Plant Biotechnology Journal (2004) **2**, pp. 101–112 Doi: 10.1111/j.1467-7652.2004.00054.x

Leaf senescence is delayed in maize expressing the *Agrobacterium IPT* gene under the control of a novel maize senescence-enhanced promoter

Paul R. H. Robson[1,*], Iain S. Donnison[1], Kan Wang[2], Bronwyn Frame[2], Sue Ellen Pegg[2], Ann Thomas[1] and Howard Thomas[1]

[1]*Department of Plant Breeding and Genetics, Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth SY23 3EB, UK*
[2]*Plant Transformation Facility, Iowa State University, Ames, IA 50011, USA*

Received 25 July 2003; revised 11 October 2003; accepted 14 October 2003.
**Correspondence* (fax 44 1970 823242; e-mail paul.robson@bbsrc.ac.uk)

Summary

We have genetically modified maize plants to delay leaf senescence. A senescence-enhanced promoter from maize (P_{SEE1}) was used to drive expression of the *Agrobacterium* cytokinin biosynthesis gene *IPT* in senescing leaf tissue. Three maize lines expressing *IPT* from P_{SEE1}, Sg1, Sg2 and Sg3, were analysed in detail, representing mild, intermediate and extreme expression, respectively, of the delayed-senescence phenotype. Backcross populations segregating for the presence or absence of the $P_{SEE1Xba}$*IPTNOS* transgene also simultaneously segregated for the senescence phenotype. At the time of ear leaf emergence, individuals of lines Sg1 and Sg2 segregating for the presence of the transgene carried about three fewer senescing leaves than control (transgene-minus) segregants, and *IPT* transcript levels were higher in leaves at incipient senescence than in young leaves. Leaves of transgenic Sg3 plants were significantly greener than controls and progressed directly from fully green to bleached and dead without an intervening yellowing phase. *IPT* transcript abundance in this line was not related to the initiation of senescence. Extended greenness was accompanied by a delay in the loss of photosynthetic capacity with leaf age. The delayed-senescence trait was associated with relatively minor changes in morphology and development. The phenotype was particularly emphasized in plants grown in low soil nitrogen. The reduced ability of the extreme transgenic line Sg3 to recycle internal nitrogen from senescing lower leaves accounted for significant chlorosis in emerging younger leaves when plants were grown in low nutrient conditions. This study demonstrates that the agronomically important delayed-senescence ('stay-green') trait can be engineered into a monocot crop, and is the first example outside *Arabidopsis* of senescence modification using a homologous senescence-enhanced promoter.

Keywords: *IPT*, promoter, *SEE1*, senescence, stay-green, *Zea mays*.

Introduction

The objective of the work described here was to modify leaf senescence in a major crop species, *Zea mays*. Senescence is the point in the plant life cycle at which most of the major determinants of crop performance converge. Senescence limits (sometimes catastrophically) crop productivity (Thomas, 1992). It is an essential response to biotic and abiotic stresses (Dangl *et al*., 2000) and drives resource recycling within the plant (Feller and Fischer, 1994), thereby contributing to nutrient use efficiency, harvest index and product quality. Environmentally and economically costly inputs, such as fertilizers, pesticides and irrigation, are essential to control senescence, as are post-harvest measures in the production and food distribution chain (King and O'Donoghue, 1995). Leaf senescence is genetically regulated (Oh *et al*., 1997; Thomas and Howarth, 2000) and therefore open to biotechnological intervention.

Although it is a phase of net loss of cell constituents, senescence is not unidirectional; new genes are expressed, new proteins are made and subcellular integrity is maintained

(Quirino *et al*., 2000; Thomas *et al*., 2003). Judged on sequence homologies, genes encoding proteolytic enzymes are prominent in the senescence-related cDNA collections from across the species range (Thomas and Donnison, 2000). One of these is *SAG12*, a senescence-associated gene (SAG) from *Arabidopsis* encoding a cysteine endopeptidase. The promoter of this gene has been isolated (Noh and Amasino, 1999) and exploited to modify senescence in dicot species.

The strategy involves the transgenic expression of the *IPT* gene from *Agrobacterium*, which encodes an isopentenyl transferase. Expression of *IPT* leads to increased levels of cytokinin through the synthesis of the first committed precursor in the biosynthetic pathway. When the expression of *IPT* is governed by a senescence-inducible promoter, elevated cytokinins are localized within senescing tissue and senescence is delayed without the induction of additional phenotypes associated with systemically high hormone levels. Gan and Amasino (1995) transformed tobacco with a fusion between *IPT* and the *SAG12* promoter of *Arabidopsis*. The lower leaves of transformant plants displayed a dramatic delay in senescence and there was an associated 40% increase in biomass and a 52% increase in seed yield. Apart from the inhibition of senescence and increase in yield, plants were similar to wild-type plants which lacked the transgene, but other effects on morphology have been noted, such as a reduction in plant height (Jordi *et al*., 2000). When compared with equivalent leaves of control plants, P_{SAG12}*IPT* tobacco retained greater levels of chlorophyll, soluble protein and Rubisco in older leaves; however, in contrast, levels in younger leaves were lower. Overall, in P_{SAG12}*IPT* tobacco plants, there was a partial inversion in the canopy N profile, compared with wild-type tobacco, caused by the inhibition of re-mobilization in older leaves (Jordi *et al*., 2000). A similar approach, using the same P_{SAG12}*IPT* construct, has been taken with other species, for example rice (Lin *et al*., 2002) lettuce (McCabe *et al*., 2001) and broccoli (Chen *et al*., 2001).

SEE1 is a maize senescence-enhanced protease from the same cysteine endopeptidase family as *SAG12* (Griffiths *et al*., 1997). We report here the isolation of the *SEE1* promoter region and its exploitation in a study that demonstrates, for the first time in a monocot species, that targeted expression of *IPT* driven by an endogenous senescence-enhanced promoter delays maize leaf senescence.

Results

Isolation of *SEE1* promoter

The putative 5′ regulatory region of the maize senescence-enhanced cysteine-protease, *SEE1*, was cloned from a maize genomic library using a pre-existing *SEE1* cDNA clone. A 3402 bp *Xho*I genomic DNA subfragment comprising the 5′ end of the gene and putative promoter was sequenced. A presumptive translational start site was assigned on the basis of sequence similarity to barley aleurain (83.5% DNA sequence identity over 1076 bp and 84.8% amino acid identity over 362 residues to a *SEE1* cDNA) and rice oryzain gamma (84.1% DNA sequence identity over 1146 bp and 83.7% amino acid identity over 363 residues) and the existence of a signal peptide as predicted by the SignalP programme (Nielsen *et al*., 1997). The sequenced upstream region shared some homology (75.6% identity over the first 43 bp immediately upstream of the translation start site, but only 38% identity over the next 500 bp) to the upstream region of the barley aleurain gene. The region –2761 bp to –2335 bp contained four MITE (miniature inverted-repeat transposable element)-like inverted transposable element sequences varying from 57 bp to 82 bp in length and this was not included in the transformation vector. A region –1006 bp to –874 bp was 66% identical to a 134 bp fragment approximately 1200 bp upstream of the transcription start site of a *Sorghum bicolor MYB* gene (accession AF470061) and this region was included in the transformation vector. The putative 5′ regulatory region of the maize senescence-enhanced cysteine-protease, *SEE1*, is termed P_{SEE1}, and 3402 bp, including the putative 5′ UTR (untranslated region) and translational start site, are available via accession number AJ494982. A 1971 bp region derived from P_{SEE1} was used to drive *IPT* expression; the construction is described in detail in 'Experimental procedures'.

Transformation of maize with $P_{SEE1Xba}$*IPTNOS*

A total of 29 transgenic events carrying the $P_{SEE1Xba}$*IPTNOS* transgene were generated using the biolistic-mediated transformation method and brought to maturity. Plants containing the $P_{SEE1Xba}$*IPTNOS* transgene were identified by polymerase chain reaction (PCR) amplification of a 500 bp fragment. Amplification was performed using oligos XBA5 and IPT1 that were designed to hybridize to $P_{SEE1Xba}$ and *IPT* such that the amplified product extended through the junction between $P_{SEE1Xba}$ and *IPT* and consequently did not amplify a product from the native genome. Incorporation of the transgene into the maize genome was confirmed by probing genomic preparations with a 978 bp *Sal*I/*Eco*RI fragment containing *IPT*/*NOS*. Restriction with *Kpn*I released the entire expression cassette from the transformation vector. Genomic DNA restricted with *Kpn*I yielded a fragment of approximately the same size as a control plasmid, confirming that the expression cassette in the genome contained contiguous

sequence (data not shown). Embryos from hybrid maize, Hill (A188xB73), were used in the transformation protocol and regenerated plants were backcrossed to parental line B73; the resulting T1 seed is heterozygous for parental alleles and segregates 50 : 50 for the presence : absence of the transgene in single-copy transformation events. A segregation frequency of approximately 50% was observed in three independent transformed lines chosen for further study; segregants within these lines that contained the transgene were referred to as Sg1, Sg2 and Sg3. The corresponding negative segregants were termed Con1, Con2 and Con3, respectively. The latter plants went through identical transformation, culture and regeneration procedures to those experienced by transgene-positive plants and represent controls when assigning phenotypic characters to the presence of the transgene.

Leaf senescence patterns in transformants

Lines 1, 2 and 3 were chosen from plants representing eight transgenic events, on the basis that they were representative of the spectrum of observable phenotypes. The distribution of pigment per unit leaf area in the three independently transformed maize lines over time and space was estimated non-destructively using a hand-held SPAD-502 chlorophyll meter. Figure 1 presents the profiles of leaf greenness measured 72 days after sowing in plants segregating for the presence of the transgene. Leaves are numbered in order of emergence during plant growth and, as the plastochron was very similar for all lines, leaf number can be treated as a continuous variable and regarded as a measure of developmental time. The oldest five or six leaves of Con1, Con2 and Con3, which lack the transgene, were completely senescent and contained no measurable chlorophyll. Leaves 7 and 8 were partially yellow and leaf 9 was at the stage of incipient senescence. The senescence profiles of plants from lines Sg1 and Sg2 were displaced in favour of a later onset of yellowing by the equivalent of about three leaves, when compared with Con1 and Con2, so that the shoot in each case carried only three fully senescent leaves and leaf 6 was the point of incipient senescence. Sg3 displayed the most extreme phenotype of the lines measured (Figure 1) when compared with Con3. All leaves of Sg3 retained pre-senescent levels of chlorophyll. Between leaves 7 and 1, the interval from incipient to complete senescence in negative segregants, the relative chlorophyll content of Sg3 leaves, far from declining, increased if anything.

Fully expanded leaves of Sg3 plants were noticeably greener than those of Con3 plants. Chlorophyll a and b were determined by methanol extraction and spectrophotometry. At 107 days after sowing, the average total chlorophyll in

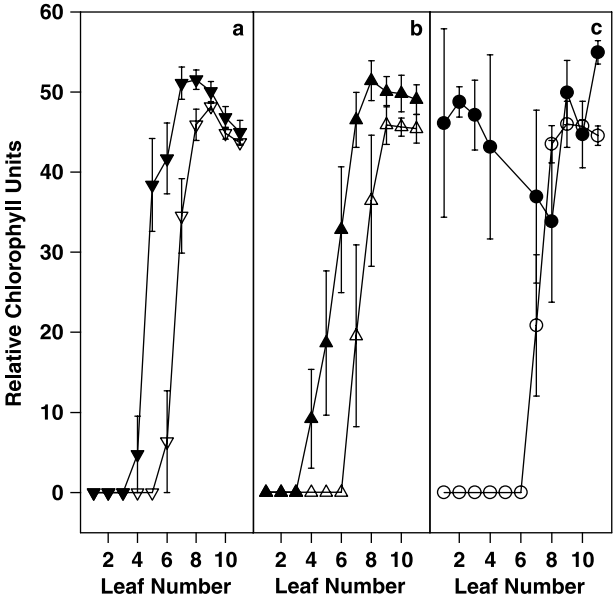

Figure 1 A stay-green phenotype was observed in three independently transformed maize lines containing the transgene $P_{SEE1Xba}$*IPTNOS*. The distribution of pigment per unit leaf area of three transgenic maize lines containing the transgene $P_{SEE1Xba}$*IPTNOS* after 72 days. In all three lines (designated Sg1/Con1, Sg2/Con2 and Sg3/Con3), in (a)–(c), respectively, the oldest five or six leaves of controls, i.e. segregants that lacked the transgene (open symbol), were completely senescent and contained no measurable chlorophyll. Leaves 7 and 8 were partially yellow and leaf 9 was at the stage of incipient senescence. The senescence profiles of plants from lines Sg1 and Sg2, (a) and (b), respectively, segregating for the presence of the transgene (filled symbol), were displaced in favour of a later onset of yellowing by the equivalent of about two and three leaves, respectively, so that the shoot in each case carried only three fully senescent leaves and leaf 6 was the point of incipient senescence. Segregants Sg3 (c), containing the transgene (filled circle), displayed the most extreme phenotype of the lines measured with all leaves retaining pre-senescent levels of chlorophyll.

the 15th leaf of Sg3 plants was 27% higher than that in the corresponding leaf of Con3 plants (Figure 2a). The average chlorophyll a : b ratio in the 15th leaf was 2.15 in Sg3 and 4.22 in Con3 (Figure 2b), indicating that the presence of the transgene and enhanced greenness in this line are associated with a relatively high light-harvesting pigment fraction. The 15th leaf of Sg1 and Con1 plants did not differ significantly in total chlorophyll content but, as in the Sg3/Con3 comparison, controls had a significantly higher ratio of chlorophyll a to chlorophyll b. Sg2/Con2 was intermediate between Sg1/Con1 and Sg3/Con3 in chlorophyll content and similar to Sg1/Con1 in the ratio of chlorophyll a to chlorophyll b (Figure 2b).

At three sample points, 72, 82 and 103 days after sowing, the number of fully senescent leaves (defined as leaves giving a SPAD-502 reading of zero) was counted on each plant in control and transgenic lines. Table 1 summarizes the data for lines Sg1/Con1 and Sg3/Con3. After 72 days, an average of

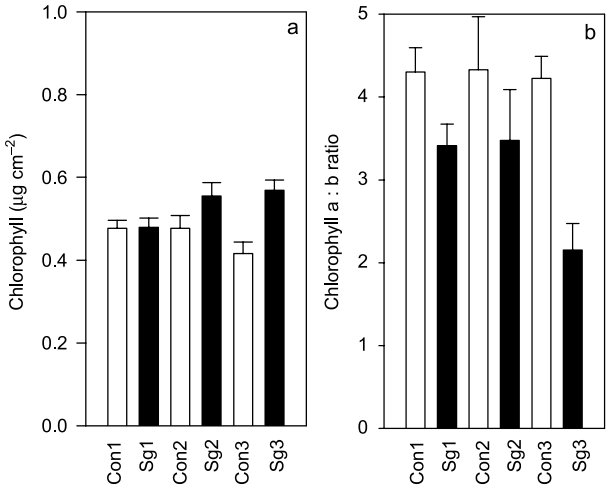

Figure 2 Chlorophyll content is altered in three independently transformed maize lines containing the transgene $P_{SEE1Xba}$*IPTNOS*. Levels of methanol-extractable chlorophyll from the 15th leaf at 107 days. Plants segregating for the presence of the transgene (filled block) had mostly higher levels of chlorophyll and lower chlorophyll a : b ratios than did plants lacking the transgene (open block). Chlorophyll in the 15th leaf of Sg3 was 27% higher (a) and the chlorophyll a : b ratio was 51% lower (b) than in the corresponding leaf of Con3. In Sg1 and Con1, the 15th leaf did not differ significantly in total chlorophyll content, but controls had a significantly higher ratio of chlorophyll a to chlorophyll b. Line Sg2/Con2 was intermediate between Sg1/Con1 and Sg3/Con3.

6.4 leaves had senesced on Con3, whereas most Sg3 plants displayed no symptoms of senescence at all (see Figure 1c) and retained their primary leaves in a fully green state. After 72 days, an average of 5.8 leaves had senesced on Con1 plants, whereas 3.8 leaves had senesced on Sg1 plants. When measured 10 days later, the difference in the number of senescent leaves per plant between transgenic and control lines of about two and more than six in lines Sg1/Con1 and Sg3/Con3, respectively, was sustained (Table 1). By 103 days after sowing, control lines carried about eight senescent leaves. At this time, Sg1 plants had one fewer and Sg3 plants three fewer senescent leaves than controls. Comparing transgenic with control plants at 82 and 103 days, it is clear that the presence of the transgene in line Sg1 is associated with a delay of about 20 days in reaching the same stage of sequential senescence as the controls. For Sg3, the differential is even greater, in excess of 30 days: even by 103 days, senescence in the transgenics had not reached the state of controls at 72 days (Table 1).

A consistent feature of the terminal stage of leaf life in Sg3 plants was the transition from fully green to bleached and desiccated without an intervening yellowing phase. In terms of the normal pattern of chloroplast pigmentation changes and physiological indices (Robson *et al*., unpublished observations), leaves of Sg3 do not senesce at all, but progress directly from productive maturity to death.

Table 1 The number of fully senescent leaves, defined as leaves with no detectable chlorophyll, present on stay-green and control maize at 72, 82 and 103 days (s.e., standard error; $n = 5$)

	Days after sowing					
	72	s.e.	82	s.e.	103	s.e.
Sg1	3.8	0.2	4.6	0.6	6.4	0.4
Con1	5.8	0.2	6.4	0.4	7.6	0.2
Sg3	0.2	0.2	0.4	0.2	4.0	0.6
Con3	6.4	0.2	6.8	0.4	8.4	0.2

Photosynthetic capacity in transformants

Photosynthetic activity was estimated by chlorophyll fluorescence. Comparisons between different leaf ages and plant lines were made based on measurements of the relative gross photosynthetic capacity (Maxwell and Johnson, 2000). Gross photosynthesis levels were calculated as the product of quantum yield, calculated from fluorescence measurements, and photosynthetically active radiation. Relative leaf chlorophyll levels in the plant canopy (Figure 3a) and the associated gross photosynthesis levels (Figure 3b) were measured in all three transgenic lines. The gross photosynthetic activity paralleled the relative chlorophyll levels and showed photosynthesis to be maintained in leaves that had no functional equivalent in control plants as measured by either relative chlorophyll levels or photosynthetic activity. Levels of photosynthetic activity measured in Sg1, Sg2 and Sg3 converged with those measured in Con1, Con2 and Con3 at around leaf 6 or leaf 7 which, as measured by the relative chlorophyll levels, is the point of incipient senescence in control plants (Figure 3a,b).

Expression of the transgene in relation to senescence phenotype

Expression of the transgene was measured in each line as the transcript abundance in total RNA from a leaf at incipient senescence and from a younger, expanding leaf taken from the same plant. In order to measure comparable leaves in the three lines, the timing of pollen shed was determined for each plant (see Figure 5b) and tissue was taken 15 days later. At this point, even line Sg1, in which senescence was extremely late, provided leaf tissue at the required stage. For each individual control or transgenic plant, incipient senescence was defined as the oldest (i.e. lowest) leaf with a SPAD-502 reading above 30. Northern analysis was carried out using a 0.75 kb *Xba*I fragment containing the *IPT* coding region

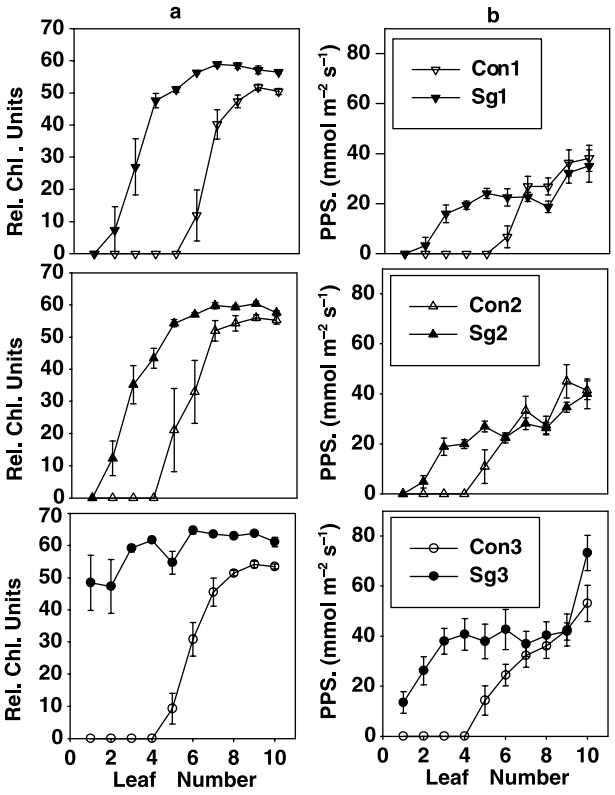

Figure 3 Photosynthetic activity parallels the retention of chlorophyll. The distribution of pigment per unit leaf area of three transgenic maize lines containing the transgene $P_{SEE1Xba}$*IPTNOS* (relative chlorophyll content, Rel. Chl. Units) (a) and associated gross photosynthetic activity (PPS) (b). In all three lines Sg1/Con1, Sg2/Con2 and Sg3/Con3, the retention of photosynthesis was coincident with the retention of chlorophyll. Photosynthetically active leaves were present on Sg1, Sg2 and Sg3 where no functional equivalent leaf was present on control plants. For example, in Sg1 plants, measurable chlorophyll was detected in all but the first leaf; gross photosynthesis was also detected in all but the first leaf; however, in Con1 plants, measurable chlorophyll and photosynthesis were not detected in leaves 1–5. Similar trends are seen in comparisons of Sg2/Con2 and Sg3/Con3.

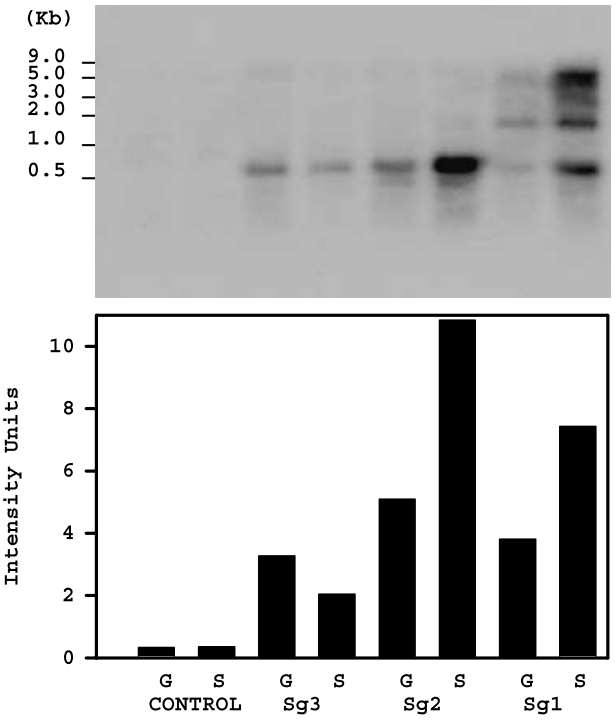

Figure 4 Senescence-associated accumulation of *IPT* transcript. Northern blot analysis of total RNA taken 15 days after pollen shed and comparing *IPT* expression between expanding green leaves (G) and leaves at incipient senescence (S). The autoradiogram (a) was digitized so that all bands remained unsaturated and a numerical value was assigned to the intensity of the individual bands corresponding to the *IPT* transcript (b). The intensities of the bands were normalized for background. No transcript from *IPT* was detected in leaves at either stage in control plants. Transcript from *IPT* was detected at both stages in leaves from transgenic plants; in Sg1 and Sg2, transcript accumulation increased in senescing leaves compared with young expanding leaves. Transcript levels in senescing leaves of Sg1 and Sg2 were 215% and 100% higher, respectively, than those of expanding leaves. Transcript abundance in expanding leaves of Sg3 was similar to, or even greater than, that in senescing leaves.

that was present in the transgenic lines in vector pBKK2 $P_{SEE1Xba}$*IPTNOS*. The *IPT* transcript was not detected in either expanding or senescing leaves of the negative segregants, but was shown to be present in all of the transgenic lines (Figure 4). Transcript abundance in expanding leaves of Sg3 was similar to, or even greater than, that in senescing leaves. Transcript levels in senescing leaves of Sg2 and Sg3 were 215% and 100% higher, respectively, than those in expanding leaves.

Phenotypes associated with delayed senescence

Sg1, Sg2 and Sg3 plants were screened for the occurrence of transgene-associated characters in addition to the retention of greenness. Foliar morphology was affected in Sg3, with leaves (mainly the fifth and sixth) occasionally displaying a scorched phenotype where the edges and a proportion of the lamina were missing or blackened. This morphology was not observed in the other two transgenic lines (data not shown). The presence of the transgene tended to be associated with a reduction in height. Measured at 72 days after sowing, Sg3 plants were, on average, 25% shorter than segregants that lacked the transgene (Figure 5a). Sg1 and Con1 were not significantly different; the behaviour of Sg2 was intermediate between that of Sg1 and Sg3. Two features that indicate delayed development were also observed in some of the transgenic lines. On average, the cob formed in the axil of the 12th leaf in Sg1 and in negative segregants of all three lines Con1, Con2 and Con3. By contrast, the cob emerged at node

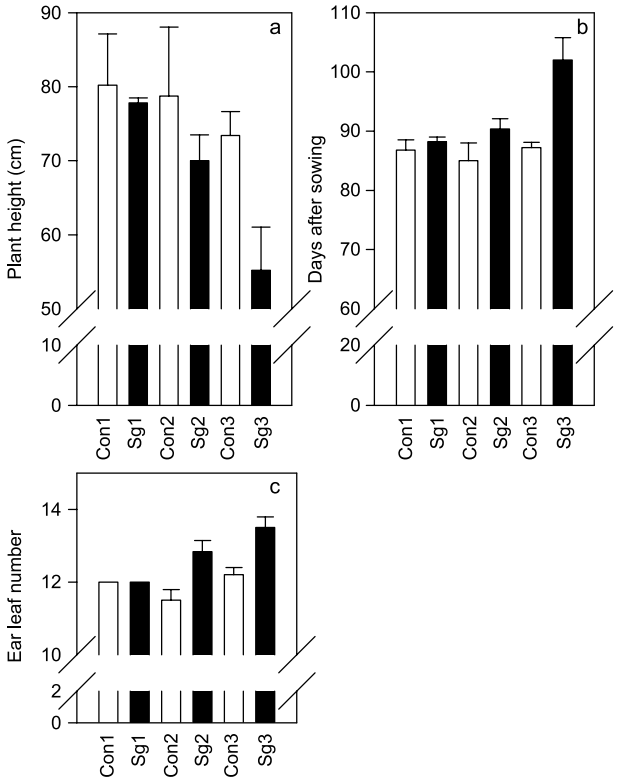

Figure 5 Plant height and development of three transgenic maize lines containing the transgene $P_{SEE1Xba}$*IPTNOS* after 72 days. A reduction in stature was associated with the presence of the transgene, but was not seen in all independent transformation events (a). Sg3 plants were on average 25% shorter than segregants that lacked the transgene. Sg1 and Con1 plants were not significantly different; the behaviour of Sg2/Con2 was intermediate between Sg1/Con1 and Sg3/Con3. Two features that indicate delayed development were also observed in some of the transgenic lines. On average, the cob formed in the axil of the 12th leaf in Sg1 and segregants lacking the transgene of all three lines (Con1, Con2 and Con3). By contrast, the cob emerged at node 13 in Sg2 and node 14 in Sg3 (c). The number of days to pollen shed was also delayed in Sg2 and Sg3, by approximately 5 days and 15 days, respectively, when compared with control segregants lacking the transgene and with Sg1 (b).

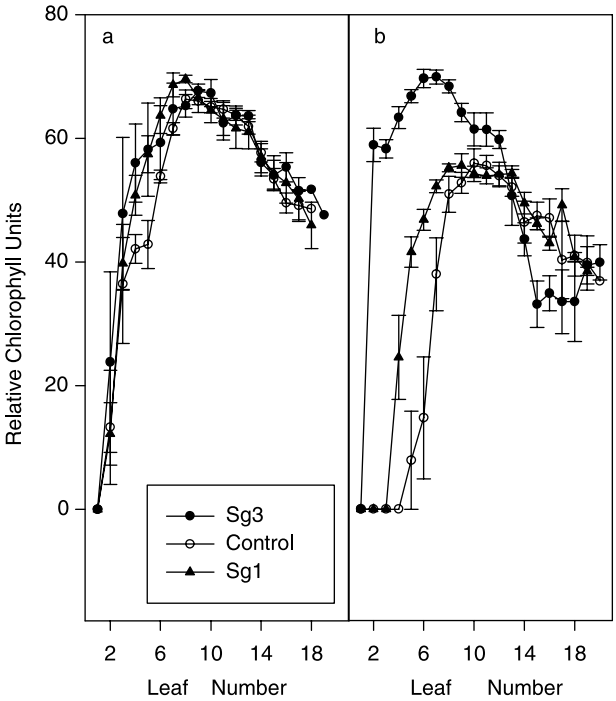

Figure 6 Nutrient treatment affects the stay-green phenotype in transgenic maize containing the transgene $P_{SEE1Xba}$*IPTNOS*. Profiles of leaf greenness after 60 days under high nutrient treatment (4.43 g nitrogen per plant) and low nutrient treatment (1.43 g nitrogen per plant). Under high nitrogen conditions (control, open circle; Sg3, filled circle; Sg1, filled triangle), plants were almost indistinguishable, each retaining a high degree of leaf greenness and bearing only one fully senescent leaf, the primary (a). Growth under low nutrient conditions differentiated between the genotypes (b). If leaves are grouped into different age classes, genotypic contrasts become clearer. The chlorophyll profile of the oldest (i.e. lowermost) 12 leaves of Sg3 was very similar under high and low nutrient regimes. Only the primary leaf had senesced and leaves displayed high levels of chlorophyll accumulation, reaching a maximum of almost 70 relative chlorophyll units. Chlorophyll in the young, expanding and newly mature leaves (leaves 13–20) of Sg3 plants in low nutrient conditions decreased to 33 units. This value was significantly below the relative chlorophyll levels of the equivalent class of leaves in either Sg1 or control plants under the same conditions. Although Sg1 plants, when grown in low nutrient conditions, did not display the dramatic stratification into very green older foliage and young leaves with reduced pigmentation seen in Sg3, senescence was clearly delayed in this population relative to the control (b).

13 in Sg2 and node 14 in Sg3 (Figure 5c). The number of days to pollen shed was also delayed in Sg2 and Sg3, by approximately 5 days and 15 days, respectively, when compared with control segregants lacking the transgene and with Sg1 (Figure 5b).

Responses of stay-green transgenics to nitrogen limitation

Genetic manipulation to delay senescence would be expected to have consequences for nitrogen nutrition and mobilization (Thomas *et al.*, 2002). Independently transformed maize lines Sg3 and Sg1 were grown under high and low nutrient regimes and profiles of leaf greenness after 60 days were analysed. In the high nutrient treatment, each plant was supplied with a total of 4.43 g nitrogen; in the low nutrient treatment, 1.43 g nitrogen per plant was applied. Under high nitrogen conditions, Con3, Sg3 and Sg1 plants were almost indistinguishable, each retaining a high degree of leaf greenness and bearing only one fully senescent leaf, the primary (Figure 6a). Growth under low nutrient conditions differentiated between the genotypes (Figure 6b). When leaves were grouped into different age classes, genotypic contrasts became clearer. The chlorophyll profile of the oldest (i.e. lowermost) 12 leaves of Sg3 was very similar under high and

low nutrient regimes. Only the primary leaf had senesced and other leaves displayed high levels of chlorophyll accumulation, reaching a maximum of almost 70 relative chlorophyll units. Chlorophyll in the young, expanding and newly mature leaves (leaves 13–20) of Sg3 plants in low nutrient conditions decreased to 33 units. This value was significantly below the relative chlorophyll levels of the equivalent class of leaves in either Sg1 or control plants under the same conditions. The decreased chlorophyll content in newly emerging leaves of Sg3 grown in low nutrient conditions was associated with the presence of chlorotic regions. Although Sg1 plants, when grown in low nutrient conditions, did not display the dramatic stratification into very green older foliage and chlorotic young leaves seen in Sg3, senescence was clearly delayed in this line relative to the control.

Discussion

Senescence-specific promoters and transgenic manipulation of senescence

In this paper, we report the isolation and functional testing of the first monocot leaf senescence-specific promoter. In *Arabidopsis thaliana* and some other dicotyledonous species, a number of promoters from SAGs have been tested (Gan and Amasino, 1995; Chung *et al*., 1997; Butt *et al*., 1998). Up-regulation of reporter gene expression during leaf senescence has also been reported for other promoters, including those from genes cloned from ripening fruits (Graham *et al*., 1992; Blume and Grierson, 1997; Lasserre *et al*., 1997; Riou *et al*., 2002). The expression of genes *SAG12* (Gan and Amasino, 1995) and *SAG2/AtALEU* (Hensel *et al*., 1993; Ahmed *et al*., 2000) is associated with senescence, whereas two other genes for C1A peptidases, *RD21A* and *RD19A*, are induced by drought stress (Koizumi *et al*., 1993).

Relation of *IPT* expression to senescence specificity of $P_{SEE1Xba}$

Northern analysis of *SEE1* expression has shown that the native maize transcript accumulates in older leaves during sequential senescence on vegetative shoots and also during reproduction-triggered monocarpic senescence of the entire canopy (Smart *et al*., 1995; Griffiths *et al*., 1997). Levels of expression of *IPT* driven by the promoter of *SEE1* should equilibrate at a relatively low level because of the operation of an autoregulatory feedback loop, as described by Gan and Amasino (1995) for P_{SAG12}*IPT* in *Arabidopsis*. In lines Sg1 and Sg2, *IPT* transcript abundance was greater in leaves at the initiation of sequential senescence than in young leaves above them (Figure 4). The accumulation of the *IPT* transcript in younger leaves may be accounted for by the onset of canopy-wide monocarpic senescence triggered by reproductive development. Transgenic line Sg3, despite exhibiting the most extreme delayed-senescence phenotype (Figure 1c), yielded the lowest abundances of transcript of the transgenic lines analysed and no evidence of senescence-related induction. We speculate that some feature of transgene integration in 48-17 has compromised the senescence specificity of the promoter, for example integration into a transcriptionally active region resulting in ectopic expression of P_{SEE1}*IPT*. Leaves of Sg3 do not senesce at all, but progress directly from productive maturity to death. Leaves of Sg1 and Sg2 do not display this characteristic. The lack of senescence-associated accumulation of *IPT* in Sg3, when compared with Sg1 and Sg2, may be explained by the suppression of senescence and consequently the weak expression of SAGs and promoters in Sg3 caused by ectopic expression of P_{SEE1}*IPT*.

Altered pigment profiles in *IPT* transgenics

Genetic variants with a delayed-senescence phenotype are often referred to as stay-greens (Thomas and Smart, 1993; Thomas and Howarth, 2000). All three transgenic lines in the present study, Sg1, Sg2 and Sg3, exhibited the stay-green trait to a greater or lesser extent. The degree to which the character was expressed depended on the stage of plant development and on the soil nutrient conditions (Figures 1 and 6; Table 1). Jordi *et al*. (2000) also found that delayed senescence in P_{SAG12}*IPT* tobacco transgenics was emphasized in low nitrogen growth conditions. In lines Sg1 and Sg2, leaves yellowed in a normal fashion once senescence was initiated: senescence modification took the form of delayed initiation rather than reduced rate. By contrast, there was no evidence that leaves of Sg3 were capable of normal senescence as defined by the kind of progressive yellowing observed in control lines or in Sg1 and Sg2. It is well established that leaf cells may follow any of a number of pathways from viability to death and some of these (such as the hypersensitivity response) do not include a senescence phase (Thomas and Donnison, 2000). In evergreens, it is the normal mode of leaf termination. Transgenics of line Sg3 behave as if terminal development of leaf cells follows a route that avoids senescence. Plants with the Sg3 phenotype offer interesting opportunities to explore the common and divergent pathways of cell death in green plant tissues.

Prolonged greenness in transgene-positive segregants is associated with the extension of photosynthetic capacity in older leaves (Figure 3), confirming that these genotypes are,

in the terminology of Thomas and Howarth (2000), functional rather than cosmetic. It is significant that delaying senescence does not slow the lifelong ontogenetic decline in photosynthesis that commences well before the onset of foliar senescence, suggesting that leaf ageing (of which this is a symptom) and senescence are mechanistically distinct processes (Thomas, 2003).

Senescence in relation to soil nitrogen supply

Up to 75% of leaf nitrogen is located in the chloroplasts. Normally, the protein content of mesophyll cells is closely correlated with the amount of chlorophyll, and leaf yellowing is generally an accurate index of the release and export of protein nitrogen (Thomas *et al*., 2002). It follows that the immobilization of nitrogen that would otherwise be available for recycling is a feature of stay-greens and has consequences for the whole plant when grown under nitrogen-limited conditions (Hauck *et al*., 1997). Sg3 plants, which were characterized by extreme suppression of normal yellowing, responded in just this fashion to a low nutrient cultivation regime (Figure 6b). Disabling senescence in the lower part of the plant had the consequence of restricting the nitrogen available to the younger growing tissues, which showed clear symptoms of nitrogen deficiency. Cytokinins may also play a more direct role in the plant's internal economy than that mediated by senescence-associated remobilization alone. Takei *et al*. (2001) reported that roots of nitrogen-depleted maize plants rapidly responded to the application of nitrate by accumulating cytokinins, principally zeatin derivatives, and exporting them via the xylem to leaves. Interactions between the degree to which senescence has been suppressed or delayed and growth conditions imply that stay-green crops based on *IPT* transgenics will need different management practices if their agronomic advantages are to be fully realized.

Phenological and morphological changes associated with transgene expression

Systemic expression of *IPT* in transgenic plants results in a range of characteristic and extreme phenotypes. The first such transformants to be reported were derived from tobacco teratomas and could form shoots but no roots, and so were grown as grafts on non-transformed rootstock (Braun and Wood, 1976). Transformant tobacco in which *IPT* expression is less extreme will form roots and can be grown normally (Yusibov *et al*., 1991). As well as difficulties in rooting, *IPT* transgenics (like cytokinin overproducing mutants; e.g. Chaudhury *et al*., 1993) are characterized by reduced apical dominance, increased branching, decreased stature and faster development. Driving *IPT* expression with a conditional rather than constitutive promoter would be expected to reduce the extent to which these aspects of the elevated cytokinin phenotype would be revealed. A senescence-related promoter should be especially effective in moderating such characters through autoregulatory feedback (Gan and Amasino, 1995). Moreover, there is good evidence from transformation experiments with *IPT* under a heat shock promoter that elevated cytokinin levels following local induction have very limited mobility (Smart *et al*., 1991). Therefore, senescence-specific *IPT* expression is likely to be confined to non-growing tissue in the terminal phase of development, spatially and temporally well separated from the sites of organ initiation, morphogenesis and differentiation. It is significant that transgenic lines, Sg1, Sg2 and Sg3, ranked in that order with respect to increasingly delayed senescence (Figure 1) and also to decreasing stature (Figure 5a), later pollen shed (Figure 5b) and position of ear leaf (Figure 5c). This may be evidence of some degree of leakiness in the P_{SEE1} promoter: it is certainly true that Sg3 showed the most extreme set of phenotypes and differed from Sg1 and Sg2 in its lack of *IPT* up-regulation at the initiation of senescence (Figure 4). On the other hand, disturbed morphology and phenology may not be primary responses to altered cytokinin status, but consequences of delayed senescence. In particular, changes in source–sink relations at the whole plant level will follow from extended carbon assimilation and slower recycling of nitrogen and other nutrients (Hauck *et al*., 1997; Thomas and Howarth, 2000; Thomas *et al*., 2002), and can be expected to modify the timing and site of developmental events over the lifetime of the whole plant.

Mode of action of cytokinins in senescence

As well as extended greenness, the presence of the transgene was associated with a reduction in chlorophyll a/b ratio in all three lines (Figure 2b). Cytokinins are known to modulate the activity of genes for light-harvesting chlorophyll-binding proteins (Flores and Tobin, 1988; Chory *et al*., 1994; Kulaeva *et al*., 1996) and to promote the reversal of senescence by stimulating the expression of genes for the redifferentiation of senescent plastids (gerontoplasts) into chloroplasts (Zavaleta-Mancera *et al*., 1999a,b). The phenotypes of maize $P_{SEE1Xba}$*IPT* lines, particularly Sg3, are consistent with the suppression by cytokinin of senescence initiation and associated catabolic processes. It is likely, therefore, that the increased amounts and stability of chlorophyll in maize *IPT* transgenics reflect cytokinin-mediated alterations in the balance of the biosynthetic and catabolic sides of metabolic turnover.

Further characterization and potential exploitation of *IPT* transgenics

We have demonstrated for the first time the genetic manipulation of leaf senescence, using an endogenous promoter, in a monocot crop species in terms of modified pigmentation profiles and associated or contingent morphological alterations. The technology that produced the maize *IPT* genotypes has been exploited in other species. The pBKK2$P_{SEE1Xba}$*IPTNOS* construct has been transformed into the pasture grass *Lolium multiflorum* and stay-green lines have been obtained with similar features to those described in the present paper for maize (Li, 2000). The P_{SEE1} promoter was shown to be active and senescence-specific in this species, indicating that it will be useful across the range of monocot crops for engineering expression of transgenes at terminal stages in organ and whole plant development with a low risk of undesirable pleiotropic effects. The transgenic plants described provide a range of stay-green phenotypes to facilitate studies of the impact of the manipulation of leaf senescence on yield under field conditions. Line Sg3 represents an extreme phenotype not available in conventionally bred stay-green maize. It is a potential tool for identifying genetic determinants of cytokinin-induced stay-green through comparisons of vegetative and stay-green leaf cDNA profiles. Such analyses would be expected to identify markers for use in conventional and molecular breeding programmes.

Experimental procedures

Plant materials

Zea mays hybrid, HiII (A188xB73), was grown in a glasshouse at 25 °C; natural day light was supplemented with 16 h of artificial light (high pressure sodium son-T plus lights). Plants were grown individually in 10 L pots, each containing 7.5 L of a mixture of soil : peat : grit : perlite (3 : 3 : 3 : 1) supplemented, unless otherwise stated, with 20 g of Osmocote (representing a total of 2.83 g of nitrogen fertilizer per plant). No further feeding with nutrients was carried out. In the experiment on plant responses to low and high nitrogen, a single dose of 10 g or 30 g of Osmocote, respectively, was added to 7.5 L of the soil : peat : grit : perlite mixture.

Promoter isolation and sequencing

A full-length *SEE1* clone was isolated by a differential screen of a λgt10 cDNA library made from early senescing maize leaves 12–20 days after pollen shed (DAPS) using ^{32}P-labelled cDNA from 0 DAPS and 16 DAPS as probes (Smart *et al*., 1995). *SEE1* cDNA was labelled with ^{32}P and used to probe a maize genomic library in λFixII (Stratagene) replicated on to nylon filters. Genomic DNA sequence analysis, using primers designed from the *SEE1* cDNA, indicated that all putative *SEE1* genomic clones were identical. A representative clone (See1-N) was restricted with *Xho*I and the resulting fragments subcloned into pBluescript II KS (Stratagene). A subclone containing the 5′ end and putative upstream regulatory sequence was identified by Southern blotting of *Xho*I-restricted plasmid DNA preparations on to nylon membrane and hybridizing with DIG (Roche)-labelled *SEE1* DNA. This plasmid clone (See1-N2) was sequenced in both directions using an ABI 373A automated sequencer (Applied Biosystems).

Vector construction

A fusion between the *Agrobacterium IPT* gene and P_{SEE1} was cloned into a novel plasmid created by inserting a linker into pBluescript II KS (Stratagene). The modified vector allows for single-site subcloning into binary vectors, such as pROK2, enables single-enzyme determination of an intact expression cassette via Southern blots, and generates the necessary *Sac*I and *Eco*RI sites to place a terminator from nopaline synthase (*NOS*) at the end of the polylinker. The linker was made from two oligos which, when hybridized, contained a *Not*I terminus at the 5′ end and a *Sac*I terminus at the 3′ end. After ligation, the *Sac*I restriction enzyme can no longer restrict the *Sac*I terminus as the hexamer contains a mismatch at the 5′ end. Internally, the linker contains four sites, from 5′ to 3′, *Sac*I, *Eco*RI, *Xho*I and *Kpn*I. The resulting plasmid (pBKK1) was restricted with *Sma*I and *Eco*RV, which generate blunt ends; after ligation, the *Pst*I site and the original *Eco*RI site were lost (pBKK2). A 300 bp PCR product tailed with *Sac*I (5′) and *Eco*RI (3′) was amplified from the *NOS* terminator region from *Agrobacterium*. The *NOS* terminator was cloned into the *Sac*I and *Eco*RI sites of pBKK2 (pBKK2*NOS*).

A single base-pair change in the promoter of *SEE1* (P_{SEE1}) allowed the generation of an *Xba*I site that would permit a fusion to be created between $P_{SEE1Xba}$ and *IPT* such that the translational start site of *IPT* was in a similar position to the putative translational start site of *SEE1*. This was produced using a single base-pair mismatched oligonucleotide (XBA3: ccatctagacggcgggactg) which amplified, in combination with oligonucleotide XBA5 (cttgaaaattggaatagattctctag), from a genomic clone of the maize *SEE1* gene, a 540 bp PCR fragment containing the novel *Xba*I sequence at position –5. The amplified fragment terminated at an upstream *Xba*I site allowing the 540 bp 5′ promoter fragment to be excised as an *Xba*I

fragment. The *Xba*I fragment was cloned into Bluescript and sequenced to confirm the presence of a novel *Xba*I site and the absence of PCR-induced mutations (pBKSP$_{SEE1Xba}$T). A *Kpn*I/*Xba*I fragment containing a further 1431 bp upstream of the 5′ *Xba*I site was cloned upstream of the PCR product (pBKSP$_{SEE1Xba}$). A 1971 bp fragment resulting from a *Kpn*I and *Xba*I partial digest of pBKSP$_{SEE1Xba}$ was ligated into *Kpn*I/*Xba*I-restricted pBKS in a triple ligation with a *Xba*I fragment containing the *IPT* coding region isolated by Goldberg *et al*. (1984) (pBKSP$_{SEE1Xba}$*IPT*). The P$_{SEE1Xba}$*IPT* fusion was excised as a *Kpn*I/*Not*I fragment and cloned into pBKK2*NOS* (pBKK2P$_{SEE1Xba}$*IPTNOS*). A PCR test for the presence of the plasmid was performed using XBA5, described above, and IPT1 (cctgtgcaagttggaccg).

Plant transformation

Immature embryos of a *Zea mays* hybrid, HiII (A188xB73), were cultured to generate embryogenic callus and transformed via Biolistic® PDS-1000/He (Bio-Rad, Hercules, CA, USA), as described previously (Frame *et al*., 2000). Embryogenic callus was cotransformed with pBKK2P$_{SEE1Xba}$*IPTNOS* containing the expression cassette, and pBAR184 (Frame *et al*., 2000) containing the selection cassette, in which a 2.9 kb fragment containing the ubiquitin promoter/intron and bar gene from pAHC25 (Christensen and Quail, 1996) was inserted into pACYC184. Transformed callus was regenerated under bialophos selection and, using oligonucleotides XBA5 and IPT1, PCR-based analysis was used to screen proliferating callus for the presence of pBKK2P$_{SEE1Xba}$*IPTNOS*. Twenty-nine independent events generated fertile plants that were backcrossed to the B73 parental line to generate 13 574 T1 seeds for experimental analysis of the construct activity in maize.

Northern analysis

RNA was extracted from maize leaves by a modification of the hot phenol method (Schünmann *et al*., 1994). Total RNA (10 μg) was resolved under denaturing conditions and blotted on to HYBOND N (Amersham Pharmacia Biotech Ltd., Buckinghamshire, UK). Blots were hybridized to a radiolabelled 0.75 kb probe containing the *IPT* coding region. Quantification of the autoradiogram was carried out on an ALPHAIMAGER 1200 (Alpha Innotech, CA, USA). A digitized image was captured at a non-saturating intensity and the intensity of each band corresponding to the correct size for the *IPT* transcript was determined and normalized for background.

Measurement of chlorophyll and photosynthesis

Non-destructive measurements of chlorophyll content were made using a SPAD-502 chlorophyll meter (Minolta, Osaka, Japan) and expressed as RChl (relative chlorophyll units) (Zavaleta-Mancera *et al*., 1999a). Ten readings were taken along both sides of the lamina and an average value per leaf was calculated. Ten plants per transgenic event were analysed and values for equivalent leaves in either the transgenic or control lines were averaged and expressed with standard errors. Chlorophyll was extracted into 1 mL methanol from two leaf discs taken from the 15th leaf using a 6 mm core-borer. Extractions were performed overnight at 4 °C in the dark. Extracted chlorophyll was measured at 665 nm and 652 nm on a Phillips PU 8720 UV/VIS scanning spectrophotometer; values were corrected by subtracting the absorption at 750 nm and the chlorophyll content was calculated according to Porra *et al*. (1989). The gross photosynthetic activity was estimated from measurements of the fluorescence and photosynthetically active radiation using a PPM Plant Photosynthesis Meter that measures fluorescence under ambient and saturating light levels (EARS, Delft, The Netherlands).

Acknowledgements

This research was funded by BBSRC (Biotechnology and Biological Sciences Research Council) under the Resource Allocation and Stress in Plants initiative (RASP project RSP07828) and by Advanta Seeds. We thank Kate Griffiths, who initiated maize senescence research at the Institute of Grassland and Environmental Research, Rosalie Metcalfe and Fiona Harris, who provided technical support, and Alan Gay for advice and discussion.

References

Ahmed, S.U., Rojo, E., Kovaleva, V., Venkataraman, S., Dombrowski, J.E., Matsuoka, K. and Raikhel, N.V. (2000) The plant vacuolar sorting receptor *AtELP* is involved in transport of NH2-terminal propeptide-containing vacuolar proteins in *Arabidopsis thaliana*. *J. Cell Biol*. **149**, 1335–1344.

Blume, B. and Grierson, D. (1997) Expression of ACC oxidase promoter-GUS fusions in tomato and *Nicotiana plumbaginifolia* regulated by developmental and environmental stimuli. *Plant J*. **12**, 731–746.

Braun, A.C. and Wood, H.N. (1976) Suppression of the neoplastic state with the acquisition of specialized functions in cells, tissues, and organs of crown gall teratomas of tobacco. *Proc. Natl. Acad. Sci. USA*, **73**, 496–500.

Butt, A., Mousley, C., Morris, K., Beynon, J., Can, C., Holub, E., Greenberg, J.T. and Buchanan-Wollaston, V. (1998) Differential expression of a senescence-enhanced metallothionein gene in

Arabidopsis in response to isolates of *Peronospora parasitica* and *Pseudomonas syringae*. *Plant J*. **16**, 209–221.

Chaudhury, A.M., Letham, S., Craig, S. and Dennis, E.S. (1993) *amp1* – a mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. *Plant J*. **4**, 907–916.

Chen, L.F.O., Hwang, J.Y., Chang, Y.Y., Sun, C.W. and Yang, S.F. (2001) Transformation of broccoli (*Brassica oleracea* var. italica) with isopentenyltransferase gene via *Agrobacterium tumefaciens* for post-harvest yellowing retardation. *Mol. Breed.* **7**, 243–257.

Chory, J., Reinecke, D., Sim, S., Washburn, T. and Brenner, M. (1994) A role for cytokinins in de-etiolation in *Arabidopsis*. *det* mutants have an altered response to cytokinins. *Plant Physiol*. **104**, 339–347.

Christensen, A.H. and Quail, P.H. (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Res*. **5**, 213–218.

Chung, B.C., Lee, S.Y., Oh, S.A., Rhew, T.H., Nam, H.G. and Lee, C.H. (1997) The promoter activity of *sen1*, a senescence-associated gene of *Arabidopsis*, is repressed by sugars. *J. Plant Physiol*. **151**, 339–345.

Dangl, J.L., Dietrich, R.A. and Thomas, H. (2000) Senescence and programmed cell death. In: *Biochemistry and Molecular Biology of Plants* (Buchanan, B., Gruissem, W. and Jones, R., eds), pp. 1044–1100. Rockville: ASPP.

Feller, U. and Fischer, A. (1994) Nitrogen metabolism in senescing leaves. *Crit. Rev. Plant Sci*. **13**, 241–273.

Flores, S. and Tobin, E.M. (1988) Cytokinin modulation of LHCP mRNA reveals the involvement of post-transcriptional regulation. *Plant Mol. Biol*. **11**, 409–415.

Frame, B.R., Zhang, H., Cocciolone, S.M., Sidorenko, L.V., Dietrich, C.R., Pegg, S.E., Zhen, S., Schnable, P.S. and Wang, K. (2000) Production of transgenic maize from bombarded type II callus: Effect of gold particle size and callus morphology on transformation efficiency. *In Vitro Cell Dev. Biol.-Plant*, **36**, 21–29.

Gan, S. and Amasino, R.M. (1995) Inhibition of leaf senescence by autoregulated production of cytokinin. *Science*, **270**, 1986–1988.

Goldberg, S.B., Flick, J.S. and Rogers, S.G. (1984) Nucleotide sequence of the tmr locus of *Agrobacterium tumefaciens* pTi T37 T-DNA. *Nucleic Acids Res*. **12**, 4665–4677.

Graham, I.A., Leaver, C.J. and Smith, S.M. (1992) Induction of malate synthase gene expression in senescent and detached organs of cucumber. *Plant Cell*, **4**, 349–357.

Griffiths, C.M., Hosken, S.E., Oliver, D., Chojecki, J. and Thomas, H. (1997) Sequencing, expression pattern and RFLP mapping of a senescence-enhanced cDNA from *Zea mays* with high homology to oryzain γ and aleurain. *Plant Mol. Biol*. **34**, 815–821.

Hauck, B., Gay, A.P., Macduff, J., Griffiths, C.M. and Thomas, H. (1997) Leaf senescence in a non-yellowing mutant of Festuca pratensis: implications of the stay-green mutation for photosynthesis, growth and nitrogen nutrition. *Plant Cell Environ*. **20**, 1007–1018.

Hensel, L.L., Grbic, V., Baumgarten, D.A. and Bleecker, A.B. (1993) Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in *Arabidopsis*. *Plant Cell*, **5**, 553–564.

Jordi, W., Schapendonk, A., Davelaar, E., Stoopen, G.M., Pot, C.S., de Visser, R., van Rhijn, J.A., Gan, S. and Amasino, R.M. (2000) Increased cytokinin levels in transgenic PSAG12-ipt tobacco plants have large direct and indirect effects on leaf senescence, photosynthesis and N partitioning. *Plant Cell Environ*. **23**, 279–289.

King, G.A. and O'Donoghue, E.M. (1995) Unravelling senescence: new opportunities for delaying the inevitable in harvested fruit and vegetables. *Trends Food Sci. Technol.* **6**, 385–389.

Koizumi, M., Yamaguchishinozaki, K., Tsuji, H. and Shinozaki, K. (1993) Structure and expression of two genes that encode distinct drought-inducible cysteine proteinases in *Arabidopsis thaliana*. *Gene*, **129**, 175–182.

Kulaeva, O.N., Karavaiko, N.N., Selivankina, S.Y., Moshkov, I.E., Novikova, G.V., Zemlyachenko, Y.V., Shipilova, S.V. and Orundgev, E.M. (1996) Cytokinin signalling systems – from whole-plant to the molecular-level. *Plant Growth Reg*. **18**, 29–37.

Lasserre, E., Godard, F., Bouquin, T., Hernandez, J.A., Pech, J.C., Roby, D. and Balague, C. (1997) Differential activation of two ACC oxidase gene promoters from melon during plant development and in response to pathogen attack. *Mol. Gen. Genet*. **256**, 211–222.

Li, Q. (2000) Studies in leaf senescence and its genetic manipulation in *Lolium multiflorum* Lam. PhD Thesis. Aberystwyth: University of Wales.

Lin, Y.J., Cao, M.L., Xu, C.G., Chen, H., Wei, J. and Zhang, Q.F. (2002) Cultivating rice with delaying leaf-senescence by PSAG12-IPT gene transformation. *Acta Bot. Sinica*, **44**, 1333–1338.

Maxwell, K. and Johnson, G.N. (2000) Chlorophyll fluorescence – a practical guide. *J. Exp. Bot*. **5**, 659–668.

McCabe, M.S., Garratt, L.C., Schepers, F., Jordi, W.J.R.M., Stoopen, G.M., Davelaar, E., van Rhijn, J.H.A., Power, J.B. and Davey, M.R. (2001) Effects of P_{SAG12}-*IPT* gene expression on development and senescence in transgenic lettuce. *Plant Physiol*. **127**, 505–516.

Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng*. **10**, 1–6.

Noh, Y.S. and Amasino, R.M. (1999) Identification of a promoter region responsible for the senescence-specific expression of *SAG12*. *Plant Mol. Biol*. **41**, 181–194.

Oh, S.A., Park, J.-H., Lee, G.I., Paek, H.H., Park, S.K. and Nam, H.G. (1997) Identification of three genetic loci controlling leaf senescence in *Arabidopsis thaliana*. *Plant J*. **12**, 527–535.

Porra, R.J., Thompson, W.A. and Friedemann, P.A. (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim. Biophys. Acta*, **975**, 384–394.

Quirino, B.F., Noh, Y.S., Himelblau, E. and Amasino, R.M. (2000) Molecular aspects of leaf senescence. *Trends Plant Sci*. **5**, 278–282.

Riou, C., Herve, C., Pacquit, V., Dabos, P. and Lescure, B. (2002) Expression of an *Arabidopsis* lectin kinase receptor gene, lecRK-a1, is induced during senescence, wounding and in response to oligogalacturonic acids. *Plant Physiol. Biochem*. **40**, 431–438.

Schünmann, P.H.D., Ougham, H.J. and Turk, K.S. (1994) Leaf extension in the slender barley mutant: delineation of the zone of cell expansion and changes in translatable mRNA during leaf development. *Plant Cell Environ*. **17**, 1315–1322.

Smart, C.M., Hosken, S.E., Thomas, H., Greaves, J.A., Blair, B.G. and Schuch, W. (1995) The timing of maize leaf senescence and characterisation of senescence-related cDNAs. *Physiol. Plant*. **93**, 673–682.

Smart, C.M., Scofield, S.R., Bevan, M.W. and Dyer, T.A. (1991)

Delayed leaf senescence in tobacco plants transformed with *TMR*, a gene for cytokinin production in *Agrobacterium*. *Plant Cell*, **3**, 647–656.

Takei, K., Sakakibara, H., Taniguchi, M. and Sugiyama, T. (2001) Nitrogen-dependent accumulation of cytokinins in root and the translocation to leaf: implication of cytokinin species that induces gene expression of maize response regulator. *Plant Cell Physiol*. **42**, 85–93.

Thomas, H. (1992) Canopy survival. In: *Crop Photosynthesis. Spatial and Temporal Determinants* (Baker, N. and Thomas, H., eds), pp. 11–41. Amsterdam: Elsevier.

Thomas, H. (2003) Do green plants age and if so, how? *Topics Curr. Genet*. **3**, 145–171.

Thomas, H. and Donnison, I.S. (2000) Back from the brink: plant senescence and its reversibility. In: *Programmed Cell Death in Animals and Plants* (Bryant, J., Hughes, S.G. and Garland, J.M., eds), pp. 149–162. Oxford: Bios.

Thomas, H. and Howarth, C.J. (2000) Five ways to stay green. *J. Exp. Bot*. **51**, 329–337.

Thomas, H., Ougham, H., Canter, P. and Donnison, I.S. (2002) What stay-green mutants tell us about nitrogen remobilisation in leaf senescence. *J. Exp. Bot*. **53**, 801–808.

Thomas, H., Ougham, H.J., Wagstaff, C. and Stead, A.D. (2003) Defining senescence and death. *J. Exp. Bot*. **54**, 1127–1132.

Thomas, H. and Smart, C.M. (1993) Crops that stay green. *Ann. Appl. Biol*. **123**, 193–219.

Yusibov, V.M.II.P.C., Adrianov, V.M. and Piruzian, E.S. (1991) Phenotypically normal transgenic T-*cyt* tobacco plants as a model for the investigation of plant gene expression in response to phytohormonal stress. *Plant Mol. Biol*. **17**, 825–836.

Zavaleta-Mancera, H.A., Franklin, K.A., Ougham, H.J., Thomas, H. and Scott, I.M. (1999a) Regreening of senescent *Nicotiana* leaves. I. Reappearance of NADPH-protochlorophyllide oxidoreductase and light-harvesting chlorophyll a/b-binding protein. *J. Exp. Bot*. **50**, 1677–1682.

Zavaleta-Mancera, H.A., Thomas, B.J., Thomas, H. and Scott, I.M. (1999b) Regreening of senescent *Nicotiana* leaves. II. Redifferentiation of plastids. *J. Exp. Bot*. **50**, 1683–1689.