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Analysis of the chlorophyll catabolism pathway in leaves of an introgression senescence mutant of *Lolium temulentum*

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Abstract

Pigments, proteins and enzyme activity related to chlorophyll catabolism were analysed in senescing leaves of wild-type (WT) *Lolium temulentum* and compared with those of an introgression line carrying a mutant gene from stay-green (SG) *Festuca pratensis*. During senescence of WT leaves chlorophylls *a* and *b* were continuously catabolised to colourless products and no other derivatives were observed, whereas in SG leaves there was an accumulation of dephytylated and oxidised catabolites including chlorophyllide *a*, phaeophorbide *a* and 13^2 OH-chlorophyllide *a*. Dephytylated products were absent from SG leaf tissue senescing under a light-dark cycle. Retention of pigments in SG was accompanied by significant stabilisation of light harvesting chlorophyll-proteins compared with WT, but soluble proteins such as Rubisco were degraded during senescence at a similar rate in the two genotypes. The activity of phaeophorbide *a* oxygenase measured in SG tissue at 3d was less than 12% of that in WT tissue at the same time-point during senescence and of the same order as that in young pre-senescent WT leaves, indicating that the metabolic lesion in SG concerns a deficiency at the ring-opening step of the catabolic pathway. In senescent *L. temulentum* tissue two terminal chlorophyll catabolites were identified with chromatographic characteristics that suggest they may represent hitherto undescribed catabolite structures. These data are discussed in relation to current understanding of the genetic and metabolic control of chlorophyll catabolism in leaf senescence.

Keywords: *Lolium temulentum*; *Festuca pratensis*; Poaceae; Catabolism; Senescence; Chlorophyll; Introgression; Mutant; Chlorophyllide; Phaeophorbide; Phaeophorbide *a* oxygenase

1. Introduction

This paper describes the pattern of catabolites and activities of a key enzyme in the pathway of chlorophyll (chl) breakdown in senescing leaf tissue of a mutant genotype of *Lolium temulentum*. Chl catabolism occurs throughout the plant life-cycle and is highly sensitive to both biotic and abiotic stresses (Thomas et al., 2001). The catabolic pathway that accounts for the net loss of chl from green tissues during senescence, ripening and other terminal developmental events culminates in the accumulation of colourless products (non-fluorescent chl catabolites or NCCs – Mühlecker and Kräutler, 1996). The enzyme sequence responsible for NCC formation comprises chlorophyllase, which dephytylates chl *a*, a dechelatase activity which removes Mg from chlorophyllide *a*, and phaeophorbide *a* oxygenase (PaO), which opens the tetrapyrrole macrocycle to produce a red bilin, RCC (Thomas et al., 2001;

Abbreviations: WT: wild-type; SG: stay-green; Chl: chlorophyll; chlide: chlorophyllide; phaeide: phaeophorbide; NCC: non-fluorescent chlorophyll catabolite; FCC: fluorescent chlorophyll catabolite; RCCR: red chlorophyll catabolite reductase; Fd: ferredoxin; G6P: glucose-6-phosphate; LCHP II: light-harvesting chlorophyll protein of photosystem II.

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Hörtensteiner and Feller, 2002). A reductase normally converts RCC immediately to a colourless, fluorescent product FCC and further enzymic and non-enzymic reactions are responsible for metabolising FCC to NCCs in a species-specific manner (Thomas et al., 2001; Hörtensteiner and Feller, 2002). Recently genes for many components of the pathway have been cloned, including chlorophyllases (Jakob-Wilk et al., 1999; Tsuchiya et al., 1999), PaO (Pružinská et al., 2003), RCC reductase (Wüthrich et al., 2000) and an ABC-type transporter able to transfer FCCs into the vacuole (Tommasini et al., 1998). A number of mutations modifying chl catabolism have been described (Thomas and Howarth, 2000; Thomas et al., 2001; Pružinská et al., 2003), including one, originally identified in the pasture grass *Festuca pratensis*, which disables the normal process of yellowing in senescent leaves (Thomas, 1987). *F. pratensis* is a slow-establishing perennial and an obligate outbreeder that has to be handled as genetically heterogeneous populations rather than individual genotypes. Thomas et al. (1999) described introgression of stay-green gene from *F. pratensis* into *Lolium temulentum*, a diploid inbreeder quick to establish, fast-growing with short generation time, in which it is easy to establish isogenic lines. In the present paper we describe the consequences for the normal pathway of chl catabolism in *L. temulentum* of introducing the stay-green gene from *F. pratensis*.

2. Materials and methods

2.1. Plant material and chemical treatments

Wildtype (WT) and stay-green (SG) lines of *Lolium temulentum* L. Ceres, near isogenic for an introgression from a senescence mutant of *Festuca pratensis* Huds., were generated as described by Thomas et al. (1999). Plants were grown from seed on vermiculite, 7 plants per 5 in. pot, in a controlled environment providing a constant 20 °C and 8/16 h light/dark cycle (light flux 350 μmol m^{-2} s^{-1}). Plants were fed with a nutrient solution as described by Gay and Hauck (1994). The laminae of leaves 4 and 5 were harvested when the sixth leaf was fully expanded, approximately nine weeks after sowing and cut into approximately 3 cm long segments. The leaf tissue was allowed to senesce in darkness for 0, 3, 5 and 7 d at 20 °C. Harvested tissues were weighed, immediately frozen in liquid nitrogen and stored at −80 °C until extracted. Three replicate samples of each age were taken.

2.2. Induced senescence in intact plants

At about 9 weeks after sowing, 14 plants of each genotype (WT and SG) were transferred to continuous darkness (20 °C) while another 14 plants of WT and of SG remained in short daylength conditions. At 7 d after the start of dark treatment, leaves 4 and 5 were excised, weighed, immediately frozen in liquid nitrogen and stored at −80 °C until extracted. Three replicate samples of each age and leaf were taken.

2.3. Pigment extraction and identification

The frozen segments were placed in a mortar and ground to a fine powder in the presence of liquid nitrogen and quartz sand. The pigments were extracted with 2 ml of 80% acetone and, after centrifugation at 10,000 rpm for 10 min at 4 °C, chls and related pigments were determined by HPLC. Pigments were identified from their spectral absorption maxima and peak ratios and by HPLC co-chromatography with authentic samples. Identification of 13^2 hydroxy chlorophyllide *a* is tentative, based on spectral absorption maxima and peak ratios. On-line UV–visible spectra were recorded from 350 to 700 nm with a photodiode array detector. HPLC was performed on a Nova-Pak C18 4 μm Radial-Pak cartridge 8 mm × 100 mm column using Waters 515 HPLC pumps and a Waters model 996 photodiode array detector. The manual injection valve (Rheodyne, model 7725I) was fitted with a 20 μl loop. Separation was carried out using an elution gradient (2 ml min^{-1}) with the mobile phases (A) ion pair reagent/methanol (1:4 v/v) and (B) acetone/methanol (1:4 v/v). The ion pair reagent was 1 M ammonium acetate in water (Langmeier et al., 1993). The gradient was isocratic A 4 min, A to B 5 min, isocratic B 9 min, return to A 2 min (Siefermannharms, 1987) and detection was at 660 nm. Analytically-pure samples of chl *a* and *b* were used to obtain the calibration slopes representing the area of the peak obtained with different injected volumes of pure solutions of known concentration. The same preparation was acidified and used for calibration with regard to phaeophorbides (phaeides). For quantification of chlorophyllides (chlides) it was assumed that dephytylation does not change the spectral properties of the porphyrin moiety (Langmeier et al., 1993).

2.4. Assay of phaeophorbide a oxygenase

Phaeide *a* oxygenase was measured using a coupled assay with RCC reductase (RCCR) according to previous protocols (Rodoni et al., 1997; Hörtensteiner et al., 1995; Wüthrich et al., 2000). Reaction mixtures contained phaeide *a* as substrate and the following cofactors: ferredoxin (Fd); NADPH; glucose-6-phosphate (G6P); G6P dehydrogenase; and Fd-NADPH-oxidoreductase. Chloroplast membranes isolated either from canola cotyledons (PaO; Hörtensteiner et al., 1995) or from *Lolium temulentum* (SOL) were used as a source of PaO. The source of RCCR was either Arabidopsis

RCCR expressed in *E. coli* (Wüthrich et al., 2000) or soluble chloroplast fraction from *Lolium temulentum* (S1). After incubation for 1 h at 25 °C, the reactions were terminated with methanol at a final concentration of 60%. The production of pFCC from phaeide *a* was followed by reverse-phase HPLC (Rodoni et al., 1997; Wüthrich et al., 2000) using an isocratic gradient: 20%A:80%B, where solvent A was 50 mM potassium phosphate, pH 7.0 and solvent B was water:methanol:potassium phosphate (10:10:80, v:v:v). Under these conditions the reaction product pFCC-2 eluted after 7.0 min and was quantified as integrated fluorescence units (FU_{pFCC}; Hörtensteiner et al., 1995).

2.5. Analysis of non-fluorescent chlorophyll catabolites

Fresh material was homogenised in liquid nitrogen and extracted into 3 volumes of 0.05 M Tris–HCl, pH 8.0:methanol (1:4, v/v). After centrifugation, the supernatant was analysed by HPLC (Ginsburg and Matile, 1993) on a Hypersil ODS C18 5 μm, 4.6 mm × 250 mm column. Non-fluorescent chl catabolites (NCCs) were eluted with a linear gradient of solvent B (20% (v/v) 25 mM potassium phosphate buffer, pH 7.0, and 80% methanol) in solvent A (50 mM potassium phosphate, pH 7.0) as follows: 50% to 100% over 15 min, 100% solvent B for 8 min, return to 50% solvent B in 3 min and finally 50% solvent B for 4 min. NCCs were identified by their spectral absorption and comparison with those from *Brassica napus* (Mühlecker and Kräutler, 1996).

2.6. Protein extraction and electrophoresis

Frozen leaf material was ground to a fine powder with liquid N_2 in a mortar and pestle. The powder was homogenised with 4 ml g^{-1} fresh weight of extraction buffer (EB; 20 mM Na phosphate pH 7.5 containing 0.1% (v/v) 2-mercaptoethanol, 1.0% (w/v) polyvinylpyrrolidone). The homogenate was filtered through two layers of miracloth. The extract was divided into two parts. One half was centrifuged 2 min at 15,000*g* to separate soluble proteins (supernatant) and membrane proteins (pellet). After resuspending the pellet in EB at the original volume, all fractions were mixed with 25% (v/v) 125 mM Tris–HCl pH 6.8 containing 5% (w/v) SDS, 25% (v/v) glycerol, 0.1% (w/v) bromophenolblue and 20% (v/v) 2-mercaptoethanol. The fractions were heat-denatured by boiling for 5 min, cooled and centrifuged at 12,500 × g for 2 min. Proteins were fractionated on 12.5% SDS polyacrylamide gels (Laemmli, 1970) using a Protean II-gel system (BioRad). Gels were stained with Coomassie Brilliant Blue.

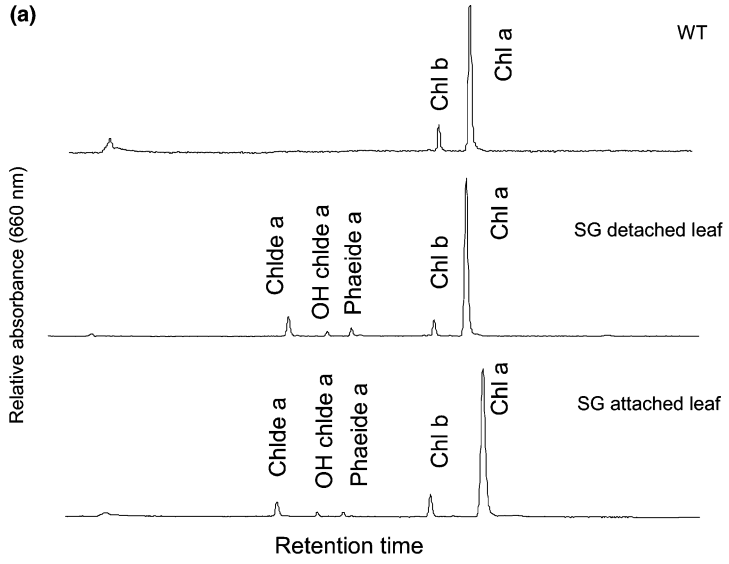
3. Results and discussion

3.1. Chlorophyll and derivatives in senescent leaves

The pigment complements of *Lolium temulentum* WT and SG leaves change in a characteristic way during senescence. Fig. 1 presents typical chromatograms of chls and phaeide pigments in senescing tissue and summarises the chromatographic and spectroscopic characteristics of all the major pigments detected. During senescence of excised (Fig. 1) or intact (not shown) WT leaves, chls *a* and *b* are continuously catabolised to colourless products and consequently no other derivatives were observed. In SG leaves, on the other hand, there was an accumulation of chl catabolites, notably chlide *a*, phaeide *a* and (tentatively) 13^2 hydroxy chlide *a*. The pattern in excised tissue was qualitatively identical to that of senescing leaves on the intact plant (Fig. 1). Slight differences in retention times for chlorophyll *a* in the three treatments (Fig. 1(a)) are due to small variations in column pressure and external temperature between different runs; calculation of the retention factor *k* for each compound eliminates this variation (Fig. 1(b)). Spectroscopic properties of each pigment (peak wavelengths and peak height relationships; Fig. 1(b)) were the same for all tissues and treatments where the pigment was detected.

3.2. Senescence of excised leaf tissue

Senescence was induced by incubating excised leaf tissue in darkness. At the time of harvest, leaf 6 was fully expanded, leaf 5 had achieved maturity about a week earlier and leaf 4 was a further week older and beginning to senesce. Thus segments of WT leaves 4 and 5 would include a proportion of tissue in which yellowing was underway and would be expected to yield lower initial pigment contents than SG at day 0. Table 1 shows that this was the case. Over the course of 7 d, chl *a* and *b* declined steadily in WT and no other related pigments were detected, whereas in SG leaves there was a progressive accumulation of dephytylated chl catabolites (Table 1). Chlide *a* was the first catabolite to accumulate, followed later by phaeide *a*. Based on enzyme measurements, Vicentini et al. (1995) proposed that the metabolic lesion in the SG mutant of *Festuca pratensis* is located in PaO or a regulator. Phaeide *a*, the intermediate immediately upstream of the blockage, would therefore be expected to be the first product to accumulate, but this was not observed. The earlier appearance of chlide *a* could be the result of a negative feedback regulation from the PaO step of chl catabolism. No dephytylated derivative of chl *b* was observed at any stage during senescence. This result agrees with the proposal that, at some early point in the catabolic sequence, chl *b* is transformed into chl *a* prior to its



(b)

Pigment	k[a]	Position of peak (nm)			Peak height relationship[b]
		I	II	III	
Chlorophyllide *a*	4.27	432	616	664	1.3
OH chlde *a*[c]	5.10	432	616	664	1.3
Phaeophorbide *a*	5.63	410	505	665	1.8
Chlorophyll *b*	7.40	466	600	650	3.3
Chlorophyll *a*	8.10	432	616	664	1.3

Fig. 1. HPLC analysis of chlorophylls from senescent leaves of *Lolium temulentum* wild-type (WT) and stay-green (SG). (a) Chromatograms, (b) chromatographic and spectroscopic characteristics of chlorophyllous catabolic pigments.

Table 1
Pigment contents of excised leaf segments senescing in darkness

Pigments	WT			
	0 days	3 days	5 days	7 days
Chlde *a*				
Chlde *a*′				
OH chlde *a*				
Phaeide *a*				
OH chl *a*	1.85 ± 0.14			
Chl *b*	153.68 ± 11.31	124.30 ± 15.77	77.36 ± 17.13	51.31 ± 0.27
Chl *a*	442.73 ± 35.28	354.54 ± 48.04	218.27 ± 58.34	152.77 ± 7.12
Total	598.26 ± 46.73	478.84 ± 63.81	295.63 ± 75.46	204.08 ± 6.85
Chl *a*/*b*	2.89 ± 0.03	2.85 ± 0.02	2.81 ± 0.01	2.98 ± 0.15
	SG			
Chlde *a*		13.28 ± 1.64	22.15 ± 0.44	15.77 ± 9.00
Chlde *a*′			2.55 ± 0.20	2.79 ± 0.59
OH chlde *a*			4.26 ± 0.17	8.85 ± 3.44
Phaeide *a*			15.81 ± 0.75	24.11 ± 3.09
OH chl *a*	3.23 ± 0.23			
Chl *b*	261.96 ± 20.08	203.36 ± 8.60	119.62 ± 20.09	124.24 ± 4.93
Chl *a*	761.95 ± 56.41	698.78 ± 32.60	414.39 ± 94.43	431.50 ± 3.23
Total	1027.14 ± 76.72	915.42 ± 42.83	577.53 ± 116.08	607.26 ± 24.28
Chl *a*/*b*	2.91 ± 0.04	3.44 ± 0.01	3.45 ± 0.21	3.48 ± 0.11

Abbreviations: Chlde *a*: chlorophyllide *a*, OH chlde *a*: 13^2 hydroxy chlorophyllide *a*, phaeide *a*: phaeophorbide *a*, OH chl *a*: 13^2 hydroxy chlorophyll *a*, chl *b*: chlorophyll *b*, chl *a*: chlorophyll *a*, total: total chlorophylls, chl *a*/*b*: ratio chlorophyll *a*/*b*, WT: wild-type, SG: stay-green. Pigment contents given as μg g^{-1} fwt ± SD.

degradation, most probably through the activity of chl *b* reductase rather than chlide *b* reductase (Scheumann et al., 1996, 1999). Although pigment catabolism is severely disrupted in the SG genotype, there was significant loss of chl over the period of the experiment, not all of which was accounted for by the appearance of dephytylated, dechelated and oxidised products (Table 1). Chl *b* was proportionately less stable than chl *a* and this was reflected in a significant increase in the chl *a*/*b* ratio in SG, whereas in WT the ratio remained relatively stable and increased only slightly at 7 d.

3.3. Proteins in senescent leaves

Previous studies of stay-green genotypes have found that retention of chl is accompanied by selective retention of leaf proteins (Bachmann et al., 1994; Thomas et al., 1999). Total, soluble and membrane proteins were extracted from WT and SG leaf tissue at various times after excision and separated by gel electrophoresis. Gel separations are presented in Fig. 2. Based on many previous studies of *Lolium* and *Festuca* leaf proteins, correlating total protein patterns with immunoblots, the

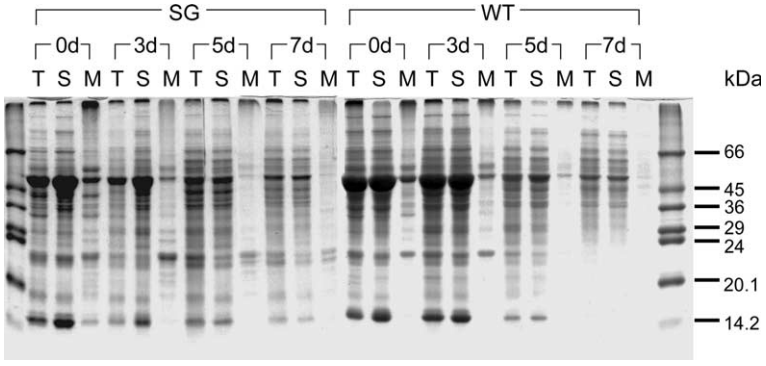


Fig. 2. SDS–polyacrylamide gel electrophoresis separations of proteins from WT and SG *L. temulentum* excised leaves during senescence. T: total protein, S: soluble protein, M: membrane protein.

Table 2
Pigment contents of attached leaves senescing in the dark (leaf 4[a])

Pigments	WT			
	LIGHT		DARK	
	Day 5	Day 7	Day 5	Day 7
Chlde *a*				
OH. chlde *a*				
Phaeide *a*				
OH chl *a*	2.08 ± 0.44	1.07 ± 0.62		
Chl *b*	95.08 ± 27.00	44.95 ± 2.73	97.40 ± 22.48	30.13 ± 14.13
Chl *a*	229.69 ± 62.58	96.27 ± 22.77	278.07 ± 60.43	69.49 ± 41.76
Total	325.82 ± 90.02	142.29 ± 26.01	375.47 ± 82.91	99.58 ± 55.88
	SG			
Chlde *a*			16.38 ± 9.63	54.40 ± 5.95
OH chlde *a*			6.80 ± 0.57	12.29 ± 2.69
Phaeide *a*			3.05 ± 0.21	49.44 ± 16.29
OH chl *a*	3.93 ± 0.03	3.04 ± 0.00		
Chl *b*	246.13 ± 3.55	180.88 ± 21.06	219.72 ± 44.87	187.51 ± 20.50
Chl *a*	658.46 ± 1.98	481.81 ± 66.24	737.11 ± 160.75	613.72 ± 21.23
Total	919.71 ± 5.56	665.73 ± 87.30	983.06 ± 216.03	917.36 ± 66.66

Abbreviations: Chlde *a*: chlorophyllide *a*, OH chlde *a*: 13^2 hydroxy chlorophyllide *a*, phaeide *a*: phaeophorbide *a*, OH chl *a*: 13^2 hydroxy chlorophyll *a*, chl *b*: chlorophyll *b*, chl *a*: chlorophyll *a*, total: total chlorophylls, WT: wild-type, SG: stay-green. Pigment contents given as μg g^{-1} fwt ± SD. LIGHT: plants grown in short daylength conditions for nine weeks and senesced in short daylength conditions for 5 or 7 days more. DARK: plants grown in short daylength conditions for 9 weeks and senesced in dark conditions for 5 or 7 days more.

[a] Similar results in leaf 5.

identities of major components can be assigned. The soluble protein fraction is dominated by Rubisco, the large subunit of which appeared as a densely-stained band at about 50 kDa. The total amount of this polypeptide decreased progressively during senescence, at a similar rate in WT and SG. In the membrane fraction, the main constituents correspond to the light-harvesting proteins of photosystem II (LHCP II) and appear as a tight group of polypeptides between 20 and 24 kDa. LHCP II disappeared completely after 5 d of senescence in WT tissue, whereas in SG these components were still clearly visible after 7 d. Total proteins represented the sum of both fractions (soluble and membrane), and as a consequence the differences between genotypes were more evident after 5 d of senescence than earlier. These results agree with the pattern found in the *F. pratensis* mutant that was the source of the introgressed SG gene (Thomas, 1977). Protein behaviour in *L. temulentum* WT and SG is consistent with the proposition that retention of chl during senescence in SG stabilizes the proteins of the pigment–proteolipid complexes of thylakoids (Thomas, 1997).

3.4. Dark-induced senescence in intact plants

The possibility that excision might introduce misleading artefacts into the pattern of chl degradation was investigated by comparing leaves of intact plants that had been transferred to darkness for up to 7 d with those of plants remaining under the control photoperiod. Pigment complements of the two genotypes (Table 2) were similar to those obtained with detached leaves (Table 1). SG plants kept in darkness retained higher levels of chl pigments than the leaves of WT plants, which had become visibly yellow by 7 d compared with WT plants under light. The SG genotype accumulated dephytylated chls during dark senescence, and their amounts increased with treatment time, whereas only chls *a* and *b* were detected in WT leaves. Significantly, no traces of dephytylated chls were found in SG leaves from the light treatment, not only over the timescale of the present experiment (Table 2) but even in older tissue showing other symptoms of senescence (data not shown). The fate of chl derivatives in SG leaves under illumination is currently under investigation.

Detached or excised leaf tissues are convenient subjects for studies of foliar senescence, but there is a long history of scepticism about the validity of such model systems. Nevertheless it is certain that the mechanism of degradation of chl, a central process of senescence, is essentially identical in excised and intact leaves. Ginsburg and Matile (1993) showed that the enzymology of chl catabolism is the same in canola cotyledons whether they yellowed during normal development of whole seedlings or else were detached and incubated in darkness. The data of Fig. 1 are consistent with this proposition. Leaves of the mutant line described in the present paper express the stay-green trait in intact plants (Hauck et al., 1997), excised entire laminae (Thomas and Matile, 1988) or leaf segments. Any artefact introduced by excision will be unrelated to the SG lesion and therefore will be shared by both mutant and WT tissue. This means that comparison of the two genotypes will reveal mechanisms and sensitivities directly related to the process of chl and pigment-protein breakdown in all modes of leaf senescence.

3.5. PaO activity in L. temulentum genotypes

Accumulation of chlide and phaeide (Fig. 1, Table 1) confirms that SG *L. temulentum* is competent with regard to chlorophyllase and Mg dechelatase. It has been shown previously (Vicentini et al., 1995) that the lesion in the SG genotype of *F. pratensis* is located in PaO or in a PaO regulator. To investigate this further, we measured PaO activity from WT and SG *L. temulentum*. No PaO activity was detected in SG tissue at day 0, whereas WT tissue yielded 5 times the activity of young leaves of the same genotype (Table 3). This is consistent with the data in Table 1 suggesting that WT leaves at day 0 had already initiated senescence. PaO activity was detected in SG tissue at day 3 but the activity was of the same order as that in young pre-senescent WT leaves and less

Table 3
PaO activity in *Lolium temulentum* leaves

	PaO source		RCCR source		FU_{pFCC}
	SOL	PaO[a]	S1	RCCR[b]	
Control	–	+	–	+	39,752
SG 0 days	SG 0 days	–	SG 0 days	–	–
WT 0 days	WT 0 days	–	WT 0 days	–	10,231
SG 3 days	SG 3 days	–	SG 3 days	–	3669
WT 3 days	WT 3 days	–	WT 3 days	–	31,279
WT young leaves	WT young leaves	–	–	+	2033
	WT 3 days	–	SG 3 days	–	30,417
	SG 3 days	–	WT 3 days	–	3332

Abbreviations: [a]PaO: phaeophorbide a oxygenase from canola cotyledons (Hörtensteiner et al., 1995), used as a standard for FCC production, [b]RCCR: RCC reductase from Arabidopsis RCCR expressed in *E. coli*, SOL: solubilized thylakoids from *Lolium temulentum* used as PaO source, S1: first supernatant from *Lolium temulentum* chloroplast extractions used as RCCR source. $FU_{pFCC:}$ integrated fluorescence units.

than 12% of WT tissue at day 3. There may be a basal PaO capacity present throughout leaf development in both genotypes, perhaps associated with chl turnover not directly associated with senescence (Thomas, 1997) and a senescence-related elevation of (either the same, or a qualitatively different) activity which is responsible for yellowing in WT and which is missing from SG. The residual basal PaO in senescing SG leaves would account for partial net loss of chl in this genotype. Pružinská et al. (2003) also found low but detectable PaO activity in pre-senescent Arabidopsis leaves. It is necessary to draw conclusions from these experiments with caution, since the complex assay system, which comprises a PaO-enriched membrane fraction and a soluble protein source of RCC reductase as well as a cocktail of cofactors (Rodoni et al., 1997; Hörtensteiner et al., 1995; Wüthrich et al., 2000), does not give absolutely quantitative results in classical enzymology terms. To exclude the possibility that the stay-green phenotype in *L. temulentum* was due to a mutation in RCCR, we performed a crossover experiment, using PaO from WT and RCCR from SG and vice versa. The results (Table 3) show that the RCCR source did not influence the total amount of PaO activity, and the PaO activity measured with RCCR from 3d SG was the same as with RCCR from 3d WT.

3.6. Terminal chlorophyll catabolites in wild-type and stay-green L. temulentum

The presence of two NCCs in *L. temulentum* senescent leaves (Fig. 3(a)) was deduced from their typical absorbance spectra (Fig. 3(b); Kräutler et al., 1991). The structures of these catabolites remain unresolved, but their retention times on HPLC, indicating that they are relatively polar and do not co-migrate with canola or maize NCCs, suggested they represent novel NCC structures. In accordance with the naming convention for catabolites introduced by Ginsburg and Matile (1993), the *L. temulentum* catabolites were tentatively identified as *Lt*-NCC-1 and -2, respectively. NCCs were found both in WT and SG genotypes, which is consistent with other data presented here that the chl catabolic pathway is partially operational in *L. temulentum* SG, in contrast to other stay-greens (Bachmann et al., 1994; Pružinská et al., 2003).

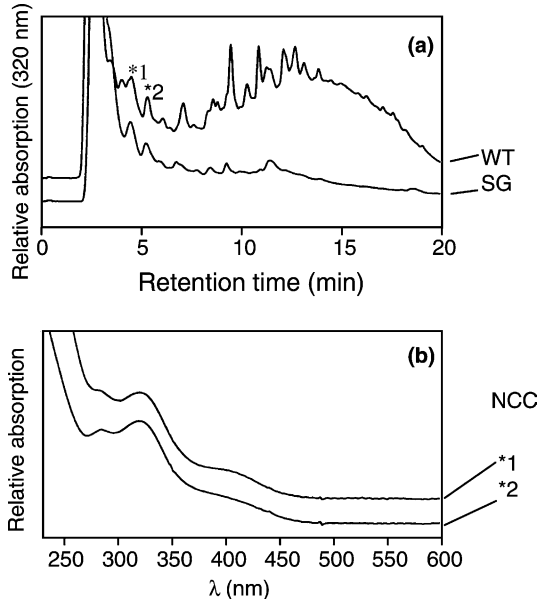


Fig. 3. Non-fluorescent (terminal) chl catabolites in senescent leaves of *Lolium temulentum* wild-type (WT) and stay-green (SG). (a) Chromatogram, (b) spectrum, *1, *Lt*-NCC-1; *2, *Lt*-NCC-2.

4. Conclusions

Introgression of a stay-green gene from *F. pratensis* changes chl catabolism during leaf senescence in *L. temulentum* in a characteristic way. In darkness chl *a* and *b* are no longer continuously metabolised but instead accumulate as a series of catabolites upstream of the macrocycle-opening step in the breakdown pathway. Enzyme measurements confirm that there is a deficiency in the activity of phaeide *a* oxygenase. The gene for this enzyme has recently been cloned (Pružinská et al., 2003) but initial evidence indicates that this gene does not co-locate with the stay-green locus on the *Lolium* genetic map (I. Donnison, personal communication). Genetic and metabolic control of the ring-opening step of chl catabolism is turning out to be more complex than previously thought and further studies underway on the SG locus in *Lolium-Festuca* are revealing new mechanisms.

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