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RESEARCH PAPER

Physiological characterization of 'stay green' mutants in durum wheat

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Abstract

Four mutants with delayed leaf senescence were selected from seed of durum wheat mutagenized with ethylmethane sulphonate. Changes in net photosynthetic rate, efficiency of photosystem II and chlorophyll concentration during the maturation and senescence of the flag leaves of both mutant and parental plants were determined under glasshouse conditions. The four mutant lines maintained photosynthetic competence for longer than the parental line and are therefore functionally 'stay green'. The mutant lines also had higher seed weights and grain yields per plant than the parental line.

Key words: Photosynthesis, stay green, *Triticum durum*, yield.

Introduction

The average global grain yield per unit area of the major cereal crops, wheat, rice and maize, more than doubled between 1940 and 1980, but more recently the rate of increase has slowed (Evans, 1993, 1998). To meet the demand for food from the growing world population, a significant increase in the world grain production is required, particularly in crops grown in developing countries. As suitable land and existing water supplies are now largely exploited, increasing the maximum yield potential per unit land area and per unit resource must be a part of any strategy for achieving yield increases.

Historically, the yield potential of cereals per unit land area has been increased by repeated selection and cross-breeding of the most productive strains (Richards, 2000). This largely resulted from exploiting genetically-determined variation in the partitioning of dry matter to the grain relative to the straw, so that the ratio of the grain to the total above-ground biomass (i.e. the harvest index) has increased (Nelson, 1988), despite little or no increase in total biomass (Evans, 1998). However, the harvest index of many crops, such as wheat, is considered to be approaching a maximum (Nelson, 1988), and further increases in yield potential may therefore require increases in crop biomass. In other words, an increase in total net photosynthesis is required.

The total photosynthesis over the life of annual crops can be increased by extending the duration of active photosynthesis. Furthermore, maintaining the supply of assimilated carbon to grain during the grain-filling period of determinate crops ensures that the mass per grain is maximized. Delaying leaf senescence is one of the ways in which this can be achieved. Indeed, in plants such as *lolium temulentum* it has been calculated that delaying the onset of senescence by only two days will result in an increase in carbon fixed by the plant of about 11% (Thomas and Howarth, 2000). Delaying leaf senescence may be particularly advantageous under conditions, such as high temperature, that tend to accelerate senescence and thus decrease the supply of assimilates to the grain.

Genetic variation exists in the timing and rate of leaf senescence, both between species and genotypes. Furthermore, mutants also occur whose leaves remain

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green for longer than those of the parental genotypes: these are defined as 'stay green' and examples of this phenotype have been identified in a number of crop species, including cereals (Thomas and Smart, 1993).

The 'stay green' phenotype can arise in different ways. Alteration of the genetic processes determining the initiation of senescence and its rate of progress results in a phenotype which continues to photosynthesize for longer than normal (i.e. is 'functional stay green') and which might therefore be expected to result in a higher yield. By contrast, other types of 'stay green' mutant remain green due to retention of chlorophyll resulting from lesions in its catabolism, but lack photosynthetic competence (Thomas and Howarth, 2000). The most extensively described 'stay green' mutant is an allele of the *sid* (*senescence induced degradation*) locus in the grass *Festuca pratensis* (Thomas and Stoddart, 1975), which is inherited as a single recessive nuclear gene (Thomas, 1987). Green tissues of individuals homozygous for *sid*^{y} retain chlorophyll more or less indefinitely, but this is not associated with an extended duration of photosynthetic CO_2 assimilation capacity (Hauck *et al*., 1997). The *sid* mutant is therefore a type C or cosmetic 'stay green' (Thomas, 1982; Hauck *et al*., 1997).

A similar phenotype has been observed in other species (Thomas *et al*., 1999), including maize (Gentinetta *et al*., 1986; Bekavac *et al*., 1995) and sorghum (Duncan *et al*., 1981; Tao *et al*., 2000). It has not been observed in bread wheat (*Triticum aestivum*), presumably, because it is a hexaploid of recent origin (approximately 10 000 years ago). Hence, related genes are expressed on two or more of the genomes and it is not possible to identify and exploit recessive mutations. By contrast, pasta wheat (*Triticum durum*) is an ancient tetraploid in which diploidization of some genes has occurred (García-Olmedo *et al*., 1978), meaning that it is theoretically possible to identify recessive mutations.

In the present paper, the selection and photosynthetic characteristics of 'stay green' mutants of durum wheat are described and the potential for exploiting this trait to improve the yield and performance of this important crop is discussed.

Materials and methods

Mutagenesis and selection of mutants

10 000 seeds of *Triticum durum* Desf. (cv. Trinakria) were soaked for 2 h in 0.3 M ethylmethane sulphonate (EMS) and sown in the field at the Experimental Institute for Cereal Crops (ISC) Foggia, Italy. One seed of each M_2 plant was sown in the field and the plants selfed for two generations to give M_5 lines. Initial screening was then carried out by visual examination of the degree of leaf yellowing during the later stages of crop maturation. Four independent mutants were chosen for detailed study based on their similar timing of flowering, but delayed timing of senescence. Individual M_5 plants were grown in the field. Control plants had not been treated with the mutagen.

Growth of plants

For the induction of senescence, plants were grown in a glasshouse at Long Ashton Research Station (Bristol, UK) in 25 cm pots of compost containing slow-release fertilizer (3.5 kg Osmocote m^{-3} with 15-11-13 NPK plus micronutrients). The photon flux (photosynthetically active radiation) was *c*. 750 µmol m^{-2} s^{-1} (supplied by 400 W sodium lamps) with a 16 h light period at 20–25 °C, 14–16 °C during darkness and 50–70% relative humidity.

For measurements of gas exchange and yield, plants were grown in a glasshouse at Rothamsted Research (Harpenden, UK) in 20 cm diameters pots of compost plus 3.5 kg m^{-3} Osmocote slow-release fertilizer and 0.5 kg m^{-3} PG mix (14-16-18 NPK granular fertilizer plus micronutrients). The photosynthetically active photon flux averaged *c*. 750 µm s^{-1} m^{-2}, supplied by 400 W sodium lamps with a 16 h light period at 18–20 °C, and 14–16 °C during darkness and 50–70% relative humidity.

Induction of senescence in detached leaves

Incubation of detached leaves in darkness was used to accelerate senescence in a consistent manner in the fifth youngest, fully-expanded leaves from both parental and mutant plants. After booting, the leaves were excised, placed in 500 ml beakers filled with tap water and incubated in the dark at *c*. 20 °C for 6 d (Vincentini *et al*., 1995). Leaves were sampled every 3 d for chlorophyll content. The tips and basal parts of the leaves were discarded and three replicate samples of each stage taken for chlorophyll determination.

Determination of chlorophyll content

Three discs (*c*. 20 mm diameter) were taken from different areas of each leaf sampled for induction of senescence in the dark, immediately frozen in liquid nitrogen and stored at –80 °C. Chlorophyll was extracted by grinding in 1 ml of cold 80% (v/v) aqueous acetone and centrifuging at 11 000 *g* for 15 min at 4 °C. This was repeated twice and the combined supernatants diluted 10-fold with 100% acetone and the A_{645} and A_{663} were measured (Buchanan-Wollaston and Ainsworth, 1997). Chlorophyll *a* and *b* concentrations were calculated by the equations of Hill *et al*. (1985).

The relative chlorophyll contents of leaves of the plants used for gas exchange analysis were determined *in situ* using a hand-held chlorophyll meter (SPAD-502, Minolta, UK), averaging readings from ten sampling positions on the leaves of individual plants. The measurements are given as relative units.

Gas exchange measurements

Water vapour and carbon dioxide exchange were measured weekly on attached flag leaves from flowering until full senescence, from eight different plants of each line. Measurements of P_n were at ambient CO_2 concentration (350 µmol mol^{-1}) in a multi-chamber gas-exchange system with automatic data collection and analysis under 800 µmol m^{-2} s^{-1} PAR, in chambers with forced ventilation, and temperature regulated to 20±1 °C. Photosynthetic rates and substomatal CO_2 concentrations were calculated as described by Habash *et al*. (1995).

Measurement of chlorophyll a *fluorescence*

Chlorophyll *a* fluorescence from PSII was measured with a modulated fluorescence meter (OSLOG-100, OPTI Sciences, USA) in flag leaves attached to the plant immediately after gas exchange. To ensure maximum oxidation of electron acceptors and full dissipation of the transthylakoid proton gradient, leaves were dark-adapted for at least 40 min. Minimum fluorescence (F_o) and maximum fluorescence (F_m) were measured in the dark-adapted leaves, using a 2 s light pulse (3000 µmol photons m^{-2} s^{-1} in the range from 350–700 nm) to saturate all PSII reaction centres. The photochemical efficience of PSII was calculated as the ratio of

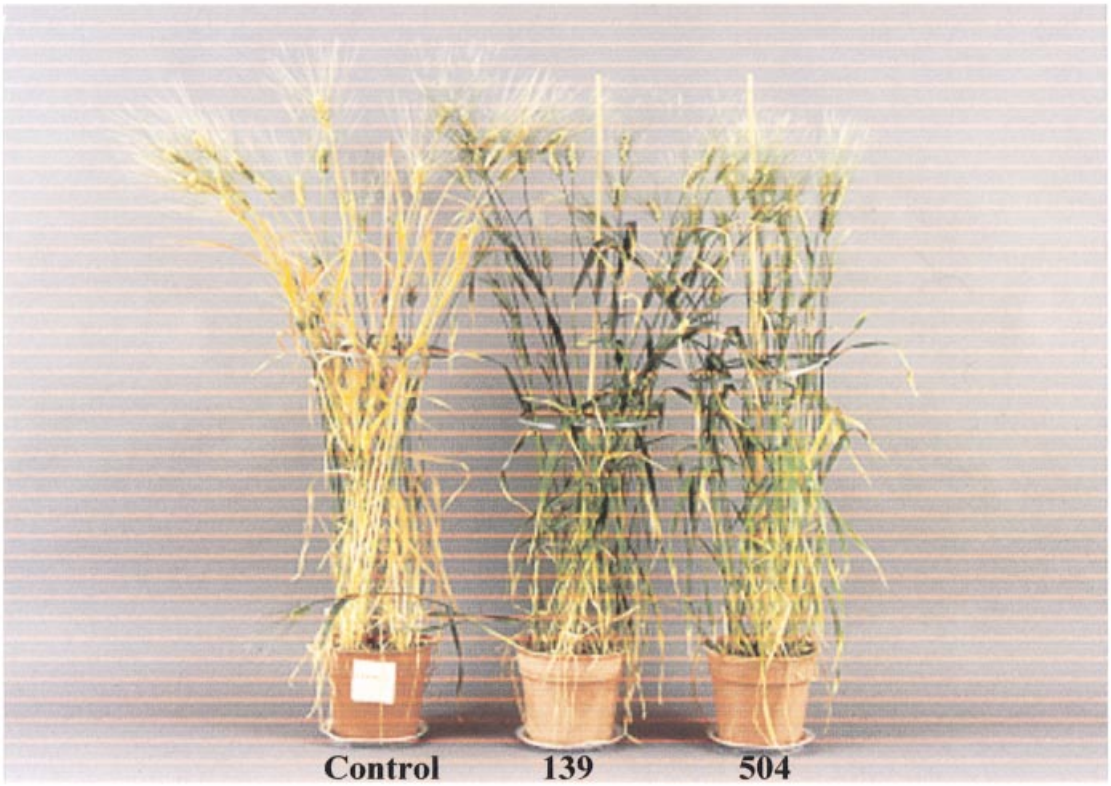

Fig. 1. The 'stay green' phenotype in durum wheat. Control plant, cv. Trinakria; 139, mutant 139; 504, mutant 504.

variable fluorescence (F_v=F_m–F_o) to maximum fluorescence (F_m) (Genty *et al*., 1989).

Grain weight, yield and nitrogen content

The number of ears per plant was determined from the experiment at Rothamsted. 1000 mature seeds were dried at 110 °C for 24 h and their weight determined after removal of embryos. Total nitrogen content of the milled grain (0.2 mm particle size) was determined by Kjeldahl analysis (Benton-Jones, 1991)

Results

Identification of durum wheat mutants with delayed leaf senescence

Four mutant lines (139, 142, 196, 504) were selected based on their similar timing of flowering but delayed senescence and experiments were carried out on M_6 plants grown in the glasshouse at Long Ashton Research Station and Rothamsted Research, UK.

The onset of chlorophyll loss was delayed in the leaves of the mutants, by about 10 d compared with the parental genotype (Fig. 1). Flowering time, as measured by the appearance of pollen, was three days earlier in mutants 139 and 142 than in the control plants, while mutants 504 and 196 had similar flowering times to the control plants. Therefore, the leaf age was measured, taking the flowering time as the starting point.

Senescence progressed normally in the control plants, with the flag leaves becoming senescent before the stem. However, in the 'stay green' mutants this phenotype was inverted, with the flag leaf remaining almost unchanged in colour, while the stem became completely senescent.

In vitro *chlorophyll breakdown*

Senescence of detached, youngest, fully expanded leaves in darkness was faster in the parental line than in the mutants, with a lower chlorophyll content after 3 d (Table 1). The chlorophyll contents, measured using a SPAD meter, of mature flag leaves of control plants grown in the glasshouse also decreased much earlier in the parental line than in the mutants; starting *c*. 20 d after flowering (DAF) (Fig. 2a), with visible yellowing being observed at about 20–25 d and almost all chlorophyll being lost by 30–40 d. By contrast, the chlorophyll contents of the leaves of mutant plants only decreased after about 30–35 d. Two of the mutants (139 and 142) retained about 50% of their initial chlorophyll content after 40 d, while the others remained slightly green.

Photosynthesis and photochemical efficiency during leaf senescence

Net photosynthesis decreased (Fig. 2b) over the period of measurement in flag leaves of the parent plants, falling to

Table 1. *Changes in the total chlorophyll content of senescing leaves of parental (cv. Trinakria) and mutant plants incubated in continuous darkness for 6 d (0 d→6 d)*

Leaves were excised and placed in the dark to induce senescence. LSD (P=0.05) = 0.236.

	Chl *a* (μg cm^{-2})			Chl *b* (μg cm^{-2})			Total Chl (μg cm^{-2})		
	0 d	3 d	6 d	0 d	3 d	6 d	0 d	3 d	6 d
Trinakria	76.2	40.1	3.3	56.1	28.4	1.2	132.3	68.5	4.5
139	75.7	55.0	34.7	54.8	33.1	17.2	130.5	88.1	51.9
142	75.1	58.0	38.4	54.9	36.1	22.0	130.0	94.1	60.4
504	76.5	60.1	38.2	55.5	35.9	20.9	132.0	96.0	59.1
196	76.1	57.8	37.8	55.2	36.0	21.2	131.3	93.8	59.0

zero at about 40 DAF. By contrast, P_n decreased later in the mutants with lines 139 and 142 retaining about 50% of their initial rate at 40 d. The CO_2 concentration within the leaf (C_i) increased after flowering in all lines but became greater in the parental than in the mutant plants as senescence progressed (Fig. 2c).

Maximum values of 0.5–0.6 were obtained for the photochemical efficiency (F_v/F_m ratio) of leaves of both mutant and control plants (Fig. 2d). However, whereas $F_{v/}$ F_m decreased steadily in the control plants until it reached zero at 40 DAF, it only decreased after about 25 DAF in the mutants. F_v/F_m was much lower in mutants 142 and 139 than in mutants 196 and 504 during the later period of maturation.

Grain weight, yield and nitrogen content

The parental and mutant lines had the same average number of ears per plant (Table 2). Increases in 1000 seed weight (by 10–14%) occurred in all the mutants but the embryo weights were lower than in the control plants (Table 2). Grain of the four mutants also had lower total nitrogen contents than those of the parent when expressed as mg g^{-1} dry wt. but differed when expressed as mg grain^{-1}, being slightly higher in 139 and 509 but lower in 142 and 196.

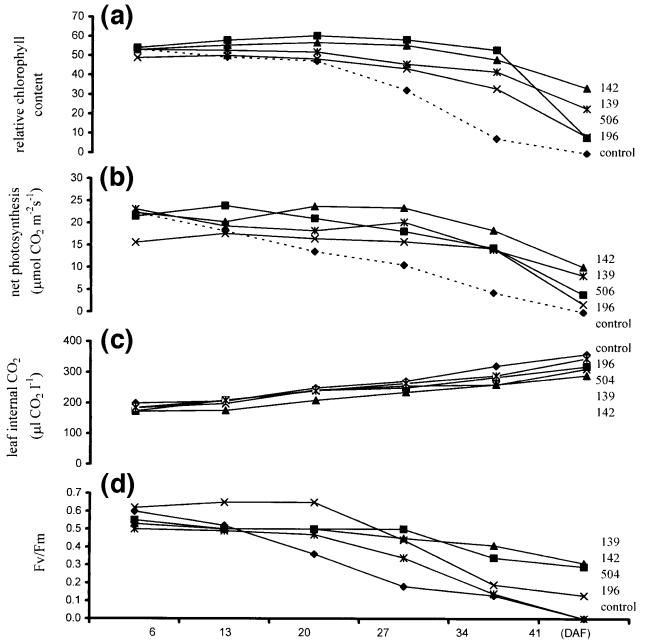

Fig. 2. Changes in biochemical parameters during senescence of mutant (lines 139, 142, 196, 504) and control (cv. Trinakria) plants of durum wheat. (a) Relative chlorophyl content; (b) net photosynthesis; (c) leaf internal CO_2 concentration; (d) efficiency of photosystem II. Values are the means of readings taken on the flag leaves of eight individual plants of each line from flowering until senescence. DAF, days after flowering. LSD (P=0.05) for comparing mutant and control plants are 5.25, 3.85, 19.3, and 5.25 for (a), (b), (c), and (d), respectively.

Discussion

Four mutants of durum wheat were selected which showed marked delay in chlorophyll loss from leaves compared to the parental lines, even after only 3 d of dark-induced senescence. However, chlorophyll loss is not always associated with the maintenance of photosynthetic competence (Thomas and Smart, 1993). Consequently, the photosynthetic competence of the mutants was also determined by measuring parameters related to photosynthesis from flowering until full senescence: chlorophyll content, P_n, C_i and the efficiency of PSII (F_v/F_m). These parameters changed at different rates between the mutants and control plants, with P_n being maintained longer in the mutants than in the parent line. The C_i was also lower in the mutants than in the parental line towards the end of the growing season, because the rate of CO_2 assimilation was maintained. Similarly, the chlorophyll content was also maintained over a longer period in the mutants. However, P_n decreased before, and more strongly than, the chlorophyll content (Fig. 2) in both control and mutant plants. Differences in the rates of decline of chlorophyll content and P_n during senescence also occur in barley (Friedrich and Huffacker, 1980; Humbeck *et al*., 1996).

Photosynthesis depends on the function of the light-harvesting and electron transport systems within the chloroplasts which is indicated by the photochemical efficiency, measured as the F_v/F_m ratio. This ratio (0.7–0.8) was smaller than expected in both parental and mutant

Table 2. *Grain yields, weights and nitrogen contents of mutant and parental (cv. Trinakria) control plants*

Lines	1000 seed wt. (g)	1000 embryo wt. (g)	Total grain yield per plant (g)	N content per grain (mg)	Total N (mg g^{-1} dry wt)	Ears per plant
Trinakria	54.0	5.8	12.0	1.80	33.0	5
mutant 139	62.0	4.0	14.0	1.86	30.0	5
mutant 142	60.0	3.4	13.5	1.68	28.0	5
mutant 504	59.0	3.2	13.3	1.86	31.5	5
mutant 196	56.5	4.0	12.9	1.70	30.0	5
LSD (p=0.05)	0.80	0.50	0.20	0.21	0.17	0.10

leaves at the start of measurement, probably because the leaves had emerged some days before flowering. The F_v/F_m ratio decreased progressively in flag leaves, but much earlier in the parent than in the mutants. Loss of photosynthetic competence can result from several processes including the breakdown of proteins (especially of PSII and Rubisco) and the destruction of membranes by lipid degradation (Thomas, 1982, 1987). It is concluded that the mutants of durum wheat retained photosynthetic competence for longer than the control plants and therefore have a functional 'stay green' phenotype (Thomas and Smart, 1993).

The 'stay green' mutants of durum wheat had 10–12% increases in seed weight (Table 2) when grown in the greenhouse, which was presumably related to the extended duration of photosynthesis resulting in increased translocation of photoassimilate to the grain. This may be particularly important during the later phase of grain filling when shortage of assimilates, resulting from leaf senescence, limits the final accumulation of starch in the endosperm to below the potential capacity. The lower total N content per gram dry matter in the mutants is partly due to dilution by accumulated carbohydrates, but two of the mutant lines (139 and 504) also had slightly lower N contents per grain. This suggests that the remobilization of nutrients from the leaves to the grain during senescence had a limited effect on grain protein content. Chlorophyll and thylakoid proteins represent about 25% of the total nitrogen in a mature leaf (Evans, 1988), so if a significant proportion of protein N is retained in leaves of 'stay green' mutant plants there is a risk that the supply of N may limit grain growth and the accumulation of proteins. Consequently, it may prove necessary for farmers to provide additional nitrogen fertilizer in order to produce grain with sufficiently high protein for making bread and pasta.

It can be concluded that the 'stay green' mutants of durum wheat are characterized by delayed senescence, but not uncoupling of chlorophyll content from photosynthetic competence. The extended period of flag leaf photosynthetic competence is associated with the production of larger grains, presumably as a consequence of increased carbohydrate content. Extending the capacity of the plant to photosynthesize and produce assimilates during the later phase of grain filling by delaying the onset of senescence may therefore, increase the potential grain yield in durum wheat. Consequently, transferring the 'stay green' trait to commercial lines in order to increase the sink strength is a worthwhile goal to increase the distribution of assimilate to the grains. However, it is necessary to verify the effects of the stay green mutation on yield of durum wheat under field conditions, particularly under drought stress which is common especially during the grain-filling period.

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