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Research Article

Identification of Differentially Senescing Mutants of Wheat and Impacts on Yield, Biomass and Nitrogen Partitioning[F]

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Abstract

Increasing photosynthetic capacity by extending canopy longevity during grain filling using slow senescing stay-green genotypes is a possible means to improve yield in wheat. Ethyl methanesulfonate (EMS) mutated wheat lines (*Triticum aestivum* L. cv. Paragon) were screened for fast and slow canopy senescence to investigate the impact on yield and nitrogen partitioning. Stay-green and fast-senescing lines with similar anthesis dates were characterised in detail. Delayed senescence was only apparent at higher nitrogen supply with low nitrogen supply enhancing the rate of senescence in all lines. In the stay-green line 3 (SG3), on a whole plant basis, tiller and seed number increased whilst thousand grain weight (TGW) decreased; although a greater N uptake was observed in the main tiller, yield was not affected. In fast-senescing line 2 (FS2), yield decreased, principally as a result of decreased TGW. Analysis of N-partitioning in the main stem indicated that although the slow-senescing line had lower biomass and consequently less nitrogen in all plant parts, the proportion of biomass and nitrogen in the flag leaf was greater at anthesis compared to the other lines; this contributed to the grain N and yield of the slow-senescing line at maturity in both the main tiller and in the whole plant. A field trial confirmed senescence patterns of the two lines, and the negative impact on yield for FS2 and a positive impact for SG3 at low N only. The lack of increased yield in the slow-senescing line was likely due to decreased biomass and additionally a possible sink limitation.

Keywords: Wheat; senescence; stay-green; grain-filling; yield; nitrogen.

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Introduction

Global production of grain crops has increased nearly three-fold since 1960 (FAO 2010). However, it will be a challenge to continue raising crop productivity to keep track with the growing population. A major consideration for the sustainable increase in productivity is the dependency on nitrogen fertilizer, which is a substantial cost factor and a potential environmental pollutant (Good et al. 2004). It is estimated that $1.1 billion could be saved annually by increasing nitrogen use efficiency (yield per unit of available N) by just one per cent (Kant et al. 2011). Optimising carbon assimilation by the plant whilst minimising nitrogen inputs would be highly beneficial.

In wheat, canopy senescence is required to recycle minerals present in vegetative organs to the grain to meet its demand for resources such as nitrogen (Gregersen et al. 2008). The proportion of nitrogen in wheat grain originating from remobilization

of pre-anthesis stored nitrogen is estimated to be over 70% illustrating the importance of this remobilization (Kichey et al. 2007). Nitrogen remobilization from the flag leaf is positively correlated with nitrogen yield per spike and per area, and the amount of nitrogen contributed by the flag leaf was found to be about 18% (Wang et al. 2008). The efficiency of leaf lamina N remobilization in wheat is estimated to be 76% (Pask et al. 2012).

Plant nitrogen status has a major impact on the onset and progression of leaf senescence. In both barley (*Hordeum vulgare*) and *Arabidopsis thaliana,* nitrogen deprivation results in accelerated leaf senescence, and when additional nitrate is supplied at the start of senescence, senescence can be halted or even reversed (Schildhauer et al. 2008).

Extending the duration of photosynthesis is a possible means to increase total photosynthesis, biomass and yield (Richards 2000). Total flag leaf photosynthesis, chlorophyll content, the onset of senescence (at low nitrogen availability), and green leaf duration have all been found to be positively correlated with wheat grain yield (Kichey et al. 2007; Wang et al. 2008; Gaju et al. 2011). Although under optimal conditions wheat crops may not be limited by assimilate supply during grain-filling (Borrás et al. 2004), under abiotic stress yields may be limited by photosynthetic capacity, so extending the duration of photosynthesis may in these cases increase yield. One approach to achieve a longer photosynthetic period is the use of functional stay-green phenotypes, which show either a delayed onset or a slower rate of senescence whilst maintaining photosynthetic activity (Thomas and Howarth 2000). Stay-green phenotypes have been described in cereal crop species such as sorghum (Borrell and Hammer 2000), rice (Fu and Lee 2008), maize (Rajcan and Tollenaar 1999; Ding et al. 2005; Martin et al. 2005; Echarte et al. 2008), and durum wheat (Spano et al. 2003).

Stay-green phenotypes and broader genetic variation in senescence have been reported in hexaploid wheat (Silva et al. 2000; Verma et al. 2004; Gong et al. 2005; Luo et al. 2006; Blake et al. 2007; Joshi et al. 2007; Christopher et al. 2008; Chen et al. 2010; Derkx et al. 2010; Bogard et al. 2011; Chen et al. 2011; Naruoka et al. 2012). The stay-green trait has been reported to increase yields (Gong et al. 2005; Luo et al. 2006; Christopher et al. 2008; Chen et al. 2010), and there were positive correlations to nitrogen use efficiency (Gaju et al. 2011), water use efficiency (Górny and Garczyński 2002; Christopher et al. 2008), spot blotch resistance (Joshi et al. 2007) and yields under heat and drought (Naruoka et al. 2012). However, less favourable effects have been observed, such as a decreased harvest index (Gong et al. 2005), more nitrogen remaining in the straw (Chen et al. 2011), and a reduced grain number (Naruoka et al. 2012). These inconsistent phenotypes indicate the interaction of the stay-green trait with the environment is likely to be strong.

In this study stay-green and fast-senescing lines of wheat were identified and characterised. The effects of the different senescence patterns on yield and nitrogen partitioning and their interactions with nitrogen nutrition levels were examined. A stay-green line was able to maintain nitrogen allocation to the grain longer under N-limiting conditions, while accelerated senescence resulted in lower yield and N allocation to the grain.

Results

Identification of stay-green and fast-senescing lines

54 ethyl methanesulfonate (EMS) mutant lines of wheat (pre-selected in a field screen to have a range of flowering and physiological maturity dates at the John Innes Centre, data not shown) were grown in the glasshouse and the relative chlorophyll content and photosynthetic rate of the flag leaf were measured at two time points, independently of the developmental status of the individual lines. The first time point was when the majority of the lines were between heading and anthesis (all plants fully green) and the second time point was six weeks later. Anthesis time for all lines was recorded. Highly significant differences in the retention of photosynthetic capacity and greenness were found (**Figure 1A, B**; $P < 0.001$**)**. Early flowering types had a very variable ability to retain leaf greenness; many were slow senescing whilst others senesced rapidly. The later flowering types tended to have senesced during the duration of this experiment **(Figure 1B)**. A similar pattern was seen for the functional assay of photosynthesis **(Figure 1A)**. The stay-green and maintenance of photosynthesis traits correlated with one another **(Figure 1C)**. Based on differences in maintenance of photosynthesis six lines with similar anthesis dates were selected for further experiments: three stay-green lines that maintained over 60% of their flag leaf photosynthesis (SG1–3), two fast-senescing lines that maintained less than 25% of their photosynthesis (FS1–2) and wild-type (WT) which had an intermediate phenotype.

The post-anthesis senescence processes of these six selected lines were studied in further detail, confirming the findings of the initial screening (**Figure 2**). Leaf greenness declined slower in the stay-green lines ($P < 0.01$). At 42 dpa lines SG1 and SG3 retained a significantly higher leaf greenness than FS2. Similarly, SG1 and SG3 had significantly higher photosystem II efficiencies (measured by fluorescence) than FS2 and WT over the entire senescence period. At 42 dpa SG1, SG2 and SG3 had lost less of their Photosystem II capacity. Overall, these results showed that SG1, SG2 and SG3 are not just cosmetically stay-green with impaired chlorophyll breakdown, but functional stay-green mutants.

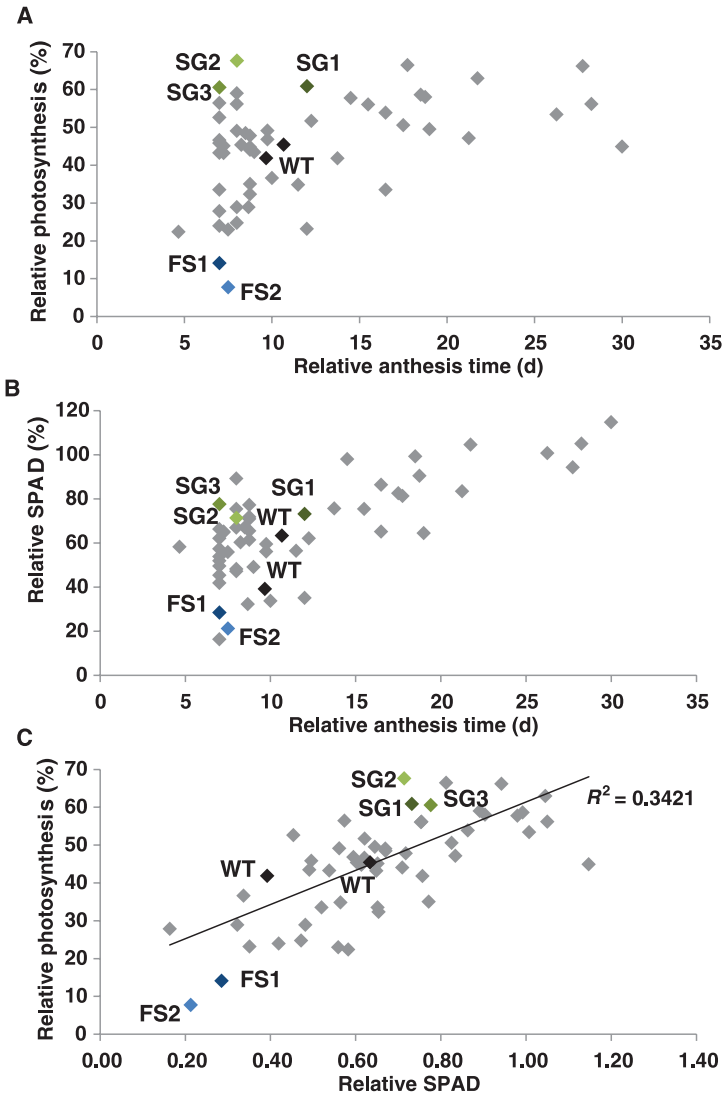

Figure 1. Flag leaf senescence in mutant wheat lines during a six-week period.

(A) Fraction of photosynthesis remaining after a 6 week period from around anthesis as a function of anthesis date.
(B) Fraction of leaf greenness (measured with a SPAD meter) remaining after a 6 week period from around anthesis as a function of anthesis date.
(C) Correlation between relative maintenance of photosynthesis (**A**, above) and relative chlorophyll content of the flag leaf (**B**, above).
The first measurement was done between heading and anthesis, when the plants were green (same date for all plants irrespectively of developmental stage), and the second measurement six weeks later when senescence was well established. All data are the means of 4 replicates. Relative performance values were plotted against anthesis date to correct for differences in developmental stage. The lines used for further experiments are marked: three stay-green lines (green, SG1–3), two fast-senescing lines (blue, FS1–2) and wild-type (black, WT).

Effect of nitrogen nutrition on senescence

Since nitrogen nutrition was expected to have effects on the senescence phenotype, yield and nitrogen economy, an experiment was set up to assess the performance of a fast and a slow senescing line under high and low N supply. For this experiment the fastest senescing line FS2, the slowest senescing line SG3 and WT were used. The high nitrogen treatment was lower than used in the previous experiments but sufficient to grow green tillering plants, whilst the low nitrogen treatment contained 10% nitrogen of the high N treatment and resulted in plants with reduced green area (lower NDVI) that hardly tillered. An assessment of whole pot senescence by using a Crop Canopy Sensor (**Figure 3**) showed that SG3 stayed green for longer at high N, with the WT and FS2 lines senescing at similar rates,

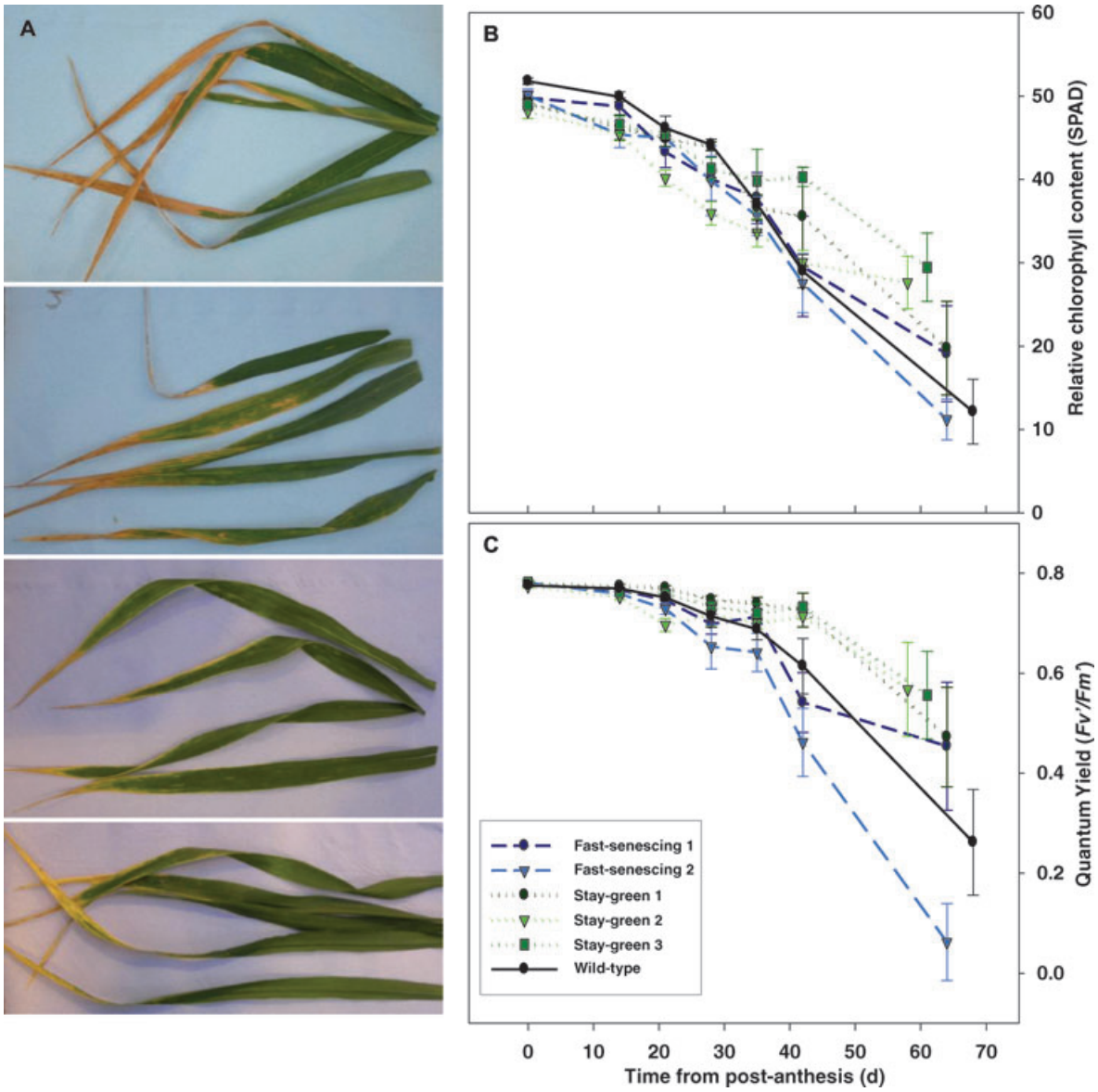

Figure 2. The progression of flag leaf senescence of selected mutant lines.

(A) Flag leaves 42 days post-anthesis of FS2 (fast senescing), WT (wild-type), and SG1 and SG3 (stay-green) respectively.
(B) Relative chlorophyll content as determined with a SPAD meter. LSD = 7.16.
(C) Quantum Yield (QY = *Fv'*/*Fm'*), which is a measurement of Photosystem II maximum efficiency, as determined by a handheld chlorophyll fluorescence meter. LSD = 0.120.
Indicated are the means of four replicate pots ± *SE*.

while at low N there were no significant differences between any of the lines.

Whole plant yield characteristics

Yield under low N nutrition was about a fifth of that under high N. FS2 had a significantly lower yield than SG3 and WT under both N conditions (**Figure 4A**). Total aboveground biomass showed a similar pattern (**Figure 4B**), suggesting it was an important determining factor for grain yield.

Even though SG3 and WT had similar grain yields, they showed consistent significant differences in yield components under both high and low N supply. SG3 had more tillers (**Figure 4C**) resulting in a significantly higher grain number (**Figure 4E**), while WT had a significantly higher thousand grain weight (**Figure 4D**). Under both N regimes FS2 had both a low

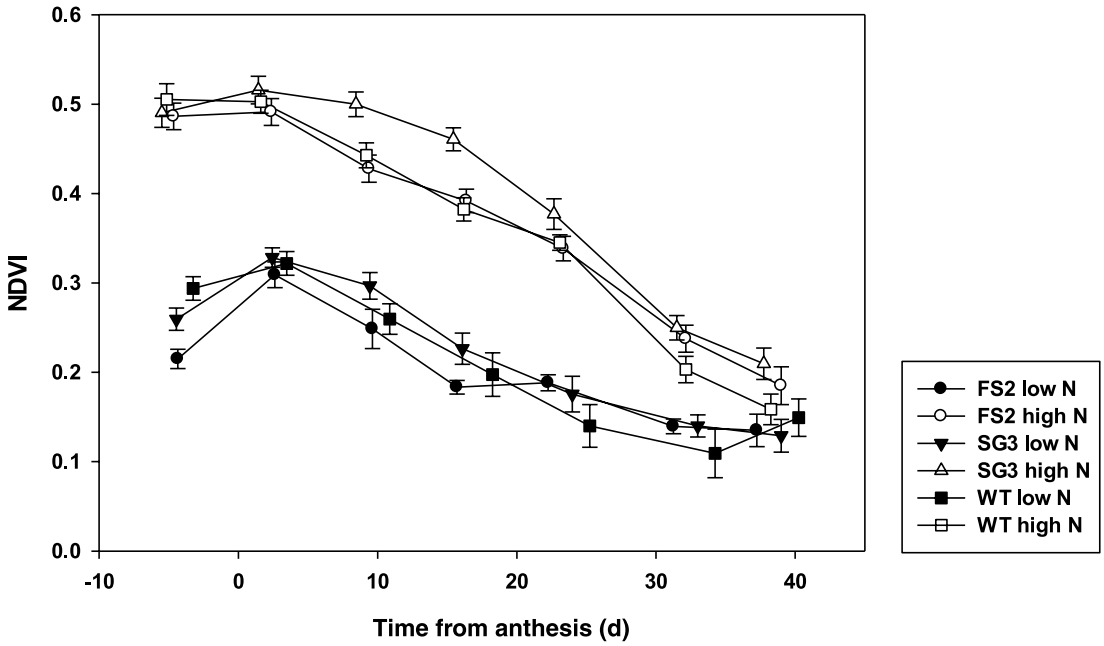

Figure 3. Progression of senescence of whole pots of lines FS2, SG3 and WT grown under high and low nitrogen nutrition.

Measurements were done with a Crop Canopy Sensor once a week; the graph is corrected for the different anthesis dates of the pots. NDVI = Normalised Difference Vegetation Index. Pots contained five plants. Indicated are the means of four replicate pots ± *SE*.

grain number and a low thousand grain weight, resulting in a low yield. Unlike grain and tiller numbers, grain size was not affected by N availability.

Both the grain N concentration and grain total N content were significantly higher in plants grown under high nitrogen supply (**Figure 4F, G**). The N concentration in grain of WT was significantly lower than that of the other two lines at both N levels. While there were no differences in N content between the lines grown at low N, SG3 had a significantly higher grain nitrogen content than WT or FS2 at high N.

FS2 had a significantly lower harvest index than SG3 and WT under both N conditions (**Figure 4H**). In addition, the nitrogen harvest index (NHI) of FS2 was significantly lower at both N levels (**Figure 4I**). SG3 maintained its NHI at low N, while the NHI of FS2 and WT were significantly lower than SG3 at low N.

Biomass and nitrogen accumulation and allocation in the main tiller

To determine the distribution of biomass and nitrogen between tissues, main shoots (not tillers) of the WT, FS2 and SG3 lines, at the two nitrogen concentrations, were separated into their plant components at anthesis and maturity. The main shoots of plants grown under low nitrogen had accumulated significantly less biomass than those of plants grown under high nitrogen conditions at both anthesis and maturity (**Figure 5A, B**). Under low N nutrition there were no significant differences in biomass of the main shoot, while at high N, WT accumulated significantly more biomass than both FS2 and SG3 (**Figure 5B**). The tissue with most biomass at anthesis was the stem, while at maturity the biggest fraction was the grain. Although stem biomass was greater at high N (**Figure 5A**), the proportion of biomass allocated to the stem was significantly greater at low N (**Figure 5C**). WT had the highest grain yield per shoot at high N (**Figure 5B**), but SG3 had proportionally allocated more of its biomass to the grain at both N levels (**Figure 5D**). Both in actual biomass and proportionally, SG3 had allocated significantly less of its biomass to the lower leaves and more to the flag leaves, especially at high N (**Figure 5A, C**).

At low N there were no significant differences in total N content of the shoot between the lines, while at high N SG3 had a significantly lower total N content at both anthesis and maturity compared to the other lines (**Figure 5E, F**). Most nitrogen, between 77.7 and 88.0%, was found in the grain at maturity. Grain total N at high N of SG3 was significantly lower than that of WT but not of FS2, while there were no differences between the lines at low N (**Figure 5F**). Although the proportion of N allocated to the grain was lower than that of WT at high N, the relative grain N content of SG3 was higher than that of FS2 at both N levels. (**Figure 5H**). In fact, SG3 was the only line that did not show a significant difference in the proportion of N allocated to the grain between N levels, maintaining grain N at low N supply as is indicated by the high NHI (**Figure 4I**).

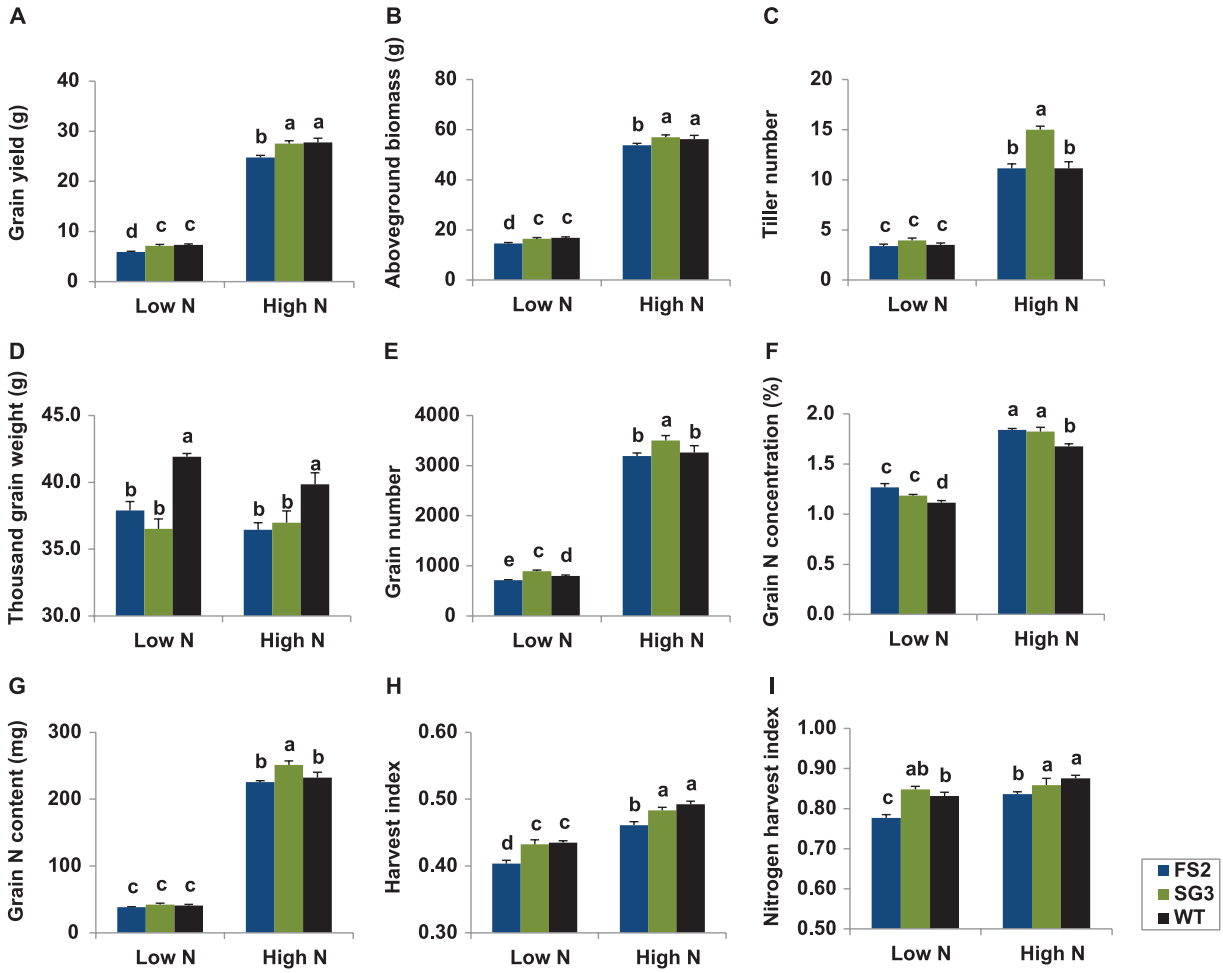

Figure 4. Yield characteristics of lines FS2, SG3 and WT grown under high and low nitrogen nutrition.

(A) Grain Yield (g) per pot.
(B) Aboveground biomass (g) per pot.
(C) Number of tillers per pot (5 plants).
(D) Thousand Grain Weight (g).
(E) Number of grains. Determined from the pooled grain of five pots.
(F) Grain nitrogen concentration (% dry matter).
(G) Grain total nitrogen content (mg) per pot.
(H) Harvest Index.
(I) Nitrogen Harvest Index.
A total of 20 pots were available per line x nitrogen level combination, so bars represent the means of either 4 or 20 replicates depending on the variable measured. Error bars denote standard errors. Letters indicate significant differences at 0.05 level as determined using the LSD.

For all vegetative tissues the total N content at anthesis was significantly higher in plants grown at high N compared to low N, which was reflected in grain N at maturity due to effective remobilization at both high and low N (**Figure 5E, F**). At high N at anthesis, SG3 had a significantly lower N content than FS2 and WT in all vegetative tissues except the flag leaf, reflected in the lower grain N at maturity (**Figure 5E, F**). However, since the total N content of SG3 was also low, the proportion of nitrogen in most tissues of SG3 did not differ significantly from that of the other lines (**Figure 5G, H**). SG3 allocated relatively more N to the flag leaf at anthesis (**Figure 5G**), explaining the stay-green phenotype of the flag leaf (**Figure 2**). This higher flag leaf N content is explained by differences in relative flag leaf biomass (**Figure 5C**), since N concentrations in the flag leaf did not differ significantly between the lines (data not shown), which was also indicated by similar SPAD readings at anthesis (**Figure 2A**). In

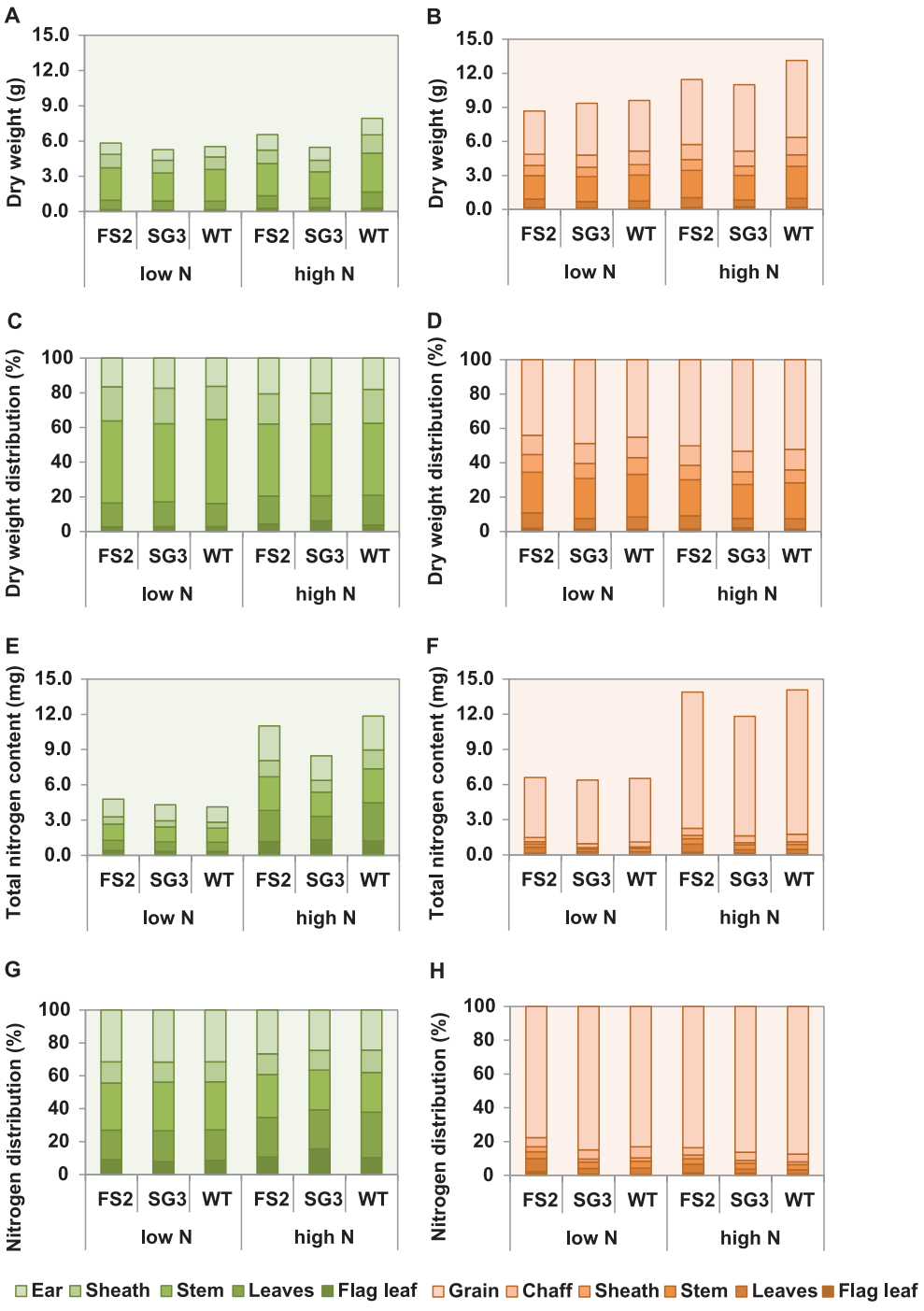

Figure 5. Dry matter and nitrogen accumulation and allocation of main shoots of lines FS2, SG3 and WT grown under high and low nitrogen nutrition.

(A, B) Dry weight (g) of the tissues of main shoots at anthesis (**A**) and physiological maturity (**B**).
(C, D) Distribution of dry matter (%) over the tissues of main shoots at anthesis (**C**) and physiological maturity (**D**).
(E, F) Total nitrogen content (mg) of the tissues of main shoots at anthesis (**E**) and physiological maturity (**F**).
(G, H) Distribution of the total nitrogen content (%) over the tissues of main shoots at anthesis (**G**) and physiological maturity (**H**).
Bars represent means of 2 main shoots of 4 replicate pots. Statistical analyses of the four datasets were performed per tissue but encompassing both time points.

contrast, FS2 had both a significantly higher total N content and relative N allocation in vegetative tissues at maturity compared to SG3, while having similar values at anthesis (**Figure 5E–H**), suggesting there was little N remobilization from the leaves.

The post-anthesis changes in biomass and N allocation for the main tiller are summarized in **Table 1**. Comparing FS2 and SG3, FS2 had less post-anthesis biomass gain (uptake) and the greater remobilization, whilst SG3 had the greater uptake and lesser remobilization at both high and low N. N uptake and remobilization followed similar patterns at high N but not low N where the lines behaved similarly.

Field validation

The FS2 and SG3 lines, along with the Paragon wild-type were grown in a field trial at two nitrogen fertilizer rates (100 and 200 kg N/ha applied) and progress of canopy senescence and final yields were determined **(Figure 6 and Table 2)**. A visual senescence score was initiated on the 15th June by which time

Table 1. Biomass and nitrogen content at anthesis and maturity, and post-anthesis uptake and remobilization of lines FS2, SG3 and WT

	Biomass (g)					Nitrogen (mg)				
	Total		Uptake	Grain	Remobilization	Total		Uptake	Grain	Remobilization
	A	M	M-A	G	G-(M-A)	A	M	M-A	G	G-(M-A)
Low N										
FS2	5.84	8.67	2.84	3.82	0.98	4.78	6.60	1.82	5.13	3.31
	(0.33)	*(0.17)*		*(0.11)*		*(0.39)*	*(0.26)*		*(0.22)*	
SG3	5.27	9.36	4.09	4.57	0.47	4.31	6.39	2.08	5.42	3.34
	(0.23)	*(0.47)*		*(0.25)*		*(0.11)*	*(0.28)*		*(0.27)*	
WT	5.54	9.28	3.74	4.46	0.72	4.11	6.53	2.42	5.41	3.00
	(0.65)	*(1.16)*		*(1.26)*		*(0.46)*	*(1.89)*		*(1.51)*	
High N										
FS2	6.56	11.46	4.91	5.74	0.83	11.01	13.89	2.88	11.62	8.74
	(0.60)	*(0.70)*		*(0.34)*		*(0.77)*	*(0.58)*		*(0.51)*	
SG3	5.47	11.01	5.55	5.87	0.32	8.47	11.82	3.35	10.19	6.84
	(0.26)	*(0.21)*		*(0.12)*		*(0.22)*	*(0.97)*		*(1.00)*	
WT	7.94	13.41	5.46	6.78	1.32	11.86	14.06	2.20	12.30	10.10
	(0.61)	*(0.32)*		*(0.14)*		*(0.45)*	*(0.22)*		*(0.08)*	

All values are means of 4 replicates (each of 2 main shoots). A, anthesis; M, physiological maturity; G, grain. Standard errors (*SE*) of the means of the measured parameters are indicated in parentheses. Since uptake and remobilization could not be measured because of destructive sampling, but were derived from anthesis and maturity values, *SE* cannot be given.

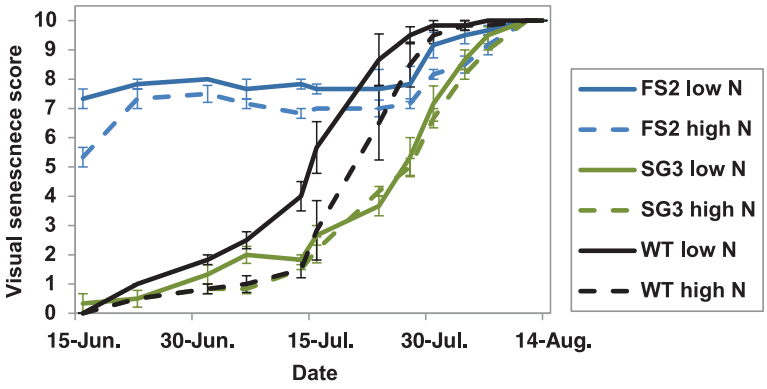

Figure 6. Senescence in field trials for the the FS2 and SG3 and WT lines.

Trials were run in 2009 at Church Farm, Bawburgh, Norwich, UK, with 3 replicate 6 m^2 plots at 100 (low N) and 200 (high N) kg N/ha. A visual senescence score of 0 (fully green) to 10 (completely senesced) was applied. Error bars represent the standard errors of the means.

Table 2. Yields of FS2 and SG3 and WT lines in a field trial at 100 (low N) and 200 (high N) kg N/ha

	Yield (t/ha)			Difference to wild-type (t/ha)	
	FS2	SG3	WT	FS2	SG3
Low N					
Mean	3.91	7.76	7.12	−3.22	0.64
SE	0.28	0.85	0.03	0.29	0.82
High N					
Mean	5.35	8.87	9.74	−4.39	−0.87
SE	0.49	0.47	0.45	0.20	0.11

Trials were performed in 2009 at Church Farm, Bawburgh, Norwich, UK, with 3 replicate 6 m^2 plots. Yields are t/ha at 85% dry matter. The means of 3 replicates and standard errors (*SE*) are indicated.

FS2 was already highly senesced. In contrast SG3 was only around 50% senesced by the 30th July **(Figure 6)**. WT was 50% senesced by the 15th July. Low nitrogen enhanced the rate of senescence in WT and FS2 but not for SG3. There was a negative impact on yield for the SG3 yield compared to WT and only a small increase in yield at low N for SG3 **(Table 2)**. FS2 yield was much lower than that of WT and SG3 under both N regimes.

Discussion

Slow (stay-green) and fast-senescing phenotypes of mutant wheat lines SG1, SG2, SG3, FS1 and FS2 were identified by the rates of decrease in chlorophyll content (greenness) and measures of photosynthesis (**Figures 1 and 2**). Importantly the slow-senescing lines were functional stay-green lines rather than cosmetic mutants merely failing to slow chlorophyll breakdown. At the whole-plant level, SG3 showed a stay-green phenotype at high N, while FS2 was fast-senescing (**Figure 3**). Stay-green line SG3 had high grain and tiller numbers, a high grain total N content at high N supply and was able to maintain its NHI under nitrogen-limiting conditions. However SG3 was not able to accumulate more biomass or to increase its yield (**Figures 4–6**), as has been reported for other stay-green wheat germplasm (Gong et al. 2005; Luo et al. 2006; Christopher et al. 2008; Chen et al. 2010; Chen et al. 2011). Accelerated senescence was even more disadvantageous, lowering yield, biomass, harvest index, post-anthesis biomass accumulation and N uptake, N remobilization from the leaves and nitrogen harvest index (**Figures 4–6, and Tables 1 and 2**).

The stay-green trait and the interaction with nitrogen supply varied under the different environmental conditions used in this study. Whilst FS2 always has a yield penalty, SG3 had a positive effect on yield at low N in the field alone (**Figures 4 and 5, and Tables 1 and 2**). It is possible that plants grown in pots in the glasshouse are more likely to be sink-limited than plants grown in field conditions.

The EMS mutagenesis procedure could have resulted in multiple mutations that diminished fitness of the mutants, both stay-green and fast-senescing plants, adversely affecting yield. However, since wheat is a hexaploid species it is unlikely background mutations occurred in all three copies of a gene simultaneously. Visible mutants are most likely to be gain-of-function mutations or mutations in genes that are already knocked out on the other genomes. So the background mutation load of the EMS procedure is unlikely to be high in a hexaploid species such as wheat.

Unexpectedly the results indicate that the accumulation and allocation of carbon (biomass) to the grain was negatively associated with the stay-green phenotype. SG3 had a high allocation of both C and N to the flag leaf and also expressed a high flag-leaf SPAD value (indicative of leaf N concentration) relative to anthesis date during grain filling compared to the WT and fast-senescing line FS2. Flag leaf N concentration of SG3 at anthesis though was similar to WT and FS2. Previous investigations in sorghum hybrids showed stay-green was linked to changes in the balance between N demand and supply during grain filling resulting in a slower rate of N remobilization from the leaves to the grain compared with senescent genotypes (Borrel and Hammer 2000; Van Oosterom et al. 2010a, 2010b). In wheat onset of senescence amongst genotypes was negatively correlated with the efficiency with which aboveground N at anthesis was remobilized to the grain (Gaju et al. 2011). Therefore, the physiological basis of the stay-green phenotype for SG3 compared to WT and FS2 may have been linked to a low grain N sink demand, since grain N supply traits such as shoot N uptake or flag leaf N concentration at anthesis were not increased for SG3 compared to WT and FS2.

It can be speculated that a reduced grain N sink size for SG3 may have been associated with a low potential grain weight, since grain number was slightly higher for SG3 compared to WT and FS2 under both high and low N conditions. Wheat seed weight hardly responds to photosynthesis levels during seed filling, indicating a lack of source limitation for seed growth with a standard grain filling period (Borrás et al. 2004). In a wheat stay-green variety, with an extended grain filling period of 6–7 days, the harvest index was reduced, indicating it was relatively inefficient in remobilization of carbon; most of the extra photosynthesis products remained in the vegetative parts instead of being trans-located to the grain (Gong et al. 2005). Under the conditions used in the current study, the grain may not have had sufficient demand for extra carbon-products, so that extra partitioning to the grain therefore did not take place. The extra post-anthesis photosynthetic capacity of SG3 may have resulted in additional allocation of assimilate to other plant

parts such as the roots. Consequently, in these circumstances, enhancing photosynthetic production did not increase grain yield as expected.

Generally grain nitrogen content is considered to be source-limited (Martre et al. 2003). In this study a lack of nitrogen may have resulted in a sink limitation for grain yield and an inability to exploit the extra carbohydrate provided by the stay-green phenotype. It has been suggested that a supplemental investment of nitrogen in the photosynthetic machinery (as in the stay-green phenotype) may be detrimental to the transfer of nitrogen to the grain and thus to final yield, at least if nitrogen uptake does not increase as well (Sinclair et al. 2004). For example, nitrogen concentration in straw of a stay-green line of wheat remained higher than controls, thus requiring more nitrogen uptake to achieve a grain protein content comparable to wild-type (Chen et al. 2011). However in this study, SG3 reached a grain nitrogen concentration similar to or higher than wild-type in all experiments under all levels of nitrogen nutrition, indicating that nitrogen was not limiting at maturity. Grain yield and grain total nitrogen content appeared to be limited by carbon remobilization, perhaps caused by a limitation to sink development due to a shortage of nitrogen earlier in grain filling.

Materials and Methods

Experimental conditions

54 ethyl methanesulfonate (EMS) mutant wheat lines (*Triticum aestivum* L. cv. Paragon, generated under the Defra-funded Wheat Genetic Improvement Network at the John Innes Centre, Norwich, UK) were grown in the glasshouse (18 °C day and 14 °C night temperature with a photoperiod of 16 hours) with five plants per pot (20 cm diameter) in a staggered randomised block design consisting of four blocks. The follow-up experiments were performed in a randomised block design with four replicate blocks. Lines used were FS1 (862a), FS2 (2514a), SG1 (2056a), SG2 (1389a) and SG3 (555a).

For the initial screening and further senescence measurements, plants were grown in a compost mix (Rothamsted Prescription Mix): 75% peat, 12% sterilised loam, 3% vermiculite, 10% grit (5 mm, lime free), to which 3.5 kg m^{-3} Osmocote Exact (Scotts UK Professional, UK), 0.5 kg m^{-3} PG mix (Hydro Agri Ltd, UK), lime to pH 5.5–6.0, and 200 mLm^{-3} Ultrawet wetting agent (Vitax, UK) were added. For the nitrogen nutrition experiment plants were grown in nutrient poor soil (Rothamsted Nematode Mix): 80% sterilised loam, 15% 2EW sand, 5% grit (5 mm). Both soil mixes were prepared for Rothamsted Research by Petersfield Products (Leicester, UK). Nutrients were applied in the form of full nutrient solution with 4.0 mM (high N) or 0.4 mM (low N) $Ca(NO_3)_2$ as the sole N source. 0.5 litre of solution was applied 12 times between planting and anthesis, amounting to a total of 67.2 and 672 mg N for low and high N treatments respectively. The pots were placed on saucers to keep the supplied nutrients in the pots. Additional demineralised water was supplied to maintain soil moisture when required.

The field validation experiment was run at Church farm, Bawburgh, Norwich, UK in 2009 on 6 m^2 plots in a randomised block design with three replicate blocks. Two levels of nitrogen fertilizer were applied: 100 and 200 kgN/ha.

Senescence measurements

Leaf greenness was assessed using a SPAD meter (SPAD-502, Minolta, UK). For the population screening two measurements were taken approximately in the middle of each flag leaf either side of the midvein. For the successive experiments two readings were taken of each individual flag leaf: one at one-third and a second at two-third down the leaf. The values of the flag leaves of the main shoot of all 5 plants were averaged to get one value per pot.

Photosynthetic rate (CO_2 fixation in μmol CO_2 m^{-2} s^{-1}) was measured using the Li-6400 Portable Photosynthesis System (LI-COR Inc, Lincoln, NE, USA). Conditions for all measurements were kept as constant as possible: the LED light source set to 1000 μmol m^{-2} s^{-1}, the relative humidity in the sample chamber at 65%, a 385 μmol mol^{-1} sample CO_2 concentration, and the leaf temperature set between 23.8 °C and 24.2 °C by adjusting the block temperature. The reading was taken when photosynthesis had stabilised. Measurements were performed on one flag leaf per pot.

Quantum Yield measurements were done on light-adapted leaves, in which case QY is equivalent to Fv'/Fm'. Measurements were done with a FluorPen FP100s handheld chlorophyll fluorometer (Qubit Systems Inc, Canada). Two readings were taken of each individual flag leaf; one-third and two-thirds down the leaf.

The Normalised Difference Vegetation Index (NDVI) was determined by using a Crop Circle ACS-210 crop canopy sensor (Holland Scientific, Lincoln, NE, USA) attached to a closed box, so that the sensor could only pick up reflections originating from the plants. Measurements were made weekly from just before anthesis until there was no further decrease in NDVI.

In the field senescence was assessed visually. The visual scores applied ranged from 0 (fully green) to 10 (completely senesced).

Yield characteristics

Grain yield, aboveground biomass, tiller number and the Harvest Index (HI) were determined per pot. Thousand Grain

Weights (TGW) were determined on the pooled grain of five pots (all pots per block of each nitrogen x genotype combination). These pooled samples were also used to count the total number of grains. In the field trial yields were combined, and recorded as 85% dry matter.

Nitrogen measurements

The pooled grain of the five pots per block of each nitrogen x genotype combination was used for grain nitrogen analysis. Nitrogen concentrations were determined by milling the dry tissues, oven-drying overnight at 80 °C, and then analysing 300 mg samples using a LECO N-analyser (Dumas combustion method). Total nitrogen contents were calculated according to the formula: "total N content = %N x dry weight". The Nitrogen Harvest Index (NHI) was determined from nitrogen contents of grain and the whole shoot of two single main shoots per pot.

Statistical analyses

Data from the population screening were analysed by Canonical Variates Analysis (by Dr Stephen Powers, Rothamsted Research). All other results were analysed by Analysis of Variance (ANOVA) using GenStat® software (Payne et al. 2009). The Least Significant Difference (LSD) at 5% level was used to assess whether two individual values were statistically different. If data were found not to be normally distributed, they were log-transformed or square-root-transformed. Correlations were calculated using Microsoft Office Excel 2007.

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