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Effects of a *Stay-green* Mutation on Plant Nitrogen Relations in *Lolium perenne* During N Starvation and after Defoliation

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The *stay-green* mutation of the nuclear gene *sid* results in inhibition of chlorophyll degradation during leaf senescence in grasses, reducing N remobilization from senescing leaves. Effects on growth of *Lolium perenne* L. were investigated during N starvation (over 18 d) and after severe defoliation, when leaf growth depends on the remobilization of internal N. Rates of dry matter production, partitioning between shoots and roots, and re-partitioning of N from shoots to roots were very similar in *stay-green* and normal plants under N starvation. K_m and V_{max} for net uptake of NH_4^+ were also similar for both genotypes, and V_{max} increased with the duration of N deprivation. The mutation had little effect on recovery of leaf growth following severe defoliation, but *stay-green* plants recommenced NO_3^- and K^+ uptake 1 d later than normal plants. Import of remobilized N into new leaves was generally similar in both lines. However, *stay-green* plants remobilized less N from stubble compared with normal plants. It was concluded that the *sid* locus *stay-green* mutation has no significant adverse effect on the growth of *L. perenne* during N starvation, or recovery from severe defoliation when plants are grown under an optimal regime of NO_3^- supply both before and after defoliation. The absence of any effect on leaf dry matter production implies that the difference in foliar N availability attributable to this mutation has little bearing on productivity, at least in the short to medium term.

Key words: Ammonium uptake, defoliation, flowing solution culture, *Lolium perenne* L., nitrogen remobilization, nitrogen starvation, perennial ryegrass, *stay-green* mutant.

INTRODUCTION

The non-yellowing or *stay-green* phenotype originally identified in a population of the S215 cultivar of *Festuca pratensis* Huds. results from a mutation in a single nuclear gene, *sid*, leading to suppression of the degradation of chlorophyll and pigment-binding thylakoid proteins in senescing leaves (Thomas, 1982, 1987; Davies *et al*., 1990; Thomas *et al*., 1999). However, functional senescence proceeds on a normal time scale. Several applications for this mutation have been suggested in forage production and amenity grassland systems (Thorogood, 1996; Humphreys and Thomas, 1998; Thomas and Howarth, 2000).

Chlorophyll and thylakoid proteins account for about 25% of the total N content of mature leaves (Evans, 1988; Peoples and Dalling, 1988). Abnormally high senescent leaf N concentrations have been reported in *stay-green* mutants of *F. pratensis* Huds. (Hauck *et al*., 1997) and *Lolium perenne* L. (Bakken *et al*., 1997) under a range of N supply regimes. However, the impact of the enhanced retention of leaf N in this *stay-green* mutant on whole plant N relations and growth has not been investigated in detail (see Thomas and Smart, 1993). A negative effect might be expected when the biosynthetic requirement for N in new tissue depends, at least temporarily, on the availability of N recycled from mature and senescing tissues (Bakken *et al*., 1997); for example, following termination of the external supply of N, or after severe defoliation. In the first case, comparison of *stay-green* and normal lines of *L. perenne* revealed no differences in dry matter production after 12 d without NO_3^-, although both V_{max} for net uptake of NO_3^- and the 'sink strength' of shoots for N were increased in the *stay-green* line (Bakken *et al*., 1997). Similarly, no differences in growth or NO_3^- uptake were observed between *stay-green* and normal lines of *F. pratensis* grown in flowing nutrient solutions at constant concentrations between 5 and 100 μM NO_3^-, although *stay-green* plants had slightly lower growth rates in conventional solution culture (Hauck *et al*., 1997). Whilst these results suggest this *stay-green* mutation has little impact on growth rate, the severity of N limitation may have been insufficient for differences in availability of internal N between *stay-green* and normal plants to become limiting.

Uptake of NH_4^+-N is significant in grasses grown under grazing management and subjected to episodes of NH_4^+-dominated N supply (Ryden *et al*., 1984; Jarvis *et al*., 1989). The impact of the *stay-green* mutation on the performance of plants subjected to NH_4^+ nutrition has not been investigated. It would be expected that any increase in V_{max} for net uptake of NH_4^+ attributable to prior N starvation (Lee and Rudge, 1986) would be greater in *stay-green* compared with normal plants as a consequence of lower levels of transport-regulating N compounds in their roots.

Following severe defoliation by herbivores or agricultural machinery, uptake of mineral N by grasses declines very rapidly and, in many grass species, the N required for synthesis of new leaf tissue is supplied by the remobilization of N from remaining tissues for several days, until uptake of

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N recovers (Ourry *et al*., 1994; Volenec *et al*., 1996; Cliquet *et al*., 1997). Temporal patterns of N remobilization from amino acids and proteins in the remaining leaves, stubble and root following defoliation have been described in *L. perenne* (Ourry *et al*., 1988; Millard *et al*., 1990; Wilkins *et al*., 1997).

The objectives of the present work were to determine whether the *stay-green* mutation affects growth and plant N relations under (*a*) prolonged N starvation and (*b*) after severe defoliation, by comparing *stay-green* and normal lines of *L. perenne* grown under controlled nutritional conditions in flowing solution culture. The experimental conditions imposed during the N starvation study (expt 1) differed from those of Bakken *et al*. (1997) in that (1) the duration of the experimental period following termination of N supply was greater and (2) N was previously supplied as NH_4^+ rather than NO_3^--N. In the second experiment (expt 2), remobilization of N and recovery of leaf growth following severe defoliation by *stay-green* and normal lines were compared under non-limiting conditions of mineral N supply. Steady-state $^{15}NO_3^-$-labelling prior to defoliation discriminated between leaf N remobilized from tissues remaining after defoliation and that derived from uptake of mineral N (supplied as NO_3^-) after defoliation. It was anticipated that leaf growth by *stay-green* plants would be slower, due to their reduced capacity for N remobilization from the stubble tissues remaining after defoliation. Consequently, uptake of NO_3^- by *stay-green* plants was expected to recover earlier.

MATERIALS AND METHODS

Plant material

The *sid* mutant, originally characterized in *F. pratensis* (Thomas and Smart, 1993), was transferred to perennial ryegrass (*L. perenne* L.) using *Festuca–Lolium* intergeneric crossing procedures (Humphreys and Thorogood, 1993). A homozygous *sid* mutant donor genotype of *L. perenne* (provided by D. Thorogood, IGER, UK) was crossed into a high water soluble carbohydrate accumulating line of perennial ryegrass, based on a cross between the cultivars Aurora and Melle (Humphreys, 1989), and a segregating F_2 population was produced. From the F_2 population 45 *stay-green* and 45 yellowing phenotypes were selected and polycrossed for seed production. The recessive *sid* mutant was fixed in the *stay-green* phenotype selection but among the plants selected for a yellowing phenotype homozygous non-mutant and heterozygous plants were expected in a 1 : 2 ratio. Therefore, further selection based on progeny tests was made within the yellowing polycross progeny for homozygous normal plants and within *stay-green* polycross progeny for confirmed homozygous *sid* mutant genotypes. Seed from these lines (*stay-green* IGER ref. 94/7; normal IGER ref. 94/17) was used in both experiments.

Growth conditions

In both experiments seeds of the normal and *stay-green* lines of *L. perenne* were sown in two separate plant culture units of a system of flowing solution culture inside a glasshouse (Clement *et al*., 1974; Hatch *et al*., 1986). Each culture unit held 200 l of recirculating nutrient solution and 24 culture vessels, within each of which seedlings were thinned on emergence to give 12 plants. Plants were grown under natural light until 28 d after sowing, at which time artificial lighting was provided each day (0800–1800 h) by a 400 W HPI-T and 400 W SON-T (Philips) lamp over each culture unit (550 ± 50 μmol m^{-2} s^{-1} PAR at the top of the canopy). Air temperature was 20–23 °C/15 °C day/night; solution temperatures were 20–21 °C.

Experiment 1. The initial composition of the flowing nutrient solutions was (μM): NO_3^-, 250; K^+, 250; Ca^{2+}, 344; SO_4^+, 550; Mg^{2+}, 100; $H_2PO_4^-$, 50; Fe^{2+}, 5·4; with micronutrients as in Clement *et al*. (1978*a*). Plants depleted these concentrations over 28 d following sowing by approx. 75%, but the solution pH was maintained at 6·0 ± 0·2 by daily addition of 500 mM H_2SO_4 as required. On day 28 the culture units were drained and refilled with fresh nutrient solutions having initial concentrations (μM): K^+, 20; Ca^{2+}, 220; SO_4^+, 330; Mg^{2+}, 100; $H_2PO_4^-$, 50; Fe^{2+}, 5·4; with micronutrients as before. The N supply was changed to 20 μM NH_4^+ at this time. Thereafter, the concentrations of NH_4^+ and K^+ were monitored automatically on a 27 min cycle by an ion selective electrode system (Hatch *et al*., 1986) and flame photometer (modified EEL mod. 100, Evans Electroselenium Ltd, London, UK), respectively, and maintained automatically at 20 ± 2 μM by delivery of $(NH_4)_2SO_4$ and K_2SO_4. All other nutrients were supplied automatically in fixed ratios to the uptake of NH_4^+. Following termination of the NH_4^+ supply at the start of the N starvation period on day 35, the supply of other nutrients was linked in a similar way to the supply of K^+. From day 28 onwards, a pH of 6·0 ± 0·05 was maintained by automatic delivery of $Ca(OH)_2$ or H_2SO_4 as required.

Experiment 2. The composition of the nutrient solution was as for expt 1 until day 28, except that N was supplied as ^{15}N-labelled NO_3^- at an initial concentration of 250 μM (measured as 1·932 ± 0·001 atom % ^{15}N). The culture units were drained and refilled on day 28 as for expt 1, except that N was supplied subsequently as 20 μM NO_3^- (1·932 atom % ^{15}N). Thereafter, concentrations of NO_3^- and K^+ were maintained automatically at 20 ± 2 μM by delivery of $Ca(NO_3)_2$ (1·932 atom % ^{15}N) and K_2SO_4, as described for expt 1. All other nutrients were supplied in fixed ratios to the supply of NO_3^-.

Net uptake of NH_4^+ (until the supply was terminated) and K^+ in expt 1, and of K^+ and NO_3^- in expt 2, was given by the quantities delivered to each culture unit to maintain the set-point concentrations. Time courses for cumulative uptake of NO_3^- were fitted by fourth order polynomials (Ross, 1987), differentiated to give net daily uptake rates and then divided by root fresh weights, interpolated from exponential functions fitted to harvest data, to estimate mean daily unit absorption rates of NO_3^-.

Measurements during N starvation

Changes in the kinetics of NH_4^+ uptake during N starvation (days 0, 4, 7, 11, 14 and 18) were assessed in expt 1 by a 'short-term' depletion technique adapted from Laine *et al*. (1993). On each occasion three randomly selected culture vessels (36 plants) of each genotype were transferred from flowing solution culture into separate polyethylene containers with their roots submerged in either 1 l (days 0, 4) or 2 l (days 7, 11, 14, 18) of vigorously aerated ^{15}N-labelled solution, with an initial concentration of 250 μM NH_4^+ (11·0 atom % ^{15}N) and 10 mM MES buffer (pH 6·0), made up in nutrient solution (–N) identical to that in the flowing culture units. The containers were placed in a temperature-controlled water bath (21–22 °C) and uptake of $^{15}NH_4^+$ was measured by depletion from 250 μM under illumination similar to that in the culture system, beginning 4 h into the photoperiod to coincide with the maximum uptake phase of the diurnal cycle. Samples of uptake solution (3 ml) were taken every 5–15 min, depending on the depletion rate, and analysed concurrently by automated colorimetry (Skalar Analytical) for NH_4^+ using a modified Bertholet reaction (Verdouw *et al*., 1977), until the concentration was < 1 μM. The time required for total depletion varied with run and vessel, ranging between 3 and 8 h. Transpiration was calculated as the difference between initial and final weight of solution (± 0·1 g), allowing for the volume sampled.

Growth parameters, N content and concurrent translocation of absorbed ^{15}N were determined by harvesting plants at the end of each depletion run in expt 1. Plants were immediately separated into three fractions on a per vessel basis: (1) roots, (2) laminae (defined as all foliage removed by cutting at a height of 5 cm above the shoot/root junction), and (3) stubble (consisting of leaf sheaths and stem base enclosing shoot meristematic tissue). Additionally, 12 senescent and 12 young expanding laminae were selected at random from each vessel harvested on days 11 and 14. Fresh weights were recorded and all fractions were freeze-dried, reweighed, and ground in a Moulinex coffee mill and/or ball-milled. Total N and ^{15}N (Barrie and Workman, 1984) were measured in sub-samples (n = 1) using a continuous flow isotope mass spectrometer (Twenty-twenty, Europa Scientific Ltd, Crewe, UK) linked to a C/N analyser (Roboprep CN, Europa Scientific Ltd, Crewe, UK).

Kinetic parameters (V_{max} and K_m) for net uptake of NH_4^+ were calculated on a root fresh weight basis from the depletion data for each culture vessel of plants. Uptake measured between successive sampling times, and expressed as a mean rate, was plotted against the corresponding NH_4^+ substrate concentrations, taken as the means of successive start and end concentrations. The simple Michaelis–Menten model was fitted directly (Fig. P, Ver. 2·7, BIOSOFT), giving best fit values of V_{max} and K_m, although there were several occasions when the model was inappropriate for the data. As a precaution against curve-fitting artefacts arising from 'free-space' and 'transplant shock' (Bloom and Sukrapanna, 1990) phenomena when plants were first transferred into the ^{15}N-labelled solutions, the first two sample intervals were omitted routinely from all data sets prior to fitting the model. Uptake and translocation of the ^{15}N during each 'depletion run' were calculated as described by Bakken *et al*. (1997).

Defoliation and N remobilization

All plants in expt 2 were defoliated *in situ* at 1700 h on day 34 after sowing, by cutting at a height of 5 cm above the shoot/root junction, leaving roots and a 'stubble fraction' (consisting of leaf sheaths and stem base enclosing shoot meristematic tissue) in the culture vessels. The supply of $^{15}NO_3^-$ was terminated 3 h before defoliation, and the concentration of NO_3^- allowed to deplete by uptake (to < 1 μM after 2 h). Immediately after defoliation the NO_3^- (natural abundance ^{15}N) supply was resumed and controlled thereafter at 20 μM.

Normal and *stay-green* plants were harvested at intervals over 15 d after defoliation for analysis of growth and N content. Four vessels (48 plants) were harvested 2 h prior to defoliation on day 34 after sowing (day 0 of the regrowth period), and again on days 2, 5, 8, 12 and 15 of regrowth. On each occasion plants were separated into three parts, bulking on a per vessel basis: (1) laminar regrowth above the 5 cm defoliation height, (2) stubble, and (3) roots. Fresh weights were recorded and all fractions were freeze-dried prior to reweighing and grinding in a Moulinex coffee mill and/or ball-mill. Total N and ^{15}N in herbage samples from each replicate vessel (n = 4) were analysed as described above.

Despite steady-state labelling with $^{15}NO_3^-$ (1·932 atom % ^{15}N) from the time of germination until defoliation, the atom % ^{15}N contents of different plant parts varied significantly immediately prior to defoliation (day 0 harvest). The mean (± s.e.) values on day 0 for the *stay-green* genotype were: laminae, 1·889 ± 0·004; stubble, 1·847 ± 0·013; roots, 1·808 ± 0·006 atom % ^{15}N. For the normal genotype the corresponding values were: laminae, 1·885 ± 0·003; stubble, 1·829 ± 0·005; roots, 1·791 ± 0·010 atom % ^{15}N. These deviations in atom % ^{15}N content of the plants probably arose from the effects of unlabelled seed N and foliar absorption of N. Weighted mean values were therefore used for the atom % ^{15}N content of the combined source tissues of remobilizable N at the time of defoliation: these were 1·823 atom % ^{15}N for *stay-green* and 1·808 atom % ^{15}N for normal types.

N absorbed prior to defoliation and subsequently remobilized and/or translocated directly into new laminae was assumed to originate from both stubble and root fractions. Furthermore, isotopic fractionation was assumed to be insignificant (Hauck and Bremner, 1976). Cumulative net remobilization of N into the laminae was calculated from the measured ^{15}N contents of the different plant parts at successive harvests (Wilkins *et al*., 1997). The total N content of stubble plus root fractions immediately after defoliation was taken as the theoretical maximum of the potentially remobilizable N pool in the plant. This value was used to express the remobilized N content of laminae measured subsequently as a proportion of the maximum remobilizable pool.

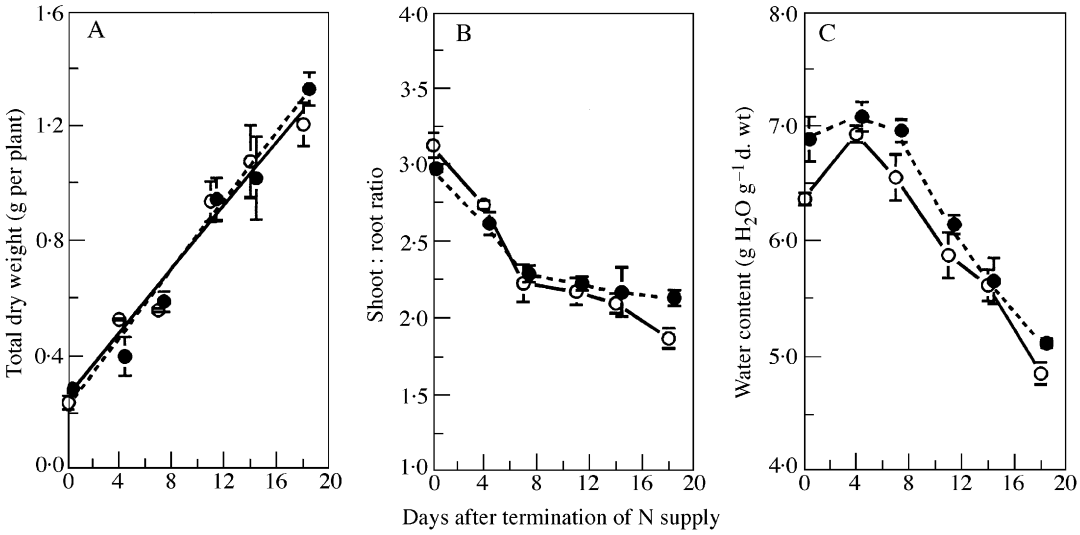

FIG. 1. Changes in total plant dry weight (A), shoot : root dry weight ratio (B), and mean water content of whole plants (C), compared for normal (open circles) and *stay-green* (closed circles) genotypes of *L. perenne* L. following termination of the N supply. Values are means of three culture vessels (36 plants), except on day 18 when nine culture vessels were harvested. Errors are ± s.e.

RESULTS

Growth and N partitioning during N starvation

The *stay-green* mutation had no significant effect on growth measured either as total fresh or dry weight gain over the 18 d of N starvation in expt 1 (Fig. 1A). *Stay-green* plants had higher total dry weights (0·287 ± 0·023 g per plant) than normal plants (0·238 ± 0·023 g per plant) when the supply of N was terminated, although the difference was insignificant (P = 0·17). Only the first leaf was fully senescent in both types. Total dry matter production increased linearly with time in both genotypes (gradient = 0·056 ± 0·005 g per plant d^{-1}, r^2 = 0·97 for normal plants; gradient = 0·060 ± 0·005 g per plant d^{-1}, r^2 = 0·98 for *stay-green*), and differences in gradient and intercept between the two regression lines were insignificant. Both types also showed a similar rapid decline in shoot: root ratios until day 15 (Fig. 1B), after which they remained constant for *stay-green,* but declined further to 1·8 on day 18 in normal plants. In contrast, fresh weight shoot : root ratios were consistently higher in wild type plants. Tissue water contents expressed on a total plant dry weight basis were consistently slightly higher in *stay-green* plants (Fig. 1C).

The partitioning of N between different organs followed a very similar pattern in both types after termination of the NH_4^+-N supply. On termination the total N content of normal plants (14·3 ± 1·3 mg N per plant) was slightly lower (P = 0·26 n.s.) than that of *stay-green* plants (16·3 ± 0·3 mg N per plant). The N content of the stubble subsequently declined until day 7 and remained almost constant thereafter. In contrast, the N content of the root increased progressively from day 7 onwards. The total N content of the leaf fraction showed no clear overall trend over time. Expressed as a proportion of the N content of the whole plant, the change in root N content (Fig. 2) occurred in three phases. Phase 1 (days 0–4) was characterized by a rapid decline in the N content of the roots; phase 2 (days 4–11) by a modest accumulation of N, and phase 3 (days 11–18) by rapid accumulation of N in the roots. The increased allocation of N to the roots as N deficiency intensified was also shown in the net translocation of ^{15}N measured during the $^{15}NH_4^+$ 'depletion runs'. Between days

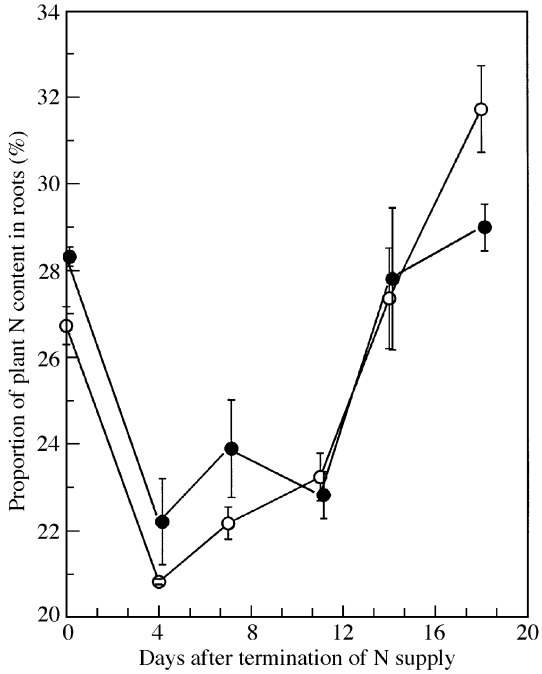

FIG. 2. Changes in the proportion of the total N content of the plant allocated to the roots of normal (open circles) and *stay-green* (closed circles) genotypes of *L. perenne* L. following termination of the N supply. Values are means of three culture vessels (36 plants), except on day 18 when nine culture vessels were harvested. Errors are ± s.e.

0 and 11, 30–40% of the absorbed NH_4^+-N was translocated concurrently to the shoots, whilst this proportion declined to < 20 % on day 14 of starvation.

The initial (day 0) concentration of N in the total plant dry matter was higher ($P = 0{\cdot}07$ n.s.) in normal (6·00 % N) than in *stay-green* (5·68 % N) plants, and this declined, respectively, to 1·44 and 1·27 % N ($P < 0{\cdot}005$) by day 18. The time course for % N in tissue dry weight was 'decay-like' in all organs analysed (data not presented), indicative of N dilution resulting from continued production of dry matter. The two genotypes were similar in this respect, although normal plants always showed slightly higher % N in the laminae.

Comparison of the % N in young expanding and senescent laminae (Table 1) indicated greater (by > 50 %) retention of N in senescing leaves of *stay-green* plants, assuming little difference in export of C between the genotypes.

Kinetics of NH_4^+ uptake during N starvation

The depletion technique for measuring net uptake over the concentration range 1–250 μM NH_4^+ gave variable goodness-of-fit for simple Michaelis–Menten kinetics (Table 2), and estimated V_{max} and K_m were ignored where the model fit was statistically insignificant. There were no significant ($P > 0{\cdot}05$) differences in K_m or V_{max} between normal and *stay-green* types at any time, although V_{max} was usually a little higher for normal plants. Values of K_m increased between days 0–7, but showed little pattern thereafter. Similarly, values of V_{max} increased between days 0–7 of N starvation, but changed relatively little thereafter.

TABLE 1. *Total N concentrations (% N in dry weight) of young expanding and visibly senescent laminae of normal and* stay-green *genotypes of* L. perenne*, on days 11 and 14 after terminating the N supply to plants*

Genotype	Day	Young laminae	Senescent laminae
Normal	11	2·42 (0·08)	1·77 (0·11)
	14	1·92 (0·14)	1·35 (0·15)
Stay-green	11	2·63 (0·16)	2·80 (0·12)
	14	1·76 (0·19)	2·58 (0·03)

Values are means ($n = 3$) of replicate samples of 12 leaves (standard deviations shown in parentheses). Differences between normal and *stay-green* plants were not significant (*t*-test, unequal variances) on either day in young laminae, but were significant ($P < 0{\cdot}01$) in senescent laminae.

Recovery in growth and N uptake following severe defoliation

The shoot morphology of the two genotypes was similar, and cutting to the same height removed similar proportions of leaf material. The *stay-green* mutation did not affect shoot growth significantly following severe defoliation in expt 2. Laminae fresh weights (Fig. 3A) over 15 d were similar for the two genotypes, as were dry weights. The same applied to root fresh and dry weights, although *stay-green* root fresh weights (Fig. 3C) were a little higher between days 2 and 8. Specific laminar regrowth rates (g g^{-1} d^{-1}), given by fitting exponential functions to dry weight data, were 0·1091 ± 0·0158 d^{-1} for wild type and 0·0896 ± 0·0319 d^{-1} for *stay-green*. The only consistent genotypic difference occurred in the stubble fraction fresh weight (Fig. 3B), which was higher in the normal type plants. This accounted for the slightly higher shoot: root fresh weight ratios in normal compared with *stay-green* genotypes. Dry weight shoot: root ratios were also very similar for the two genotypes (data not presented).

Net uptake of NO_3^- over 15 d following defoliation was 5 % higher in normal plants (1750 μmol NO_3^- per plant) compared with *stay-green* plants (1600 μmol NO_3^- per plant). The corresponding totals for K^+ uptake were 750 ìmol per plant for the normal type and 699 μmol per plant by *stay-green*. Nitrate uptake, calculated from the increment in ^{15}N content of the plants, showed good agreement with the more accurate values based on the automatic supply of NO_3^- to maintain 20 μM in the culture solutions. Uptake of NO_3^- and K^+ decreased to zero within 24 h of defoliation, and resumed the next day in normal plants, but was delayed for a further day in *stay-green* plants (Fig. 4). Normal plants had slightly higher mean daily unit absorption rates of NO_3^- than *stay-green* plants throughout most of the regrowth period. In terms of total N uptake per plant, this difference more than compensated for the slightly smaller total root mass of the wild type over part of the recovery period.

TABLE 2. *Michaelis–Menten parameters (V_{max} and K_m) for net uptake of NH_4^+ by normal and* stay-green *genotypes of* L. perenne *at intervals (days 0, 4, 7, 11, 14) following termination of the NH_4^+-N supply to plants in flowing nutrient solutions*

	Normal genotype		*Stay-green* genotype	
Day	K_m (μM)	V_{max} (μmol h^{-1} g^{-1} root f. wt)	K_m (μM)	V_{max} (μmol h^{-1} g^{-1} root f. wt)
0	29 ± 3	11 ± 1	35 ± 1	10 ± 1
4	–	–	85 ± 21	13 ± 2
7	145 ± 16	18 ± 2	195 ± 28	20 ± 1
11	106 ± 5	19 ± 1	97 ± 9	15 ± 1
14	151 ± 47	22 ± 4	127 ± 33	16 ± 3

Measurements by depletion of solutions containing initially 250 μM $^{15}NH_4^+$. Uptake by each genotype was measured separately on three replicate culture vessels each containing 12 plants. Values are means ± s.e., and are omitted where the Michaelis–Menten model gave a poor fit to the data.

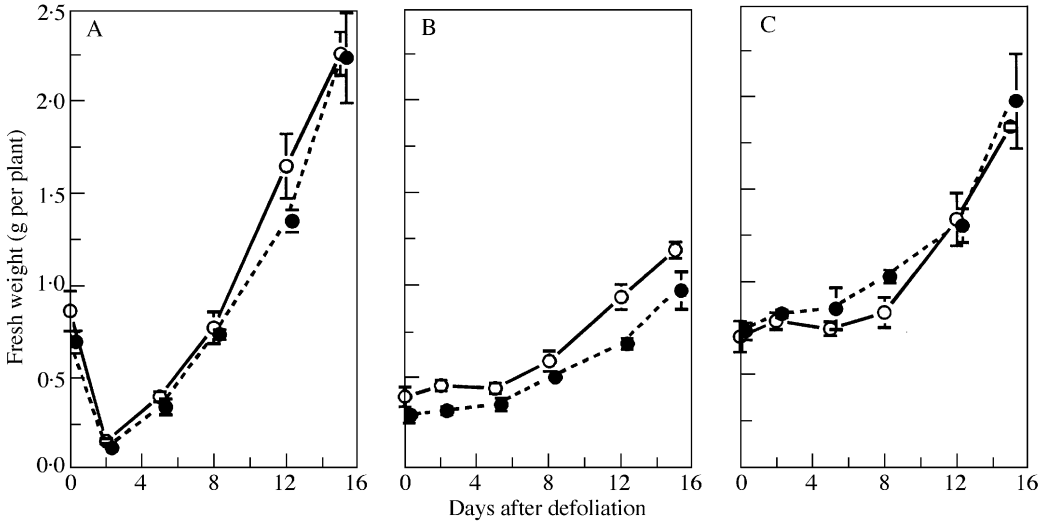

FIG. 3. Comparison of fresh weight accumulation in the laminae (A), stubble (B) and roots (C) of normal (open circles) and *stay-green* (closed circles) genotypes of *L. perenne* L. following severe defoliation. Values are means of four culture vessels (48 plants). Errors are ± s.e (n = 4).

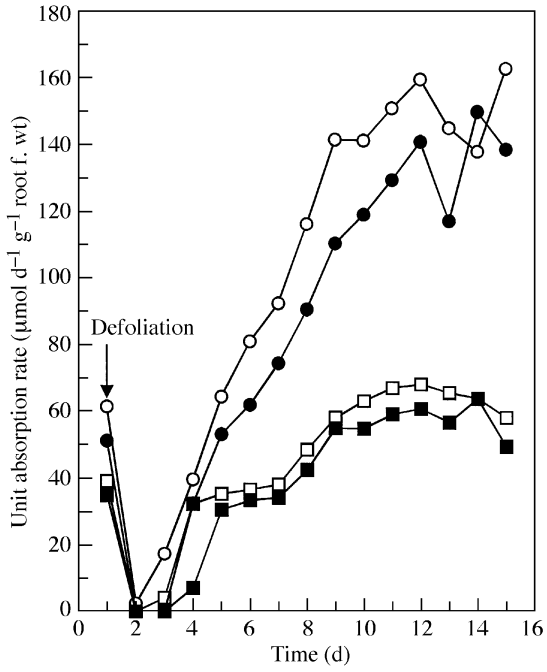

FIG. 4. Effect of severe defoliation on mean daily unit absorption rates of NO_3^- (circles) and K^+ (squares) by normal (open symbols) and *stay-green* (closed symbols) genotypes of *L. perenne* from concentrations of 20 μM NO_3^- in flowing nutrient solutions. Uptake was measured on one culture unit per genotype, each initially containing 288 plants.

Remobilization of N following defoliation

In calculating N remobilization, the harvested leaf fraction was treated as the sole 'sink' for N exported from the stubble and root fractions during the regrowth period. Complications arising from tissue heterogeneity together with the recycling of N within, or between, sources and sinks were ignored. Hence, the data described net remobilization, rather than the absolute N fluxes between the tissues. The total (source + sink tissues) plant content of N absorbed prior to defoliation was reasonably stable until day 8 after cutting (Fig. 5A), but appeared to decline subsequently in normal plants. This was probably attributable to sampling errors (i.e. a low laminae dry weight harvested on day 12) rather than loss of ^{15}N through efflux from the roots and by other processes. However, because of this variation over time in absolute N recovery, the remobilization of N was also expressed on a relative basis.

The changes in % N in tissue dry weight following defoliation were generally similar in the two genotypes, although leaf and stubble concentrations were slightly higher in *stay-green* plants, whereas root N concentrations were slightly lower compared with normal plants. A marked decline in root % N occurred between days 0 and 5, reflecting net remobilization of N from this tissue, but stubble % N remained almost constant throughout the experiment.

The absolute quantities of remobilized N in the leaf fraction of normal and *stay-green* plants were similar until day 8 (Fig. 5A), and cumulatively the differences between genotypes remained insignificant ($P > 0{\cdot}05$) until day 12. There was also very little difference between the genotypes (day 12) when the remobilized N content of the leaves was expressed as a proportion of the total N potentially available for remobilization (Fig. 5B). By day 15 the leaves of both genotypes contained 60 % of the potential maximum remobilizable N.

The contribution of remobilized N to successive increments in leaf acquisition of N declined rapidly over time in both genotypes (Table 3): from 96–97% between days 0 and 2 to 13–22% between days 5 and 8. Subsequently, almost all the N accumulated in the leaves derived from NO_3^- uptake. Overall, there was little evidence of significant genotypic differences in the dynamics of N remobilization into leaves.

The root system not only contained a larger pool of potentially remobilizable N than the stubble, but also

TABLE 3. *Changes in total N content of laminae of normal and* stay-green *genotypes of* L. perenne *between successive harvests following severe defoliation, and the percentage contribution of remobilized N from stubble and root fractions to these increments*

Interval (d)	Genotype	Increment in laminar N content	
		Total N (μmol per plant)	Proportion remobilized N (%)
0–2	Normal	62	96
	Stay-green	51	97
2–5	Normal	123	53
	Stay-green	115	62
5–8	Normal	201	13
	Stay-green	196	22
8–12	Normal	429	0
	Stay-green	646	7

Values given by the difference between successive mean laminae N contents per plant, each of which was based on ^{15}N analysis of herbage samples from four replicate culture vessels.

exported N to the leaves at a higher rate, at least until day 5 (Fig. 6A). Both genotypes had similar initial (day 0) root N contents and similar net rates of N export from the roots. This contrasted with remobilization from the stubble fraction. *Stay-green* plants had a lower initial stubble N content than normal plants and also a lower net rate of N remobilization from the stubble. Hence, although both genotypes remobilized 60–70 % of the potentially remobilizable root N over 12 d, and normal plants remobilized 60–70% of the stubble N, *stay-green* plants remobilized only 20% of their stubble N (Fig. 6B). The apparent inconsistency in this finding, given that both genotypes had similar N remobilization from the roots and import of remobilized N into the laminae, probably arose from sampling errors associated with stubble dry weights, resulting in an erroneous value for the 'potentially remobilizable N' remaining in this fraction.

The fraction of source organ N content unavailable for remobilization to the laminae was estimated by fitting a monoexponential model (Thornton *et al.*, 1993) to the decline in the proportion of the total potential remobilizable N pool remaining in source organs over time, with the form:

$$N_u(t)/N_u(0) = (1 - P_u)e^{-kt} + P_u$$

where $N_u(0)$ and $N_u(t)$ are the amounts (μmol N per plant) of ^{15}N-labelled N in the source tissues measured at times 0 and t, respectively, P_u is the apparent proportion of the ^{15}N-labelled N content not accessible for remobilization, and k is

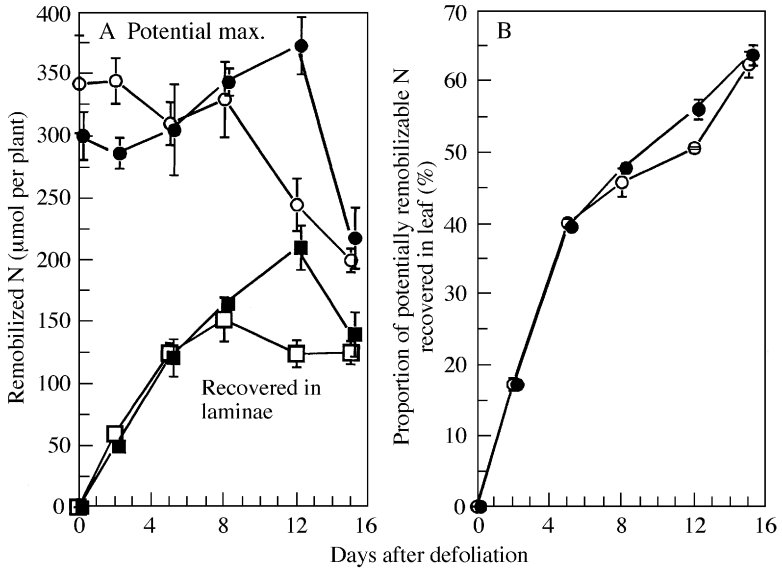

FIG. 5. Net import of remobilized N from stubble and roots into laminae of normal (open symbols) and *stay-green* (closed symbols) genotypes of *L. perenne* following severe defoliation: A, absolute quantities of remobilized N recovered in laminae (squares), and potential maximum remobilizable N content of whole plant (circles); B, remobilized N recovered in laminae as a proportion of potential maximum remobilizable N content of the plant. Means (± s.e.) of measurements on four culture vessels. The potential maximum remobilizable N content was calculated for each harvest date as the total plant content of N absorbed prior to defoliation.

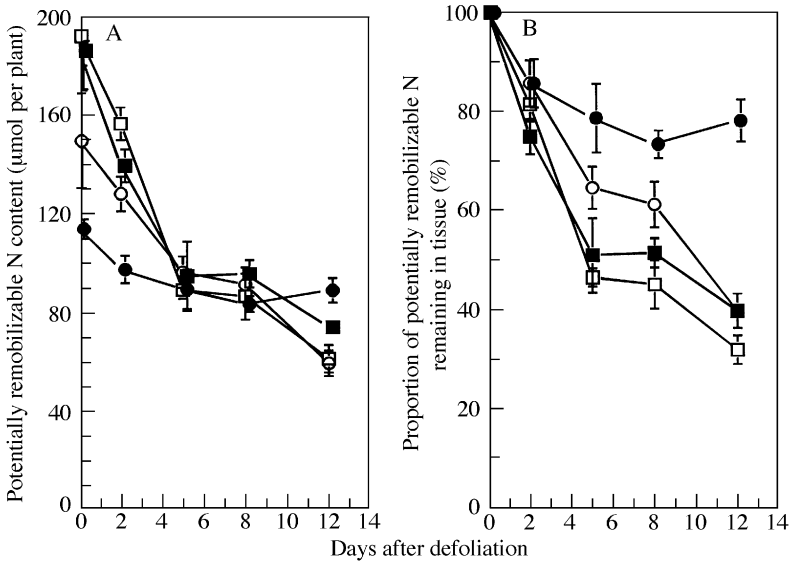

FIG. 6. Remobilization of N from roots (squares) and stubble (circles) of normal (open symbols) and *stay-green* (closed symbols) genotypes of *L. perenne* L. following severe defoliation, expressed as (A) the absolute decline in the potentially remobilizable N content of the source organ, and (B) the proportion of the initial potentially remobilizable N content of the source organ remaining on successive harvests. Means (± s.e.) of measurements on four culture vessels.

TABLE 4. *Predicted values of parameters for the proportion of the ^{15}N-labelled N content of stubble and roots (N absorbed prior to defoliation) not accessible for remobilization (P_u), and the rate of remobilization of the accessible fraction (k), assuming monoexponential decay kinetics modelled according to Thornton* et al. *(1993)*

Genotype	Organ	P_u (± s.e.)	k (± s.e.) (d^{-1})	r^2
Normal	Root + stubble	0·37 ± 0·06	0·171 ± 0·036	0·98
Stay-green	Root + stubble	0·31 ± 0·03	0·155 ± 0·018	0·99
Normal	Root	0·38 ± 0·04	0·242 ± 0·046	0·98
Stay-green	Root	0·38 ± 0·04	0·220 ± 0·036	0·99
Normal	Stubble	–*	0·059 ± 0·004	0·97
Stay-green	Stubble	–*	0·053 ± 0·005	0·94

Correlation coefficients (r^2) are for data *vs.* model.
* Model fitted assuming P_u of zero.

the rate of remobilization of the accessible fraction (d^{-1}). The value of $N_u(t)/N_u(0)$ for the combined root + stubble fraction at each harvest was calculated as the recovery of label in this fraction divided by the recovery of label in the whole plant at the same time (as opposed to total recovery at t_0). The model did not provide a good fit to the stubble-only data (there was no indication of an asymptote), and in this case the parameter P_u was set at zero. For the other plant fractions the predicted values of P_u (0·31–0·38) and k were not significantly different between genotypes (Table 4). However, predicted remobilization rate constants were four-times higher for roots than for stubble, although this must be treated with caution given that $P_u = 0$ in the latter case. Despite the statistical insignificance of genotypic differences, *stay-green* plants appeared to have a lower fractional content of stubble + root N unavailable for remobilization than normal plants. This was contrary to expectation, and is difficult to reconcile with the observation that the proportional remobilization of N from the stubble was lower in *stay-green* plants (Fig. 6B).

DISCUSSION

Stay-green and the maintenance of growth during N starvation

Results from expt 1 showed that the *sid* gene *stay-green* mutation has little or no impact on the growth of *L. perenne* during prolonged N starvation, either in terms of total or shoot biomass production. Similar conclusions were reached under conditions of milder N stress (Bakken *et al.*, 1997; Hauck *et al.*, 1997). The absence of a significant effect of *stay-green* on growth may reflect the relative insensitivity, compared with other species, of dry matter production by perennial ryegrass to termination of NO_3^--N supply when plants have been grown previously under high N nutrition. For example, 9 d of NO_3^- starvation had no effect on shoot growth by cultivar S23, although root growth was enhanced (Clement *et al.*, 1979). Likewise, Jarvis and Macduff (1989) reported only a slight depression in total dry matter production by the same cultivar over 11 d, despite a halving of the concentration of N in the shoots. Even greater insensitivity to N deprivation might be expected under NH_4^+-N nutrition, given the higher concentrations of insoluble reduced N in the roots of NH_4^+-fed plants (Clarkson *et al.*, 1992).

Nevertheless, the absence of measurable effects on growth is surprising in view of the significantly higher levels of N measured in the senescent leaves of *stay-green* plants, and the several-fold decline in average tissue N concentrations that occurred over the 18 d of N starvation. Presumably the level of internal N stress remained insufficient for any diminution in the flux of N from the senescent leaf fraction of *stay-green* plants to affect the rate of

synthesis of new tissue. Furthermore, although the re-allocation of N between shoots and roots during N starvation was substantial, it was not clear from the data exactly when, if ever, mobilization of N from thylakoid proteins in leaf tissue was potentially rate-limiting on growth. There is also the possibility that diminished availability of thylakoid protein N for remobilization in *stay-green* plants was compensated for by enhanced remobilization of N from other sources.

NH_4^+ uptake during N starvation

The present study suggests that the *stay-green* mutation does not affect net uptake of NH_4^+ over the concentration range associated with saturable HATS (high-affinity transport systems) for NH_4^+ (e.g. Wang *et al*., 1993; Kronzucker *et al*., 1996), at least in terms of K_m and V_{max} during prolonged N starvation. There was also no evidence that V_{max} for NH_4^+ uptake increases more rapidly in *stay-green* than in normal plants following the onset of N stress, as would be anticipated if there were lower internal levels of N compounds active in down-regulating N uptake (e.g. Cooper and Clarkson, 1989; Touraine *et al*., 1994). The results for NH_4^+ uptake also differ from those reported for NO_3^- uptake (Bakken *et al*., 1997), where V_{max} was higher in *stay-green* plants during N stress.

The tendency for V_{max} and K_m for NH_4^+ uptake by ryegrass to increase following the termination of N supply, at least until day 7, contrasts with the response reported for NO_3^- uptake (Bakken *et al*., 1997). Increases in V_{max} for influx and net uptake of NH_4^+ by plants previously deprived of N have been reported for several other species (Lee and Rudge, 1986; Morgan and Jackson, 1988*a*, *b*; Lee, 1993; Wang *et al*., 1993; Kronzucker *et al*., 1996). Kronzucker *et al*. (1996) argued that higher initial fluxes measured in N-deprived plants reflect the combination of absence of negative feedback and low efflux. But in the case of NO_3^-, prolonged N starvation has been shown to provoke a long-term decline in V_{max} attributed to a combination of declining transporter abundance and growth rate (Clarkson, 1986). The apparently different behaviour of NH_4^+ compared with NO_3^- could be associated with lower turn-over of the NH_4^+ HATS or uptake of NH_4^+ through K^+ transporters (Lee and Ayling, 1993). Maintenance of a high constitutive capacity for NH_4^+ uptake during N stress would be ecologically advantageous, as NH_4^+ is frequently the predominant form of mineral N in nutrient-poor ecosystems, produced by mineralization of soil organic N. Hence plants must compete effectively for its capture against the soil microbial population. However, a decrease in K_m for NH_4^+ uptake might be expected by this reasoning; the opposite of what was observed in the present study. The mean K_m values for net uptake ranged between 29 and 151 μM, similar to values reported for a number of species grown under N-sufficient conditions (Rao *et al*., 1993), but higher than those measured by Bloom and Sukrapanna (1990) in *Hordeum vulgare* L.

A note of caution should be sounded with respect to the results because relatively long-term (3–8 h) depletion procedures for estimating concentration-dependent fluxes and kinetic parameters have several weaknesses. First, Michaelis–Menten characteristics describe dependence of initial unidirectional rates (usually measured over 5–10 min) upon concentration. Hence, prolonged measurements of net uptake by the same set of plants could confound effects of varying external concentration, root storage pool concentration, and plant acclimation. Secondly, the form of the concentration/rate relationship may vary with the NH_4^+ status of the plants at the start of depletion, as reported for NH_4^+ uptake by *Triticum aestivum* L. (Goyal and Huffaker, 1986). However, values of V_{max} in the present study were derived from rates measured near the start of the depletion period, when the N content of storage pools in the roots was likely to be very low.

Growth and N dynamics following severe defoliation

Results from expt 2 indicate that extra retention of N in senescent leaves of *stay-green* plants does not inhibit leaf growth during recovery from severe defoliation, when the biosynthetic requirement for N is initially met by remobilization. However, this conclusion is qualified by the fact that the severe defoliation imposed in this study actually removed much of the senescing leaf tissue that gives rise to the difference in remobilization characteristics between the two genotypes. Furthermore, the finding may be restricted to plants grown under high N nutrition (Clement *et al*., 1978*b*) both before and after defoliation. Under conditions of limiting external N supply, a negative effect of the *stay-green* mutation might be expected, if it resulted in lower levels of soluble N in the plant compared with those in the normal genotype. Leaf growth under the present experimental conditions was almost certainly not limited by internal N availability given the high concentrations of N (5·6–7 % N in d. wt) recorded in the leaves throughout the recovery period. Moreover, these concentrations were always higher in *stay-green* plants, and notably so between days 0–5 after defoliation when remobilization was the predominant source of leaf N.

The predominance of remobilization as the main source of N for synthesis of leaf tissue, until day 5, agrees with previous studies of *L. perenne* (Ourry *et al*., 1988; Millard *et al*., 1990; Thornton *et al*., 1994; Wilkins *et al*., 1997). Unexpectedly, the down-regulation of NO_3^- uptake following defoliation (Clement *et al*., 1978*b*; Macduff *et al*., 1989) was more severe and prolonged in *stay-green* compared with normal plants. It might be inferred from this that substrate N levels in *stay-green* plants were at least as high as those in normal plants. The higher leaf N concentrations in *stay-green* plants between days 0 and 5 provide some indication that this was the case.

The quantity of remobilized N in new leaves was probably underestimated in the present study, at least until day 5, because the harvesting procedure did not account for tissue heterogeneity in the 'stubble fraction' (see Avice *et al*., 1996; De Visser *et al*., 1997). This fraction contained both source (leaf sheaths) and sink (new leaf) tissues for N, and newly incorporated N in the leaf meristem zone would take 2–3 d to appear above the cutting height for inclusion in the harvested 'leaf fraction'. Nevertheless, *stay-green* and

normal types were very similar with respect to the quantity of remobilized N recovered in the new leaves by the end of the experimental period; and the similarity was underscored by the derived values for the parameters P_u and k.

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