the mature green (MG), and breaker plus 3 (B+3) and breaker plus 10 (B+10) stages, frozen in liquid nitrogen and 2.0 g of fruit from each stage was ground for pigment extraction.

Sample	Stage	Leaf pigments (μg g^{-1} fr. wt.)			
		Chlorophyll	Chl%	Carotenoids	Car%
Wild type	MG	897.30	100	185.00	100
	AD	145.39	16	110.50	60
Green flesh	MG	1226.76	100	209.00	100
	AD	905.7	74	183.10	88

Sample	Stage	Fruit pigments (μg g^{-1} fr. wt.)			
		Chlorophyll	Chl%	Total carotenoids	Lycopene
Wild type	MG	32.44	100	9.23	0.82
	B+3	0.74	2	27.60	21.99
	B+10	0.16	0.5	36.06	28.62
Green flesh	MG	30.04	100	8.38	0.71
	B+3	25.37	84	32.83	21.69
	B+10	8.27	28	46.27	36.64

MG, mature green; AD, advanced senescence; fr. wt., fresh weight; chl, chlorophyll; Car, carotenoids; B, breaker.

Table 2. *Chlorophyll and metabolites in leaves and fruits*

(A) Chlorophylls and metabolites in ripe fruit of wild-type and *gf* plants.

	Chlorophyll (nmol g^{-1} fr. wt.)		Chlorophyllide (nmol g^{-1} fr. wt.)		Phaeophorbide *a* (nmol g^{-1} fr. wt.)
	a	*b*	*a*	*b*	
Wild type	0.36	0.21	0.0	0.0	0.0
Green flesh	12.22	5.07	0.67	0.008	0.0

(B) Chlorophylls, chlorophyllide *a* and phaeophorbide *a* in senescent leaves treated with 8-hydroxyquinoline.

Sample	Treatment with chelator	Chlorophyll *a*+*b* (μmol g^{-1} fr. wt.)	Chlorophyllide *a* (nmol g^{-1} fr. wt.)	Phaeophorbide *a* (nmol g^{-1} fr. wt.)
Wild type	−	0.20	0.5	0.0
	+	0.76	11.7	10.5
Green flesh	−	0.96	1.4	0.0
	+	1.06	15.0	6.7

after 7 d compared to 30% for control leaves (data not shown).

Pigment content

Quantitative determination of pigments supported the visual observations. A decline of only 26% in chlorophyll and 12% in carotenoids was found in ageing *gf* leaves, as compared with 84% loss of chlorophyll and 40% loss of carotenoids in comparable wild-type leaves (Table 1). In fruit, almost all chlorophyll was lost from the wild-type 3 d after the start of colour change (B+3) and carotenoids accumulated. Carotenoid production in *gf* was normal, but at a comparable stage (B+3) 84% of chlorophyll was retained. After a further week this had dropped to 28% (Table 1).

Chlorophyll degrading enzymes

Chlorophyllase activity was present in leaves and fruit of both wild-type and *gf*. Whereas chlorophyllides were not detectable in the mature fruit of the wild-type, significant amounts of chlorophyllides *a* and *b* had accumulated in the fruit of the mutant (Table 2). Phaeophorbide *a* was not detectable in mature fruit of either genotype. It was accumulated, however, in senescing leaves of both genotypes, when the ring opening oxygenase was inhibited in the presence of 8-hydroxyquinoline (Table 2B). Both genotypes were also competent with regard to the enzymic conversion of phaeophorbide *a* into a primary tetrapyrrolic product of macrocycle cleavage (pFCC-2), suggesting that they contain functional phaeophorbide *a* oxygenase and RCC reductase (Table 3).

Protein content and proteolysis during leaf senescence

Total soluble protein determinations showed that senescing leaves from both genotypes retained more than 60% of their presenescence protein level (Table 4). Detached wild-type and *gf* leaves showed a prominent increase in free amino acids during senescence (3-fold and 5-fold, respectively, data not shown), indicating that protein degradation takes place in both genotypes. The *in vitro* proteolytic activity of tomato leaf extracts was found mainly at pH 7.0, with little or no activity at pH 9.0 in either fresh or senescent leaf material (Vera and Conejero, 1990). A senescence-associated increase in proteolytic activity (1.7-fold to 2.6-fold, data not shown) was consistently observed with both wild-type and *gf*.

Analysis of total leaf protein by gel electrophoresis did not disclose major qualitative differences in components, but some quantitative differences between the wild-type and *gf* genotypes were evident (Fig. 2). In both genotypes

Table 3. *Activities of chlorophyll catabolic enzymes in leaves and fruits of wild type and* gf *genotypes*

Fully mature green leaves or ripe fruit from wild-type and *gf* plants were freshly harvested and used for *in vitro* chlorophyllase assay, with chlorophyll extracted from spinach leaves as substrate, or determination of phaeophorbide *a* oxygenase.

Sample	Chlorophyllase activity (μmol chlorophyllide h^{-1} g^{-1} fr. wt.)			Phaeophorbide *a* oxygenase (RCC-reductase) (pFCC-2 Fu min^{-1} g^{-1} fr. wt.)
	Leaves	Green fruit	Ripe fruit	Ripe fruit
Wild type	2.50	0.12	1.08	0.53
Green flesh	2.22	0.05	5.10	0.30

Fu, fluorescence units λex = 320, λem = 450 nm.

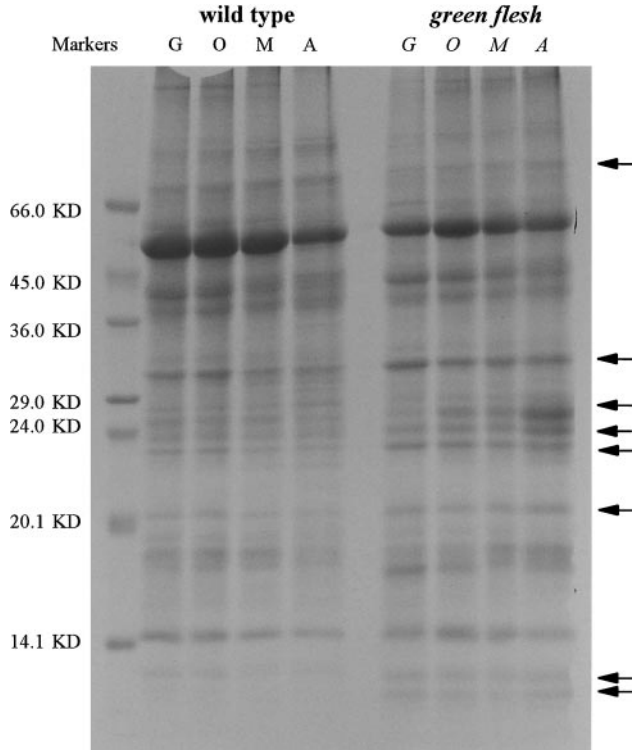

Fig. 2. Changes in proteins during senescence of wild-type and *green flesh* leaves. Aliquots of total protein (50 μg) from different stages of senescence were fractionated on a 14% polyacrylamide gel. The proteins marked with arrows show differences in *green flesh* leaves compared to wild-type. Leaves were harvested from 6-week-old plants grown in similar conditions of light and temperature. G, O, M, A, refer to stages green, onset, mid, and advanced colour change (for *green flesh*, these are shown in italics because there is no colour change). *Green flesh* leaves were harvested from equivalent position to those of wild-type leaves. Markers, position of molecular weight markers (kDa).

numerous polypeptides were reduced in abundance with the advent of senescence, while others appeared to remain unchanged. A few seemed to increase, at least with respect to other polypeptides, particularly two 24–26 kDa polypeptides in *gf*.

Western analysis of photosynthesis associated proteins

The abundance of the photosynthesis-related proteins, Rubisco small subunit (Rubisco SSU), Rubisco large subunit (Rubisco LSU) and the major light-harvesting LHCII protein was assessed by Western analysis (Fig. 3).

Table 4. *Soluble protein amounts in attached leaves: the selection of leaves for analysis was as described in Table 1*

Sample	Stage	(mg protein g^{-1} fr. wt.)	Protein (%)
Wild type	MG	5.91	100
	AD	4.04	68
Green flesh	MG	3.92	100
	AD	2.42	61

Each measurement is the average from two leaf samples. MG, mature green; AD, advanced senescence.

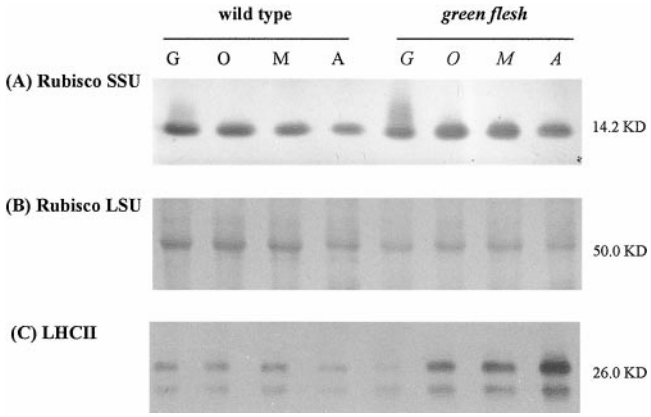

Fig. 3. Western analysis of protein in senescing leaves of wild-type and *green flesh* plants. Proteins were extracted from leaves at different senescence stages and 5 μg of total protein was loaded on a 12% polyacrylamide gel. G, O, M, A refer to the stages green, onset, mid, and advanced colour change. For *green flesh* stages are shown in italics because there is no colour change and leaves at similar positions to those in wild-type were harvested. After electrophoresis the gels were blotted onto membranes and probed with Rubisco SSU (A), Rubisco LSU (B) and LHCII (C) antibody.

All three polypeptides showed a gradual decline during senescence in wild-type, with an indication of a slower decline in Rubisco LSU in *gf*. A major difference was observed for LHCII, which persisted at a much higher relative level in *gf* compared to the control.

Expression of senescence-related mRNAs

Expression of several senescence-related mRNAs was examined in senescing leaves of wild-type and *gf* (Fig. 4). SENU3 mRNA (Fig. 4A), which encodes a protease which has been shown to be up-regulated during tomato leaf senescence (Drake *et al*., 1996), increased in both genotypes. Other mRNAs tested also showed a similar increase in wild-type and *gf* (data not shown), including SENU2 (another senescence related protease, Drake *et al*., 1996) and ACC oxidase. The accumulation of mRNA for the chlorophyll *a*/*b* binding protein Cab (LHCII) was strongly reduced during senescence in both genotypes (Fig. 4B). The expression of the ribulose bisphosphate carboxylase/oxygenase small subunit (*rbcS*) mRNA (Fig. 4C) was also strongly reduced in wild-type leaves, but less dramatically in *gf* leaves, whereas *rbcL* (Fig. 4D) remained at a relatively high level in both genotypes. The mRNA for the oxygen-evolving PSII 10 kDa polypeptide remained at similar levels throughout senescence in both genotypes (Fig. 4E).

Discussion

The *gf* mutation was reported 40 years ago (Kerr, 1956) but only its effects on tomato fruit were described. This seems to be the first study in which the effects of the *gf* mutation on leaf development have been examined. These

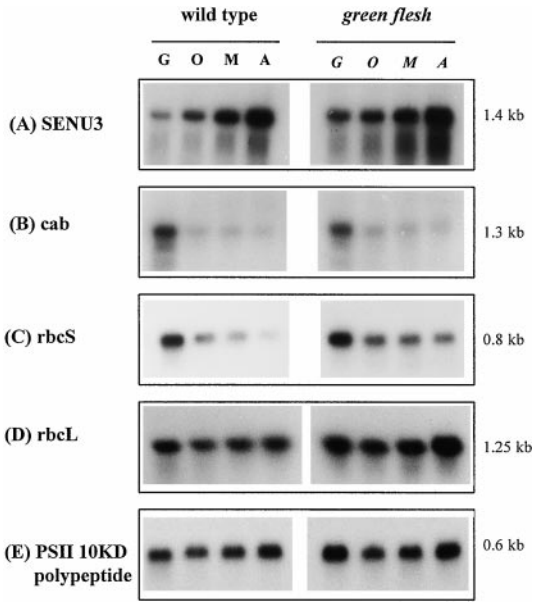

Fig. 4. Expression of mRNA during senescence in wild-type and *green flesh* leaves. Total RNA was extracted from leaves at the green (G), senescence onset (O), mid-senescence (M) and advanced senescence (A) stages (5 μg) of each sample was loaded on to a 1% agarose gel and probed with mRNAs encoding senescence protease SENU3 (A), Cab (B), RbcS (C), RbcL (D), oxygen-evolving PSII 10 kDa protein (E). The molecular sizes of the mRNAs are indicated in kb.

results show clearly that *gf* leaves exhibit an absolute stay green phenotype. The behaviour of *gf* leaves appears to be different from that of *gf* fruit, which lose a considerable portion of their chlorophyll during ripening (Ramirez and Tomes, 1964; see also Fig. 1B and Table 1). Chlorophyllase activity was found to be similar in both genotypes (Table 3). This confirms the early report by Ramirez and Tomes (1964) who measured similar chlorophyllase activities in fruit tissues of wild-type and *gf*.

According to this study's observations, the *gf* mutation does not alter the normal course of greening, etiolation and de-etiolation and does not seem, therefore, to interfere with chloroplast development. The slight differences in height between *gf* and wild-type plants evident from Fig. 1 were not consistently found. Thus, the effect of the *gf* mutation seems to be confined to the senescence phase, which includes numerous degradative events mostly associated with the disintegration of the photosynthetic apparatus. Among the most important biochemical changes are the breakdown of chlorophyll and the loss of protein. In intact or detached ageing *gf* leaves, the almost complete arrest of chlorophyll breakdown and retention of carotenoids is most conspicuous (Fig. 1; Table 1). However, differences between wild-type and *gf* in protein degradation during senescence were not so obvious (Table 4). The increase in free amino acids and the upsurge of proteolytic activity (data not shown)) suggest that senescent proteolysis occurs in both genotypes to a similar extent.

Using immunological detection (Fig. 3), only small differences were found between wild-type and *gf* in the persistence of the Rubisco SSU and Rubisco LSU polypeptides. There was some indication of a greater decline in Rubisco LSU in the wild-type, but this is difficult to measure quantitatively using antibodies. The persistence of these chloroplast polypeptides in ageing leaves is not necessarily inconsistent with the decline in their transcript levels, as previously indicated by Bate *et al.* (1991). The most notable difference was in the LHCII polypeptides which were retained at much higher relative levels in *gf*, although the mRNA abundance declined dramatically.

Northern analysis of several senescence-related mRNAs revealed identical trends for both genotypes, in all cases examined (Fig. 4). The up-regulation of SENU3 and others on one hand, and the down-regulation of *cab* and *rbcS*, on the other, suggest that the *gf* mutation does not modify the transcriptional activities of these genes during senescence. A similar conclusion has been drawn previously for another stay-green mutant (Thomas *et al.*, 1992).

These results indicate that the LHCII polypeptides in leaves must somehow be protected from senescent degradation. LHCII is a major component of PSII and retention is correlated with preservation of chlorophyll and carotenoids in the thylakoids. It seems plausible that factors which maintain overall membrane integrity (including retention of LHCII protein) are responsible for this (as suggested by Cheung *et al.*, 1993, for fruit).

Comparison with results from other stay-green mutants indicates that the precise relationship between chlorophyll and protein breakdown is still a matter of conjecture. In the *cyt*G stay-green soybean mutant, Guiamet and co-workers assumed that the mutation preferentially affects the breakdown of the Cab (LHCPII) protein, thereby holding back chlorophyll catabolism (Guiamet *et al.*, 1991). On the other hand, in the BF 993 *Festuca* mutant, it was hypothesized that a lesion in the chlorophyll catabolic pathway prevents normal degradation of the chlorophyll binding proteins (Nock *et al.*, 1992).

The loss of chlorophyll in senescent leaves is due to the stepwise degradation by chlorophyllase (producing chlorophyllides), Mg-dechelatase (yielding phaeophorbide) and phaeophorbide *a* oxygenase which cleaves the porphyrin macrocycle oxygenolytically into a colourless fluorescent catabolite (Matile *et al.*, 1996). This pathway has also been demonstrated to be responsible for the breakdown of chlorophyll in ripening fruits of *Capsicum annuum* (Moser and Matile, 1997). The *gf* mutant clearly contains enzymes capable of catalysing these reactions, including chlorophyllase, Mg-dechelatase and phaeophorbide *a* oxygenase (Tables 2, 3). Senescent leaves of the stay-green mutants of *Festuca pratensis* (Vicentini *et al.*, 1995) and of *Pisum sativum* (Thomas *et al.*, 1996) have been found to be deficient with regard to the third

catabolic step, phaeophorbide *a* oxygenase, for which the green flesh mutant of tomato is clearly competent. The conversion of phaeophorbide *a* into the primary fluorescent catabolite, pFCC-2, indicates that not only the oxygenase but also the second enzyme of the channelled reaction, RCC reductase (Rodoni *et al*., 1997), is present in *gf*. Analysis of chlorophyll breakdown products in ripe fruit showed (Table 2A) that *gf* accumulates chlorophyllides *a* and *b* but phaeophorbide a does not accumulate *in vivo*. This may indicate that the action of phaeophorbide *a* oxygenase is somehow prevented in the mutant *in vivo*, thereby blocking the breakdown of chlorophyll. Since the phaeophorbide *a* oxygenase system is present in the tissues, the lesion of *gf* is probably associated with altered accessibility or transport of components or the absence of another factor required for the reaction.

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