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Five ways to stay green

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Abstract

The relationship between carbon income and expenditure over the life of a leaf is described and related to the productivity benefits of altering the timing of senescence initiation. In genetic variants with delayed leaf senescence ('stay-greens') deconstruction of the photosynthetic apparatus during leaf senescence is partially or completely prevented. Although the stay-green phenotype is superficially similar in all species and genotypes, the genetic and physiological routes to the trait are diverse. In one type of stay-green, chlorophyll catabolism is disabled. Legumes and monocots with pigment breakdown lesions are discussed. Sorghum is presented as an example of another kind of stay-green in which perennial tendencies have been bred into a monocarpic annual crop species. Transgenic approaches are briefly discussed (enhanced endogenous cytokinins, reduced ethylene production or perception). An alternative route towards making a stay-green phenotype is through quantitative trait mapping and marker-assisted selection. Loci for greenness in pearl millet have been identified, some of which are associated with drought responses or flowering time. Finally the question of the limits on stay-green as a productivity-enhancing character is addressed.

Key words: Leaf senescence, productivity, stay-green, mutant, chlorophyll, sorghum, transgenic, pearl millet, QTL, drought.

Income and expenditure

Plant productivity is a measure of net assimilation, that is, the difference between the gross amount of resource captured and the fraction that is expended or dissipated to satisfy the requirements of thermodynamics, maintenance, defence, and other biophysical obligations. But instantaneous measurements of inputs and outputs generally do not relate in any very direct way to total production (Thomas, 1992). The reason is clear—such measures must be integrated over time and it is a feature of plant development that physiological processes, including, and perhaps especially, assimilation and maintenance, undergo large ontogenetic changes.

One way of assessing the contribution of a leaf to the photosynthetic productivity of the whole plant is to measure carbon fixation capacity from birth to death of the organ and to subtract from this the fractions of carbon destined for respiration and for investment in the structure of the leaf itself over the same time-scale. Such a study of the net contribution of the fourth leaf to the total carbon economy of *Lolium temulentum* grown under standard conditions has been made (Gay and Thomas, 1995). These authors presented a curve (the 'carbon credit contour'; Thomas, 1987*a*) describing the estimated export of carbon from leaf emergence to advanced senescence, based on which the return on the investment of raw material in making and maintaining that leaf could be calculated (Fig. 1). The net contribution over the lifetime of leaf 4 to the growth and maintenance of the rest of the plant (that is, the area beneath the curve) is 36.8 mg C. In order to make leaf 4 required the investment of about 10 mg C between initiation and the time when the leaf became autotrophically self-sufficient. Thus each fourth leaf (typical of *Lolium* leaves, in general, in size and physiology) contributes enough C to the total economy of the plant to make 3.7 more leaves, each of which will, in turn, support 3.7 more ... which means that unconstrained production of leaves from n pre-existing leaves would result in 3.7^{n-1} further leaves. It may be calculated that it would take only about 33 successive leaves to approach the estimated net annual primary production of the entire biosphere (48×10^{15} g; Potter

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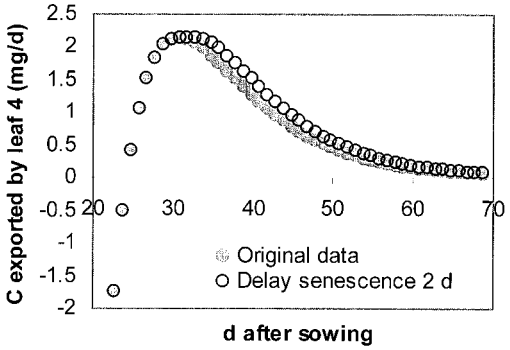

Fig. 1. Net carbon import and export from birth to death of leaf 4 of *Lolium temulentum* (based on data from Gay and Thomas, 1995). A second curve has been added, representing the trend for a leaf in which the initiation of senescence has been delayed by 2 d.

et al., 1993). So much for the simple mathematical games one can play with these data.

More relevant to the subject of the present paper is what Fig. 1 tells us about the temporal constraints on photosynthetic production. Export of C is at a maximum on day 32 and declines thereafter. Operationally, day 32 may be taken to define the point at which leaf senescence is initiated. What would be the consequence of delaying the start of senescence by, for the sake of argument, 2 d? The second curve in Fig. 1 illustrates this. The result is to increase the total contribution of C to the plant over the lifetime of the leaf by 4.1 mg, or 11%. This is achieved without altering the rates of either the establishment of maximal expanded and exporting leaf surface or of senescence once underway. The scale of the response of productivity to such a relatively small modification to the timing of senescence emphasizes the need for a thorough systematic understanding of the process, its genetic basis and the options for non-empirical manipulation.

How to stay green

The progress of senescence is normally apparent to the eye as loss of chlorophyll. Stay-green is the general term given to a variant in which senescence is delayed compared with a standard reference genotype. It has been shown how a stay-green phenotype could arise in one of four fundamentally distinct ways (Thomas and Smart, 1993). In Type A stay-greens (Fig. 2), senescence is initiated late but then proceeds at a normal rate. Assuming a tight correlation between net carbon output and chlorophyll content (an assumption by no means justified in practice), the hypothetical case shown in Fig. 1 would be classified as quantitative stay-green behaviour of Type A. Type B stay-greens (Fig. 2) initiate senescence on schedule, but thereafter senesce comparatively slowly. In Type C stay-green behaviour, chlorophyll may be retained more or less indefinitely (Fig. 2), but measures of physiological function such as photosynthetic capacity show that senescence is proceeding normally beneath the cosmetic surface of retained pigmentation. Another way of ensuring unlimited colour retention is to kill the leaf by, for example, freezing, boiling or drying. Stay-greens of the frozen spinach or herbarium specimen kind are referred to as Type D (Fig. 2) (Thomas and Smart, 1993).

Classifying stay-greens in this way is useful for understanding the kinds of modified gene or physiological process underlying the phenotype, but, in practice, particular stay-greens can be combinations of two or more different functional types. Fig. 3 gives an illustration of this point. Pigments and photosynthetic parameters were measured in two maize lines, one of which displays a delay of about 5 d in yellowing of the ear leaf during grain filling. Greenness measured non-destructively with a hand-held chlorophyll meter (Fig. 3a) followed fairly closely the progress of photosystem II function determined by the fluorescence parameter F_v/F_m (Fig. 3d). But photosynthetic capacity measured by IRGA declined identically in the two lines (Fig. 3b); neither did the increase in substomatal CO_2 during senescence (Fig. 3c) relate to the trend in leaf pigment. The late-senescing line has some features of a Type A stay-green (delayed initiation of yellowing) and some of Type C (retention of chlorophyll, but normal loss of CO_2 fixation capacity). Closer examination of the data in Fig. 3 reveals further subtleties. Leaves of the late-senescing line tend to be greener at maturity than those of the early genotype (Fig. 3a). It may be, therefore, that the late line is stay-green simply because its chlorophyll has further to fall. This kind of behaviour might be classified as Type E (Fig. 2).

Particular environmental, surgical and chemical treatments can prevent, arrest or even reverse foliar yellowing. For instance, shading a plant often greatly extends the green area duration of its mature leaves (Mae *et al.*, 1993). Removing the shoot above a senescing leaf commonly results in the arrest of yellowing, and even regreening. In the case of maize, detopping has been shown to down-regulate senescence-enhanced genes (Griffiths *et al.*, 1997). A vast array of growth regulators, metabolic inhibitors and chemicals of unknown mode of action will stop yellowing. Chelators of iron, to take just one example, are effective in retaining greenness, probably because they target an iron-dependent step in chlorophyll degradation (Hörtensteiner *et al.*, 1995).

A generalization can be made by stating that outside interference makes a plant stay green by perturbing a particular biochemical or physiological process. In turn, the process will be specified by one or more genes. It follows that manipulating such a gene will also produce a stay-green phenotype, but in this case the trait will usually be heritable. For most examples of genetically-determined stay-green, virtually nothing is known of the biochemical alterations that stand between the variant

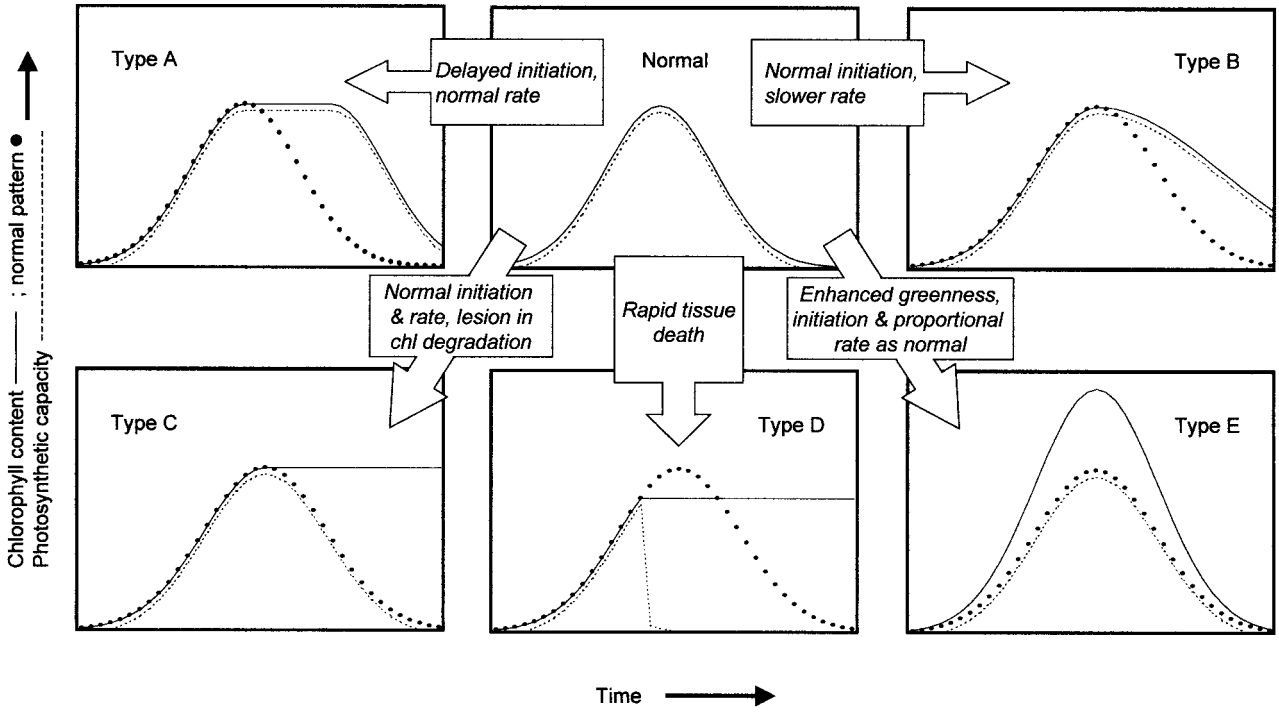

Fig. 2. Five ways to stay-green. Curves show chlorophyll content and photosynthetic capacity (arbitrary scale) for a representative leaf, whole plant or canopy. Type A stay-greens lose pigment and function at the normal rate after a delay in the start of senescence. In Type B, senescence is initiated on schedule, but subsequently proceeds more slowly. Type C stay-greens undergo functional senescence on a normal time-scale, but a lesion in pigment breakdown means they retain chlorophyll indefinitely. Type Ds are stay green because they are dead. In Type E behaviour, the photosynthetic capacity of an intensely green genotype may follow the normal ontogenetic pattern, but comparison of absolute pigment contents identifies it as a stay-green.

gene and the superficial phenotype. However, there are a few models where the gap between genotype and phenotype has been at least partially bridged and which suggest non-empirical approaches to creating useful stay-greens. Some examples are discussed below.

Knock out chlorophyll degradation

The facile way of creating a stay-green is to disable pigment degradation. In fact stay-greens have played an important part in research carried out over the last decade or so to establish the catabolic pathway for chlorophyll. To date only two steps in the sequence have been shown to give rise to heritable stay-green phenotypes when blocked by a genetic lesion. A cytoplasmically inherited mutation, *cytG*, renders the chlorophyll *b* of senescing soybean leaves more stable than chlorophyll *a* (Guiamét *et al.*, 1991). The chemical structures of all known terminal chlorophyll catabolites have the distinctive methyl group on C7 of ring B that show them to be chlorophyll *a* derivatives. The C7 formyl group of chlorophyll *b* must be converted to the *a*-type methyl structure before the macrocycle is opened (Scheumann *et al.*, 1999). It is significant in this regard that the ring-opening oxygenase is specific for phaeophorbide *a* and is competitively inhibited by phaeophorbide *b* (Hörtensteiner *et al.*, 1995). The detailed phenotype of the *cytG* mutation of soybean is consistent with a lesion in *b* to *a* conversion, but this has not been confirmed biochemically, nor has the corresponding locus been isolated and characterized.

All other known chlorophyll catabolism mutants have a lesion at the ring-opening (phaeophorbide *a* oxygenase, PaO) step of chlorophyll catabolism. The green cotyledon character of peas, one of the seven traits originally addressed by Gregor Mendel, is a pleiotropic expression of generalized foliar stay-greenness and is characterized by negligible PaO activity during senescence (Thomas *et al.*, 1996). A similar phenotype in french bean is the consequence of a mutation at a locus probably homologous to that of pea (Bachmann *et al.*, 1994). The best characterized PaO activity mutant is a stay-green originally described in the grass *Festuca pratensis* (Thomas, 1987*b*; Vicentini *et al.*, 1995).

Although these Type C stay-greens may be classified as 'cosmetic' rather than functional, in the sense that the persistence of greenness is not associated with extended photosynthesis, their cellular and physiological phenotypes are more complex than pigmentation alone might suggest. Active degradation of chlorophyll is a prerequisite for rendering the pigment-associated proteins of the thylakoid membrane available for remobilization as part of the salvage and recycling function of leaf senescence (Thomas, 1997). This means that the internal N

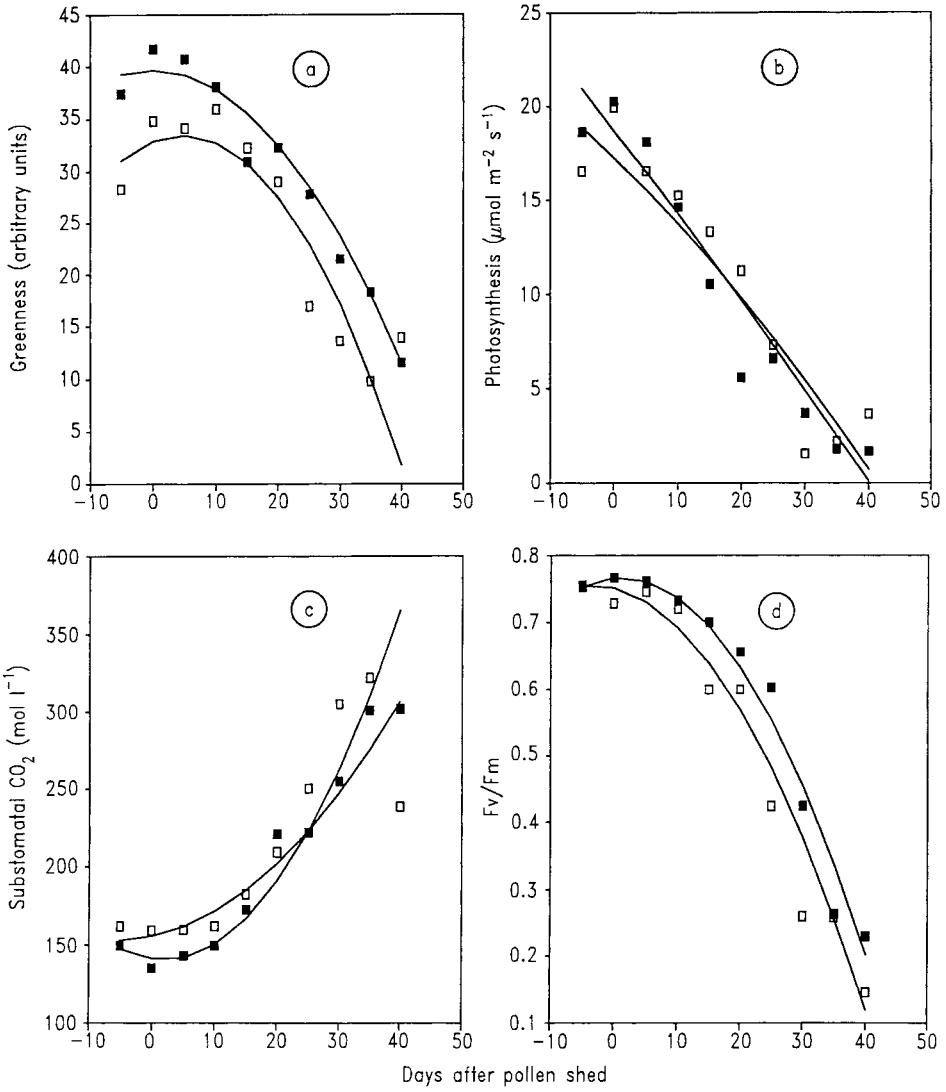

Fig. 3. Senescence of the ear leaf of an early (□) and a late (■) senescing maize line. Growth and analysis conditions as described earlier (Smart *et al.*, 1995). (a) Greenness measured with a hand-held chlorophyll meter; (b) photosynthetic CO_2 fixation rate determined at light saturation by infrared gas analysis; (c) substomatal CO_2 concentration estimated by IRGA; (d) ratio of variable to maximal chlorophyll *a* fluorescence (estimate of quantum efficiency of electron transport through photosystem II) measured at ambient temperature in dark-adapted leaves.

economy of Type C stay-greens may be compromised under conditions of low N supply to the roots (Hauck *et al.*, 1997). Close examination of the chlorophyll-associated proteins of stay-green *Festuca* reveals interesting nuances. LHCP-2 does not remain unchanged throughout senescence of the mutant. Leaves of Bf993, the stay-green genotype, progressively accumulate a proteolytic fragment, which appears to be native LHCP-2 protein minus the N terminal hydrophilic region that protrudes into the stroma (Fig. 4; Kühlbrandt *et al.*, 1994). A plausible explanation is that Rubisco and other soluble stroma proteins are degraded normally as stay-green leaves senesce and, as the supply of soluble substrates runs down, the proteolytic system in the stroma increasingly turns its attention to exposed regions of mostly buried membrane proteins. This may therefore be

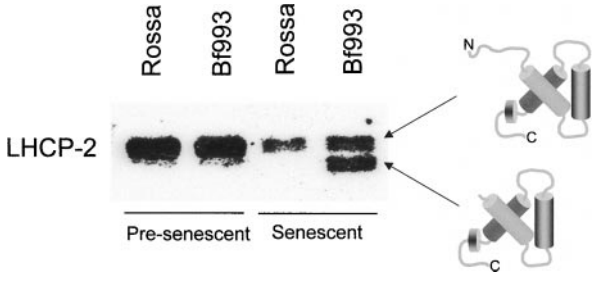

Fig. 4. Western blots of the light-harvesting protein of photosystem 2 from leaves of a normal (Rossa) and stay-green (Bf993) genotype of *Festuca pratensis*. Senescence conditions, analysis and immunoblot methodology as described earlier (Thomas *et al.*, 1999). Schematic diagrams (based on Kühlbrandt *et al.*, 1994) of likely structures of the two forms of LHCP-2 in senescent Bf993 tissue are shown.

a rare glimpse of the elusive protein-degrading mechanism of senescing plastids caught in the act.

Introduce perennial tendencies into an annual

It is possible to make wide hybrids between certain related species or genera with contrasting degrees of annuality or perenniality. The tendency to perenniality may weaken the monocarpic influence that triggers and sustains wholesale foliar senescence in an annual. Something of the sort seems to have happened in the breeding of lines of sorghum with marked stay-green and drought-tolerant characteristics. Sorghum and pearl millet were both domesticated in tropical Africa 3000–5000 years ago and although from the beginning cultivation was primarily to produce grain for food, the crop residue has always been, and remains still, an important commodity for the farmer (Kelly *et al.*, 1991). In some regions, ratooning of sorghum is used to extend the productive period of this normally annual cereal over two or more seasons (Escalada and Plucknett, 1975), exploiting the perennial tendency of some cultivars.

Stay-greenness in sorghum is genetically and physiologically complex, exhibiting a variety of expression patterns and environmental sensitivities depending on background genotype (van Oosterom *et al.*, 1996). Under normal field conditions, leaves of many typical sorghum lines senesce after grain maturity. Some genotypes, however, not only remain green (Duncan *et al.*, 1981), but also contain significantly more carbohydrates in the stem at all maturity stages than go-brown types and have a higher grain weight (McBee *et al.*, 1983). Drought during grain filling hastens leaf senescence leading to premature death (Rosenow and Clark, 1981). When water is limiting, however, stay green genotypes retain more green leaf area than do genotypes not possessing this trait, and they also continue to fill grain normally under drought conditions (Rosenow *et al.*, 1983). Moreover there is a positive association between stay green and grain yield under water-limited environments (Borrell and Douglas, 1996). Stay-green also reduces lodging, and there is good association with resistance to stem rots as well (Rosenow, 1984), suggesting that stay-green leaves remain photosynthetically active.

Sorghum genotypes vary in the timing of senescence initiation and also in the subsequent rate of leaf senescence. Greenness trends in leaf six of nine contrasting sorghum lines grown under well-watered conditions are presented in Fig. 5. By fitting linear splines (Ross, 1986) it is possible to identify the time of onset of visible senescence (location of breakpoint between the two fitted lines), and rate of yellowing can be estimated from the slope of the line to the right of the breakpoint. This allows the classification of early and late senescing types (E36-1, origin Ethiopia, and B35, Texas A&M stay-green,

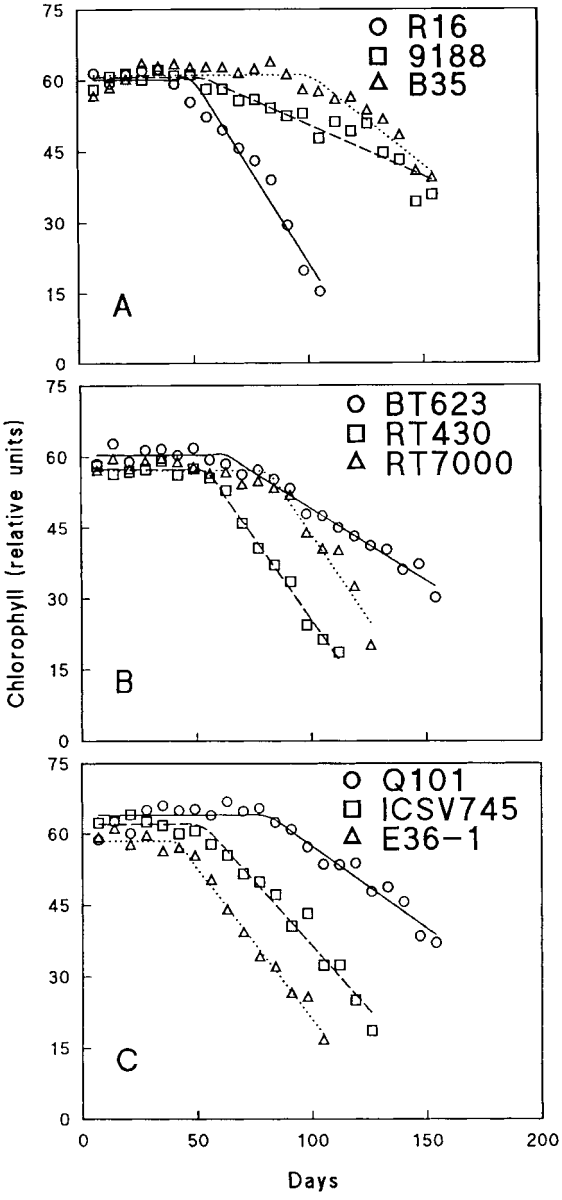

Fig. 5. Leaf senescence of sorghum lines. Plants were glasshouse-grown from seed under well-watered conditions similar to those employed for maize by Smart *et al.* (Smart *et al.*, 1995). Chlorophyll was measured in leaf 6 using a portable chlorophyll meter and splines were fitted (Ross, 1986) in order to estimate the time of senescence initiation and the rate of yellowing.

are, respectively, extreme examples) and fast (RT7000 from Texas, for example) and slow (e.g. B35) lines. Although E36-1 is a known source of stay-green, field observations suggest that the trait is only expressed under water-limiting conditions (van Oosterom *et al.*, 1996); this was confirmed in the present experiment in which plants were well-watered throughout. In a separate experiment, this time with a much more restricted supply of water, photosynthesis was measured in eighth leaves of a line showing strong expression of the stay-green phenotype (QL41, from the Queensland breeding programme

and derived partly from B35), an early and rapidly senescing line (SPV475 from ICRISAT, Hyderabad) and R16, an intermediate type (ICRISAT). Figure 6 shows that QL41 still retained almost pre-senescent levels of chlorophyll, light-saturated CO_2 fixation capacity and quantum efficiency at 85 d. Only about 20% of chlorophyll remained in SPV475 leaves at this point and there was no measurable photosynthetic activity. R16 leaves were completely senescent by 95 d, but QL41 leaves retained measurable chlorophyll and photosynthetic capacity until beyond 105 d. Compared with SPV475, R16 looks to be a type B stay-green (Fig. 2) whereas QL41 is a type A.

Other ways to stay green

Expression of senescence genes that have been cloned may be modified by transgenic intervention or mutagenesis. Genes associated with leaf senescence (*See*s) have been cloned from several species and *See* homologues have also been isolated from fruits and from flower parts (Smart, 1994; King and O'Donoghue, 1995; Buchanan-Wollaston, 1997; Nam, 1997; Medina-Suárez *et al*., 1997). Around 50 *See*s have been assigned possible functions in senescence on the basis of sequence homology (Buchanan-Wollaston, 1997). There has been no report of an altered leaf senescence phenotype based on transgenic or specific mutagenic (e.g. transposon) targeting of any *See*, and this clearly represents an important area of opportunity for current and future stay-green research.

The most dramatic transgenic intervention leading to a stay-green phenotype concerns manipulation of endogenous cytokinin status. In plants transformed with a conditional promoter fused to *ipt*, an *Agrobacterium* gene encoding a limiting step in cytokinin biosynthesis, increased amounts of cytokinins where the promoter is active are associated with delayed senescence (Smart *et al*., 1991). Gan and Amasino fused *ipt* with the promoter of *SAG12*, a *See* from *Arabidopsis* (Gan and Amasino, 1995). Tobacco transformants had an extreme stay-green phenotype as a consequence of autoregulated cytokinin production. Many laboratories are now attempting to apply this approach to making stay-greens in a whole range of species. Greenness can also be altered by down-regulating the production of a senescence-promoting hormone. Tomato plants in which ethylene biosynthesis is inhibited by antisense suppression of the gene for ACC oxidase exhibit delayed leaf senescence (John *et al*., 1995). A similar phenotype is apparent in ethylene-insensitive mutants of *Arabidopsis* (Grbic and Bleecker, 1995).

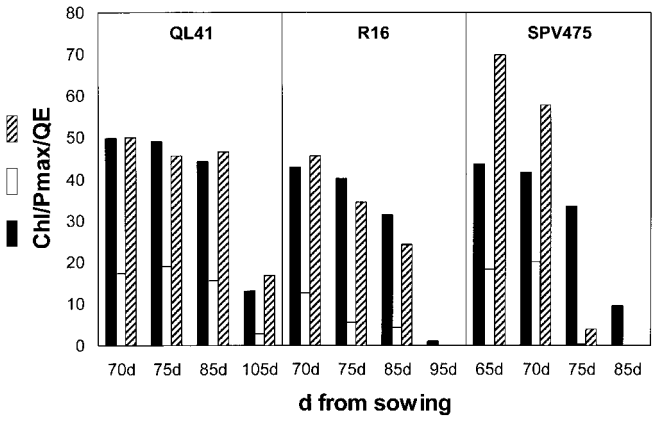

Fig. 6. Chlorophyll (arbitrary units), photosynthetic rate at light saturation (P_{max}, μmol CO_2 m^{-2} s^{-1}) and efficiency of light conversion (QE, initial slope of light-response curve of CO_2 fixation $\times 10^2$) in leaf 8 of three contrasting sorghum lines. Plants were grown under field-like conditions of water limitation in 1 m long, 25 cm diameter open-ended plastic pipes filled with a 3:3:3:1 (by vol.) mixture of soil, peat, grit, and Perlite. Holes 0.5 cm in diameter drilled at intervals along the length of each pipe provided aeration for root growth. Water was supplied from below by placing the pots in trays within which water levels were maintained at a few cm throughout the experiment. Other conditions are as described by Smart *et al.* (Smart *et al.*, 1995).

Quantitative trait (QTL) analysis and marker-assisted selection are powerful plant breeding tools that could be applied to the efficient development of lines with delayed senescence. In the few cases where greenness has been explicitly scored, several separate genetic loci have been identified. For example, up to six QTLs for leaf senescence have been observed in certain *Lolium* populations (Thorogood *et al*., 1999). QTL analysis not only provides molecular markers for efficient selection and breeding, but is also of particular value in resolving several interacting genetic and environmental effects. This is important in sorghum where, as described above, tolerance to post-flowering drought stress uses stay-green as a key trait in selection. Evaluation of stay green is not straightforward, however, as it is difficult to control the timing and intensity of drought stress in the field and there are large interactions between growth stage, flowering time and other factors. Stay-green in sorghum is currently the subject of mapping programmes in Australia and the United States.

The QTL mapping approach is also revealing details of the genetic and environmental control of leaf senescence in pearl millet. Pearl millet is an indeterminate cereal in that, although an annual plant, flowering on the main stem does not prevent further vegetative tiller development. Many pearl millet genotypes are highly tillering. Once flowering has commenced on an individual tiller, the leaves of that stem senesce, starting at the oldest node. In Fig. 7 the senescence of the top five leaves of the primary stem is shown for three time points post anthesis for two pearl millet genotypes under either irrigated control or post flowering drought stress conditions. One of these lines shows enhanced green leaf retention under control conditions. Furthermore, the same line not only displays reduced drought-induced senescence, but also maintains a higher leaf water status. By 74 d after sowing, the mean relative water content of the top two leaves on the primary stem was 85% for the

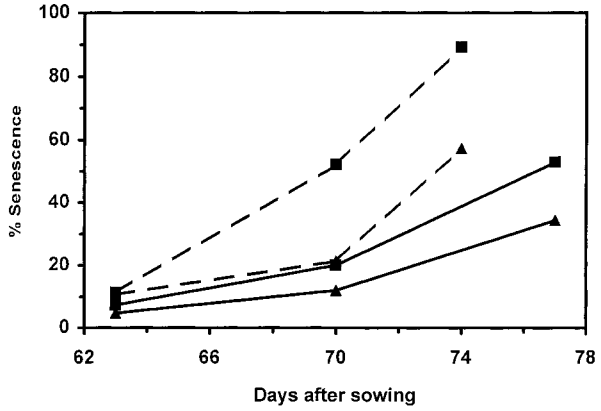

Fig. 7. Time-course of senescence of two contrasting genotypes of pearl millet in the field growing either under full irrigation (solid lines) or post flowering drought stress (dashed lines). Percentage senescence was assessed visually of each of the top five leaves of the primary stem of 18 plants and the mean value calculated. (▲), H 77/833-2 × 843A; (■), PRLT 2/89-33 × 843A. (CJ Howarth and K Skøt, unpublished results).

non-senescent line and 42% for the senescent line. A cross between these two lines (H 77/833-2 and PRLT 2/89-33) has been produced in order to develop a mapping family to study drought-related senescence and other traits further (Howarth *et al.*, 1994; Yadav *et al.*, 1999). The range of senescence of the progeny under both irrigated and post-flowering drought stress is presented in Fig. 8, indicating the quantitative nature of both responses. Transgressive segregation is also apparent. In conjunction with genetic mapping, it is now possible to identify QTLs associated with senescence and to dissect the genetic control of senescence under various environmental conditions. Interestingly, of the QTLs so far obtained, one (on linkage group 6) was found to be in common not only

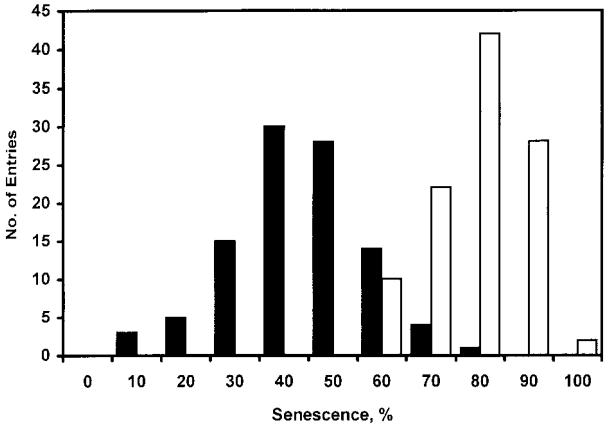

Fig. 8. Frequency distribution showing the range of senescence in the field of 104 progeny derived from a cross between H 77/833-2 and PRLT 2/89-33 grown either under full irrigation (■) or post flowering drought stress (□). The top five leaves of the primary stem of nine plants of each progeny were assessed visually for percentage senescence and a mean value calculated. Plants growing under irrigated control were assessed for senescence 77 d after sowing and those under drought stress 74 d after sowing.

under control and drought stress conditions but also to be at the same position as a major QTL for flowering time. On the other hand, QTLs were obtained that appear to be independent of flowering time and also specific to either control conditions or drought stress. It is currently being investigated whether pearl millet resembles sorghum in an association between stay green and maintenance of grain yield under post-flowering drought stress.

What are the limits and how close are they?

Plots of production against time, of the sort presented in Fig. 1, generally take the form of asymmetric bell-type curves. If the productive structure (individual leaf, whole plant, entire canopy as may be) could be established instantaneously, remain at its productive maximum for the period of its existence and then instantaneously die, the curve would make a rectangle with dimensions set by lifespan on the abscissa and maximal productive capacity on the ordinate. If it is assumed that the limits of lifespan and productive capacity are fixed, then the strategy for maximizing production is to make the curve approximate as near as possible the idealized rectangle. In practice, this equates to two major agronomic objectives, getting leaves out fast and keeping them there as long as possible. Based on a few simple generalizations Thomas calculated that it may be possible to get to within about 70% of the potential maximum (Thomas, 1992). Is it merely coincidence that the record yield for corn, obtained on an Illinois farm in 1985, according to one estimate was about 73% of the theoretical maximum (calculated as described by Tollenaar, 1985)? It is significant that the variety that yielded so prodigiously was FS854, a stay-green.

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