# Modification of nitrogen remobilization, grain fill and leaf senescence in maize (*Zea mays*) by transposon insertional mutagenesis in a protease gene

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## Summary

• A maize (*Zea mays*) senescence-associated legumain gene, *See2β*, was characterized at the physiological and molecular levels to determine its role in senescence and resource allocation.

• A reverse-genetics screen of a maize *Mutator* (*Mu*) population identified a *Mu* insertion in *See2β*. Maize plants homozygous for the insertion were produced. These *See2* mutant and sibling wild-type plants were grown under high or low quantities of nitrogen (N).

• The early development of both genotypes was similar; however, tassel tip and collar emergence occurred earlier in the mutant. Senescence of the mutant leaves followed a similar pattern to that of wild-type leaves, but at later sampling points mutant plants contained more chlorophyll than wild-type plants and showed a small extension in photosynthetic activity. Total plant weight was higher in the wild-type than in the mutant, and there was a genotype × N interaction. Mutant plants under low N maintained cob weight, in contrast to wild-type plants under the same treatment.

• It is concluded, on the basis of transposon mutagenesis, that *See2β* has an important role in N-use and resource allocation under N-limited conditions, and a minor but significant function in the later stages of senescence.

**Key words:** chlorophyll, leaf senescence, legumain, nitrogen remobilization, photosynthesis, source–sink relationship, transposon mutagenesis, *Zea mays* (maize).

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## Introduction

This paper describes the application of reverse genetics to establish the function of a senescence-associated gene in maize development. Degradation of leaf proteins during foliar senescence is required in order to remobilize leaf nitrogen (N) and export it to developing grain and other sinks (Feller & Fischer, 1994). It is therefore unsurprising that elevated levels of proteolytic activity have been reported as characteristic of leaf senescence: genes encoding proteases are among the commonest classes of genes reported to show up-regulation of expression in senescing leaves. For example, senescence-enhanced cysteine endopeptidases have been identified in many species, including maize (Smart *et al.*, 1995), perennial ryegrass (Li *et al.*, 2000), *Arabidopsis thaliana* (Hensel *et al.*, 1993; Lohman *et al.*, 1994; Grbic, 2003), tomato (Drake *et al.*,

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1996; John *et al.*, 1997), and *Brassica napus* (Buchanan-Wollaston & Ainsworth, 1997). Large-grain cereals such as maize are particularly dependent on adequate N availability during the period of grain fill (Alexander *et al.*, 1991; Ma & Dwyer, 1998; Borras *et al.*, 2004). A high proportion of the N required, especially under suboptimal N nutrition regimes, is normally supplied by leaf senescence, during which leaf protein is remobilized and amino acids are exported to the developing grain (Feller *et al.*, 1977; Borras *et al.*, 2004). Optimal regulation of the onset and rate of senescence is therefore important if grain yield is to be maximized. On the one hand, maintaining productive photosynthetic leaf area by delaying or slowing down senescence may improve yield by increasing total photosynthate available to the sink tissue (Borras *et al.*, 2004). On the other hand, under conditions where N or other nutrients are limiting, inhibition of senescence will result in nutrient deficiency for the sink organs, and grain yield will be reduced.

There is considerable genetic variation in the interaction between leaf senescence and grain fill under different N regimes (Hirel *et al.*, 2001; Gallais & Hirel, 2004; Martin *et al.*, 2005; Pommel *et al.*, 2006). A better understanding of the molecular basis of this variation therefore opens up the possibility of developing improved varieties. This improvement may be achieved by a transgenic approach or, where this is not feasible, by conventional breeding assisted by marker-assisted selection using markers closely linked to, or single nucleotide polymorphisms (SNPs) at, genes contributing to the process. The types of genetic variants known collectively as stay-greens, in which some or all aspects of the senescence syndrome are retarded or blocked, strikingly demonstrate the potential of this approach. While some stay-greens are 'cosmetic' (i.e. they retain green colour but lose photosynthetic capacity at the normal rate; Thomas *et al.*, 1999), others are 'functional' in that photosynthetic leaf area is maintained for a longer period, either by delaying the onset of senescence or by slowing the progression of the syndrome (Thomas & Howarth, 2000). It is functional stay-greens which best exemplify the possibility of manipulating the senescence–grain fill interaction, and they have been identified in a number of cereal crop species, including *Sorghum* (Xu *et al.*, 2000), wheat (Spano *et al.*, 2003) and maize (Thomas & Howarth, 2000; Kamara *et al.*, 2003).

The protease genes already identified in maize have proved a useful source of promoters for generating transgenics with modified senescence properties; for example, the maize *See1* promoter has been used to drive expression of the bacterial *ipt* gene in a senescence-specific manner in both maize (Robson *et al.*, 2004) and perennial ryegrass (Li *et al.*, 2004). The *ipt* gene encodes an enzyme responsible for cytokinin biosynthesis, and the transgenic plants exhibited a stay-green phenotype characteristic of cytokinin overexpression, comparable to that of tobacco plants expressing the *ipt* gene under the control of the promoter from the *Arabidopsis thaliana* sag12 (senescence-associated) gene (Gan & Amasino, 1995). An alternative route to manipulate senescence is to knock out senescence-enhanced genes and assess their contribution to the senescence syndrome, including the effect on N mobilization. In this paper we report on the identification of mutations in the maize *See2* genes, which encode cysteine-proteases, by a reverse genetic screen of a maize *Mutator* (*Mu*) (Robertson, 1978) population. The development and productivity of one of these mutants are compared with those of a wild-type control under two N regimes to assess the function of the *See2β* gene, one of the two maize *See2* genes, in senescence and N remobilization.

## Materials and Methods

### Isolation of *See2* cDNA and genomic clone

A partial *See2* clone was previously isolated by a differential screen of a λgt10 cDNA library made from maize (*Zea mays* L.) leaves at early senescence, 12–20 d after pollen shed (daps), using $^{32}$P-labelled cDNA from 0 daps and 16 daps as probes (Smart *et al.*, 1995). Another 255 bp *Dpn*II cDNA fragment, at the 5′ end of the gene, was identified using a modification of representational difference analysis (RDA; Lisitsyn *et al.*, 1993) for comparing cDNA (Thomas *et al.*, 1997; Hubank & Schatz, 1999). The subtracted clone (dd8) was labelled with $^{32}$P and used to probe a maize genomic library (Missouri 17) in λFixII (Stratagene). A PCR screen of putative *See2* genomic clones using primers 5dd8 (gatcatgaagaagggcggactta) and 3dd8 (gatcattggggccactgtcca) designed to amplify a *c.* 250 bp cDNA fragment, resulted in one of two differently sized bands in each positive λ clone. The primers spanned an intron, and the DNA fragments of 600 or 820 bp were cloned and sequenced. One genomic clone for each of the two PCR-polymorphisms, designated *See2α* and *See2β*, was sequenced, new PCR primers designed and 'full length' cDNA copies of each sequence amplified and sequenced.

### Screening for *Mutator* insertion in the *See2* gene

A number of forward and reverse primers were designed along the *See2* gene sequence. Single-gene-specific primers were used in combination with a primer to the terminal region of the maize *Mu* transposon to screen a maize mutant collection. DNA from pools of up to 50 individual plants was used as a template in duplicate PCR reactions with the gene-specific and *Mu* primers. The resulting PCR reactions were dot-blotted in duplicate onto nylon membrane and hybridized with DIG-labelled *See2* DNA generated using the DIG Hi-Prime method (Roche, Lewes, UK). Where cross-hybridizing spots were observed, the PCR reaction was repeated using the original pair of primers and DNA from the individual plants which made up the pool. These PCR reaction products were separated by gel electrophoresis and the parent plant which

 

contained the *Mu* insertion in the *See2* gene was identified. Ten seeds collected from the parent plant were grown and small amounts of leaf tissue sampled for DNA extraction. These DNA samples were used as templates in PCR reactions with the *See2* gene-specific and *Mu* primers to determine if the insertion had been inherited in the progeny of the original plant which had been screened in the grid. Maize plants which contained this insertion were backcrossed to the B73 genotype twice and selfed for phenotypic analysis.

### Southern and northern analyses

Maize genomic DNA was isolated using a modification of the method of Shure *et al.* (1983). Maize RNA was extracted using a standard phenol method and LiCl precipitation (Malik, 1987). DNA digestion, DNA and RNA gel electrophoresis, northern blots onto Hybond-N (Amersham, Little Chalfont, UK) and Southern blots onto Hybond-$N^+$ (Amersham) were performed using standard laboratory methods (Sambrook *et al.*, 1989). cDNA probes for northern and Southern blots were labelled with $^{32}P$ using random hexanucleotide primers and Klenow DNA polymerase (Stratagene, La Jolla, CA, USA).

### RT-PCR analyses

Total RNA was extracted from leaves taken from maize plants of increasing age as for northern analyses. *See2α*- and *See2β*-specific pairs of primers were designed (*See2α*, ccgacgaaacggacgacggc and ggtcgcatacacattgatgt; *See2β*, caaatgcagaggagagcagc and ggagcggctgaaatatttcgc) and RT-PCR performed using the Titan (Roche) one-tube system. Putative *See2* PCR products were blotted and probed with DIG (Roche)-labelled *See2* DNA probes to confirm the identity of the DNA bands, and also sequenced to confirm that the products were exclusively either *See2α* or *See2β*.

### Plant growth conditions, N treatments, developmental and growth records

Plants of maize containing a *Mu* insertion in the *See2β* gene and sibling controls were grown from seed in pots containing 7.7 l of maize mix (30% loam, 30% 1–3 mm grit, 30% peat and 10% perlite all by volume) in a glasshouse at the Institute of Grassland & Environmental Research (IGER), Aberystwyth. Plants showing delayed germination or extremely aberrant phenotypes were discarded at the seedling stage. The period of growth was from 8 January 2002 to 23 April 2002, and natural daylight in the glasshouse was supplemented with overhead lighting using 400 W high-pressure sodium lamps (Phillips son T Agro) on for 16 h daily. Minimum temperatures were maintained at 25°C (day) and 18°C (night). N treatments were applied by watering with 5.14 g $l^{-1}$ ammonium nitrate solution at days 48, 62, 69, 76 and 84 after sowing. Low-N plants received 10 ml of this solution per treatment; high-N plants received 20 ml.

Dates of tassel tip emergence (defined as the tassel becoming visible), complete emergence of the tassel (defined as the appearance of the tassel collar) and initial pollen shed were recorded for each plant based on daily observations of 18 plants per genotype and N treatment combination.

The fresh weights (FWs) of cobs, the uppermost six leaf laminae, all older leaf laminae combined, and the stems, including leaf sheaths, were recorded on four plants for each treatment and genotype combination 32 and 33 daps in the wild-type and mutant, respectively. Subsamples of known FW were taken from each tissue immediately after collection and oven-dried at 80°C and their FW : DW (dry weight) ratios used to calculate DWs of organs. Additionally, 1, 17, 23, 33 and 37 daps (mutant) and 4, 19, 25 and 32 daps (wild-type), the FWs, FW : DW ratios and area of a small subsample of known FW were recorded separately for the uppermost six leaves on each of four plants per treatment. From these DWs per leaf, leaf area ratio (area per unit dry weight) and area of each leaf were estimated. Further subsamples from the third leaf below the flag were taken and oven-dried for N and carbon content measurement, or immediately frozen and stored at −80°C for chlorophyll determinations.

### Western analyses

Protein was extracted from maize leaves as described previously (Smart *et al.*, 1995). Proteins were separated by SDS gel electrophoresis in 12.5% polyacrylamide and transferred electrophoretically to nitrocellulose membrane using a Bio-Rad Mini Trans-Blot apparatus. An antibody to a recombinant legumain derived from *Vicia sativa* was obtained from K. Muentz (IPK, Gatersleben, Germany) and immunodetection was performed as described by Hilditch *et al.* (1989).

### Photosynthesis measurements

Photosynthesis was measured half way between the tip and the ligule of the leaf on the third and fifth leaves below the flag at 0, 16, 22, 32 and 36 or 3, 18, 24 and 31 daps in the mutant and normal plants, respectively, on four replicate leaves per treatment, except on the first measurement in both treatments when only a single replicate was used. Measurements were made at a mean background [$CO_2$] of between 348 and 355 μmol $mol^{-1}$ (SE 1.0 within a day) at 0, 52, 155, 298, 559 and 1156 μmol $m^{-2}$ $s^{-1}$ PPFD using a CIRAS 1 portable photosynthesis system fitted with a narrow leaf cuvette and a tungsten halogen illumination system (PP Systems, Hitchin, Herts, UK). PPFD was varied using a series of spectrally neutral filters, and temperature was maintained at 25 ± 1°C by circulating cooled water though a heat exchanger attached to the cuvette.

**Table 1** Comparison of introns 1–8 between *See2α* and *See2β* genes in maize (*Zea mays*)

| | *See2α* | | *See2β* | | |
|---|---|---|---|---|---|
| Intron | bp | No. of bp | bp | No. of bp | Similarity (%) |
| 1 | 202–3446 | 3245 | 199–1658 | 1460 | 45 |
| 2 | 3613–4202 | 590 | 1824–2174 | 351 | 72 |
| 3 | 4359–4482 | 142 | 2332–2450 | 119 | 85 |
| 4 | 4569–4661 | 93 | 2537–2626 | 90 | 86 |
| 5 | 4862–5890 | 1029 | 2827–4476 | 1650 | 66 |
| 6 | 5939–6036 | 97 | 4526–4604 | 79 | 78 |
| 7 | 6244–6367 | 124 | 4827–4964 | 138 | 86 |
| 8 | 6575–6689 | 119 | 5171–5273 | 103 | 76 |

### Compositional measurements

Nitrogen content of dried, milled plant material was measured with a micro-Dumas combustion technique (Kirsten, 1983) using a Leco FP420 analyser (Leco Corporation, St Joseph, MI, USA). Carbon content was measured using a continuous-flow isotope ratio mass spectrometer-IRMS (ANCA-SL 20/20, Europa Scientific Ltd, Crewe, UK).

### Chlorophyll, carotenoids and proteins

Frozen leaf material was ground to a fine powder with liquid $N_2$ and sand, in a mortar and pestle. The powder was homogenized with extraction buffer (0.17 mM Tris, pH 8.0-NaOH, 5% glycerol, 1% EtSH, 1 mM PMSF, 1 mM monoiodoacetate) using 5 ml $g^{-1}$ FW and allowed to thaw before adding 20% lithium dodecyl sulphate (0.5 ml $g^{-1}$ FW) and homogenizing further. A subsample of the homogenate was taken and acetone was added to 80% for spectrophotometric chlorophyll and carotenoid analysis with values calculated as in Gay & Thomas (1995). The homogenate was heat-denatured by boiling for 5 min, cooled and centrifuged at 12 500 ***g*** for 10 min. An equal volume of 10% trichloroacetic acid (TCA) was added to the supernatant and proteins were precipitated overnight at 4°C followed by centrifugation at 12 500 ***g*** and 4°C for 5 min. The pellet was washed with 0.8 ml 5% TCA and centrifuged, washed twice with ice-cold 100% acetone followed by centrifugation, allowed to air dry for 1 h, then dissolved in 0.1 N NaOH overnight at 4°C. Total protein was determined using bovine serum albumin as standard (Lowry *et al.*, 1951).

### Statistical analyses

Statistical analyses were carried out using the Genstat statistical package for analyses of variance, using fixed models with genotypes and N treatments and their interaction as the sources of variation. These analyses are presented directly in Table 2. In Figs 7 and 8 the analyses were conducted for nearby grouped time points for wild-type and stay-green plants, and the standard errors of difference (SEDs) are presented at the mean time for the interaction. For the final time points in Figs 7 and 8, only stay-green plants were present, and the SED given is for the differences between nitrogen treatments. In Fig. 9, as there were no significant differences between nitrogen treatments, only means for genotypes are presented, together with SED for differences between genotypes analysed harvest by harvest. No comparison is available for the last harvest of the stay-green plants. In Fig. 10, the analysis of variance was conducted separately for each light intensity across harvests 1–4, N amounts and genotypes, and to simplify presentation, the SED for the three-level interaction is presented for each light intensity and applies to both Fig. 10a and 10b. When the data for the final harvest of the stay-green plants were analysed for N responses (Fig. 10b), no significant differences were found and the SED is smaller than that shown on the figure.

## Results

### *See2* gene sequence and homologies

DNA sequencing of the two versions of *See2* cDNA revealed that the sequences are similar and both contain a putative open reading frame. The *See2α* gene (ZMA131718) is predicted to encode a polypeptide of 486 amino acids with a molecular mass of 53.3 kDa, whilst the predicted polypeptide of *See2β* (ZMA131719) is five amino acids shorter and has a molecular mass of 52.8 kDa. Both the DNA sequence and the predicted amino acid sequence of the two *See2* genes show a strong homology to the legumain/haemoglobinase group of cysteine proteases. *See2* was cloned from a differential screen (Smart *et al.*, 1995) and also from a cDNA subtraction experiment as a leaf senescence up-regulated message and this has been confirmed by northern analysis (Fig. 1a). Northern analyses also revealed that the abundance of *See2* mRNA increases over the first 5 d after germination and can be detected in the roots of young seedlings grown hydroponically (Fig. 1b). RT-PCR analysis, using *See2α*- or *See2β*-specific primers, indicates that both *See2* genes are expressed in senescing leaves of maize but that the pattern of expression appears different (Fig. 2). From the cDNA sequence, the predicted amino acid sequence of *See2* is of a similar length to other legumains. Both *See2α* and *See2β* have a putative signal sequence of 21 amino acids as predicted by the SignalP program (Nielsen *et al.*, 1997). The first 47 amino acids, which include the signal peptide and the N-terminal pro-region, comprise the most highly divergent region of the *See2* and other legumain proteins. Seven amino acids downstream of the putative signal-peptide cleavage site is a highly conserved five-peptide sequence, I(/L)R(/K)LPS, perfectly conserved in all legumains similar to *See2*, and near-perfectly

 

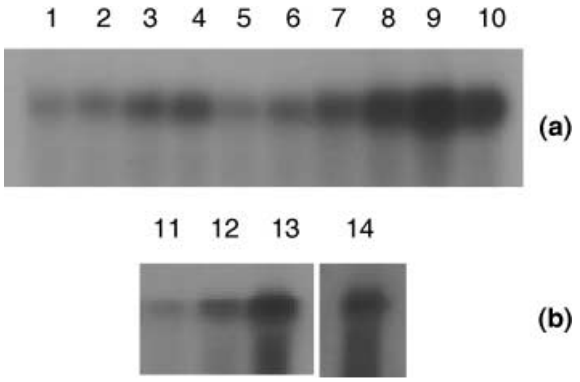


**Fig. 1** Northern analysis using RNA from maize (*Zea mays*) leaves and germinating seeds. Each lane contained 10 μg of total RNA which was hybridized at high stringency to the *See2* cDNA probe. (a) Natural senescence of mature leaves: lanes 1–10, RNA extracted at 5 d intervals from the ear leaves of mature soil-grown wild-type plants –5 to +40 d after pollen shed. (b) Seeds germinating in hydroponic conditions: lanes 11–13, RNA extracted from wild-type maize seeds 1, 3 and 5 d after germination; lane 14, roots.

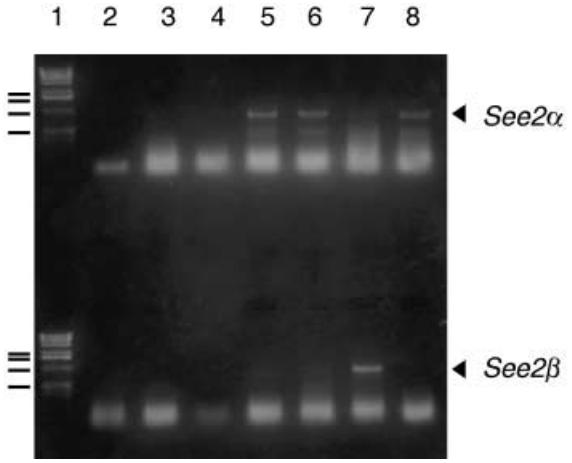


**Fig. 2** RT-PCR analysis using RNA from maize (*Zea mays*) leaves. RNA was extracted from young leaves (lane 2) and ear leaves 10 (lane 3), 15 (lane 4), 20 (lane 5), 25 (lane 6), 25 (lane 7) and 30 (lane 8) d after pollen shed. RNA was reverse-transcribed and PCR-amplified in a single tube before being electrophoresed on an agarose gel. Lane 1, 1 kb ladder.

conserved in other plant legumains. This peptide is contained in the short (approx. 20–30 amino acids) pro-region of legumains.

## Genomic DNA sequencing

The genomic DNA clones of *See2α* and *See2β* were sequenced: EMBL accession numbers AJ251453 and AJ251454, respectively. Both genes have eight introns and, by comparison of cDNA sequences and genomic DNA sequences, identical intron/exon splice sites (Fig. 3). Small differences between cDNA and genomic DNA sequences indicate allelic differences, the most notable being that the *See2β* genomic DNA sequence contained an additional 12 bp in the first exon. Introns 1, 2 and 5 were significantly longer than other introns (Table 1) and, on comparison, also more divergent in terms of both length and percentage identity. Intron 1, which is the most highly diverged intron, also contains a TATA motif 55 bp upstream of the start of exon 2 in *See2α* and 200 bp upstream of the start of exon 2 in *See2β*. In addition, cDNAs have been cloned which lack exon 1 but contain the 3′ of intron 1 at the 5′ end, exons 2–9, but no other intron sequences (Fig. 3c,d). Both *See2α* and *See2β* contain a methionine 15 bp from the start of exon 2. At the 3′ end of *See2β* intron 1, there is a tandem repeat of 48 bp. Surprisingly, however, this sequence is 71% identical to the 3′ of a castor-bean cysteine protease gene (AF050756; Schmid *et al.*, 1998) in the reverse orientation.

Intron 2 is similar between *See2α* and *See2β* with one major difference: *See2α* contains an additional 212 bp fragment which is highly palindromic and 76% identical to itself in the reverse orientation, increasing to 95% for the terminal 40 bp. Similar sequences appear in, or close to, other maize genes in the DNA databases, including AI619399 (83%), AJ223470 (82%), U09989 (77%) (Jin & Bennetzen, 1994), AI600397 (69%), AW147120 (65%) and A1612358 (82%).

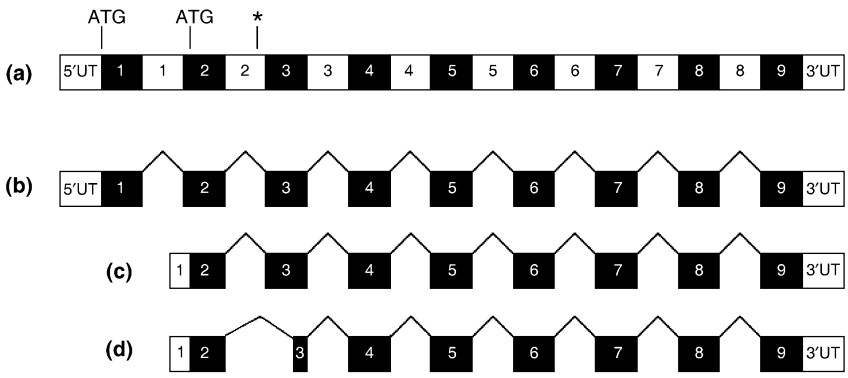


**Fig. 3** *See2* gene organization and alternative transcripts. (a) Gene organization: exons, black boxes; introns and untranslated (UT) regions, white boxes; ATG, putative translation start codons; *, the position of a MITE in *See2α*. (b, c) cDNAs cloned and sequenced from *See2β*. (b–d) cDNAs cloned and sequenced from *See2α*.

**Table 2** Dry weight and nitrogen (N) uptake of organs and whole plants of maize (*Zea mays*)

| | Wild-type | | Mutant | | SED and *P* | | |
|---|---|---|---|---|---|---|---|
| Nitrogen quantity | High | Low | High | Low | Genotype | N | Genotype × N |
| Dry weights (g) | | | | | | | |
| Stem | 66.7 | 75.5 | 27.6 | 37.2 | 8.55, < 0.001 | 8.55, 0.300 | 12.09, 0.963 |
| Lower leaves | 8.02 | 7.79 | 4.19 | 3.18 | 0.685, < 0.001 | 0.685, 0.384 | 0.968, 0.579 |
| Upper leaves | 6.69 | 7.83 | 5.92 | 6.64 | 0.662, 0.164 | 0.662, 0.186 | 0.936, 0.751 |
| Cob | 47.1 | 12.5 | 42.8 | 41.9 | 7.19, 0.106 | 7.19, 0.030 | 10.17, 0.037 |
| Total (incl. tassel) | 129.9 | 105.4 | 81.8 | 90.4 | 7.48, 0.001 | 7.48, 0.308 | 10.58, 0.047 |
| Nitrogen content (mg) | | | | | | | |
| Stem | 250 | 313 | 120 | 113 | 43.6, 0.003 | 43.6, 0.535 | 61.6, 0.439 |
| Lower leaves | 45.6 | 47.7 | 42.1 | 20.9 | 8.03, 0.083 | 8.03, 0.258 | 11.35, 0.173 |
| Upper leaves | 53.3 | 66.3 | 72.9 | 64.0 | 6.87, 0.234 | 6.87, 0.772 | 9.72, 0.139 |
| Cob | 471 | 113 | 505 | 430 | 65.9, 0.020 | 65.9, 0.007 | 93.2, 0.053 |
| Total (incl. tassel) | 830 | 551 | 752 | 638 | 41.0, 0.914 | 41.0, < 0.001 | 58.0, 0.068 |

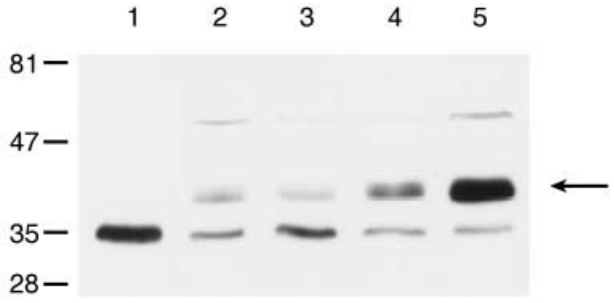


**Fig. 4** Western analysis using a recombinant *Vicia sativa* antibody to legumain. Lanes 1–5, protein extracted from maize (*Zea mays*) leaves of increasing age. Lane 1, tissue was isolated from a young expanding green leaf and for lanes 2–5, tissue was isolated from mature/senescing leaves sampled 15, 20, 25 and 30 d after pollen shed, respectively. Maize *See2* is indicated by an arrow, the two other cross-reacting proteins represent nonspecific protein binding since they also cross-reacted to preimune sera.

Related sequences were also identified in *Drosophila melanogaster* (AC007137, 61%), *Homo sapiens* Y chromosome DNA (AC006157, 65%) and *Plasmodium falciparum* (AL035475, 60%). Since this additional DNA fragment in *See2α* comprises a long inverted repeat, there is the potential for a stable secondary structure of a stem-loop type to occur at this position which is only 50 bp from the intron 2/exon 3 splice site. Moreover, one *See2α* cDNA was identified in which the first 71 bp of exon 3 was deleted whilst the remainder of exon 3 and subsequent exons were intact.

## Southern analysis and mapping

Southern analysis of maize genomic DNA digested with either *Cla*I, *Hin*dIII, *Eco*RI or *Pst*I and probed with $^{32}$P-labelled *See2* DNA reveals two major bands and, in some blots, a small number of faint bands (data not shown). RFLP mapping with *Eco*RI identifies two loci for the *See2* gene. These were mapped using 46 individuals in the T232 × CM37 recombinant inbred mapping population (Ben Burr, Brookhaven National Laboratory, Upton, NY, USA). One locus is tightly linked to marker umc2a, on chromosome 3 between umc96 and umc317, and the other is linked to marker umc89 and located between umc120 and umc93 on chromosome 8. As two bands, corresponding to the 600 and 820 bp fragments of *See2α* and *See2β*, can be PCR-amplified from the genomic DNA extracted from a single maize plant, it is likely that the two loci identified by RFLP mapping correspond to the two *See* clones.

## Western analyses

The antibody to a recombinant *Vicia sativa* legumain detected a band of approx. 40 kDa in maize (Fig. 4). Western analysis indicates that the protein is rare in young maize leaves and increases in concentration through senescence (Fig. 4).

## PCR genomic screening and northern analysis

A *Mu* insertion in the *See2β* gene was identified and this followed through to progeny derived from seed of the parent plant. This confirmed a germinal insertion event. The PCR fragment generated by the *Mu* and *See2* primer was cloned and sequenced to determine the position of the insertion in the first intron. Once this position was known, it was possible to distinguish the *See2-Mu* mutant allele from the wild-type allele using PCR. For example, the mutant allele could be detected with the original primers used to identify the mutation. By using primers that flanked the insertion, it was possible to detect a PCR product if a wild-type allele was present and therefore to distinguish homozygous mutants, homozygous wild-types and heterozygotes at the molecular level. Because of the difficulty in distinguishing between the *See2α* and *See2β* genes to demonstrate an impact on expression of the *Mu* insertion, a double *See2α* plus *See2β*

 

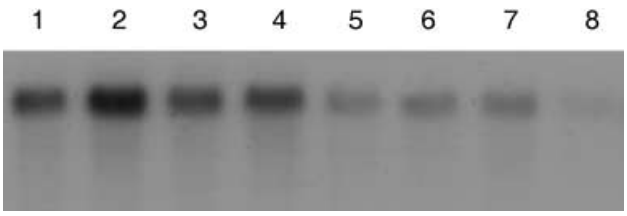


**Fig. 5** Northern analysis using RNA from senescent maize (*Zea mays*) leaves. Each lane contained 10 μg of total RNA from a single plant which was hybridized at high stringency to the *See2* cDNA probe. Lanes 1–4, wild-type control plants; lanes 5–8, *See2α* and *See2β* double mutant plants.

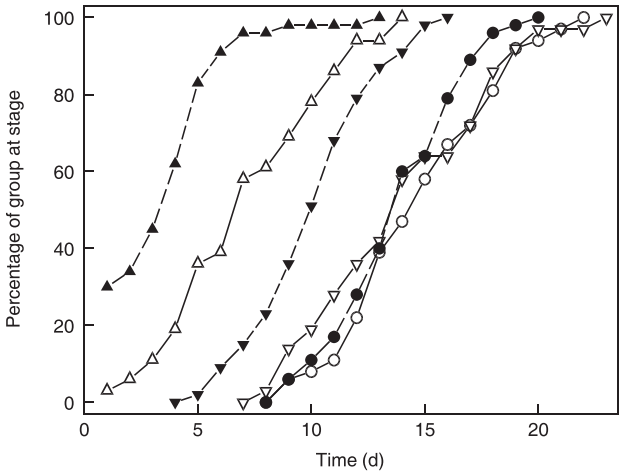


**Fig. 6** Time course of tassel tip (△, ▲) and tassel collar (▽, ▼) emergence and pollen shed (○, ●) for wild-type (open symbols) and mutant (closed symbols) maize (*Zea mays*).

insertion mutant was generated by crossing homozygous plants for both insertions. Approximately one plant in 16 was homozygous for both mutations and *See2* expression was compared between *See2* double mutant and sibling *See2* nonmutant plants using northern analysis. All wild-type plants expressed the *See2* gene, whilst double mutant plants tended also to express *See2* but to a visibly lesser degree (Fig. 5). This suggests that either the insertional events did not completely eliminate *See2* transcription, or maize contains additional *See2*-like genes.

## Reproductive development

Although there were few differences in germination of the seeds, the initial appearance of the tassel tip and collar was *c.* 3 d earlier in the mutant than in the wild-type phenotype (Fig. 6). However, the dates of pollen shed were much closer, with only a very slight delay in appearance in the wild-type plants. This means that in the mutant there was a delay of *c.* 3 d between emergence of the tassel collar and pollen shed, whilst in the wild-type plants these two stages followed on within less than 1 d. Thus, there may be an effect of the mutation on the developmental phasing or sequencing of heading.

## Plant weights and nitrogen use

Total plant weights (Table 2) were significantly higher in the wild-type than in the mutant, and there was a significant genotype × N interaction, showing considerably higher total plant weight in the wild-type plants at higher N. This was largely caused by the much smaller cobs (difference 34.6 g) produced in the low-N wild-type plants. In contrast, low N did not reduce cob size in the mutant. The pattern of N uptake by the organs (Table 2) is similar to that of their DW, but with a bias such that the proportion of the total uptake in the cob was greater. Overall, the third leaf below the flag in the mutant retained significantly higher N per unit leaf area than the control (449 and 406 mg $m^{-2}$, respectively; SED 20.2, $P = 0.037$), which is consistent with the expectation of slower release of N from senescing leaves of the mutant. Leaf size in terms of area and weight significantly ($P < 0.001$) increased from the flag leaf (area 82.4 $cm^2$, DW 0.336 g) to the fifth leaf below the flag (area 562 $cm^2$, DW 1.89 g; SED 15.7 $cm^2$ and 0.0506 g). There were no significant differences ($P = 0.133$, SED = 0.292) in mean leaf DW between mutant and wild-type control (1.174 and 1.218 g, respectively; SED 0.029, $P = 0.133$). However, the average area of the top six leaves was greater in the mutant than in the wild-type (364 and 319 $cm^2$, respectively; $P < 0.001$, SED 9.07). These differences resulted in a higher leaf area ratio in the mutant than in the wild-type control (301 and 262 $cm^2$ $g^{-1}$, respectively; SED = 4.3, $P < 0.001$). Thus, in the mutant it appears that there was increased allocation of DW towards production of leaf area, resulting in larger upper leaves, which may contribute to better grain filling in the low-N treatment.

## Nitrogen and carbon contents

In both genotypes in both N treatments, leaf N content declined to *c.* 50% of its initial value over the period following pollen shed (Fig. 7). Mutant plants consistently had a higher leaf N content than wild-type plants, the difference being most marked at the early and late stages of the post-pollen shed phase. High-N treatment resulted in a higher leaf N content than did low N treatment in wild-type plants, but this was only significant at 20 daps. The effect of high- vs low-N treatment on mutant plants was more variable.

In contrast to N, leaf carbon content changed relatively slowly as the leaves aged. Except for the last measurement (in the mutant) there was a significant (< 0.001) overall increase in carbon content in the lower N treatment (Fig. 7). There was a small decline in leaf carbon percentage over time in mutant but not in wild-type plants.

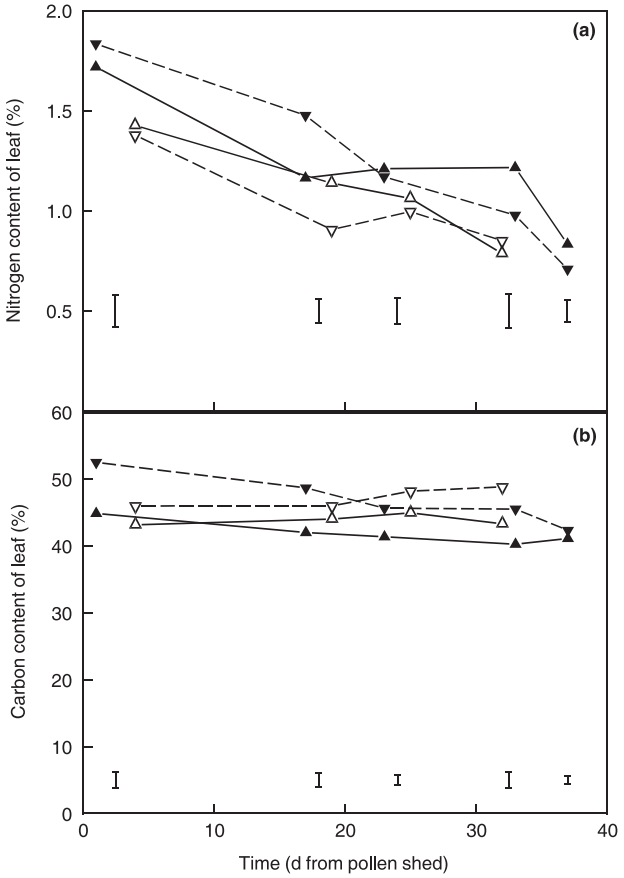


**Fig. 7** Time course of (a) nitrogen and (b) carbon content of third leaf below flag for wild-type (open symbols) and mutant (closed symbols) maize (*Zea mays*) grown at high (△, ▲) or low nitrogen (▽, ▼). Vertical bars are standard errors of difference.

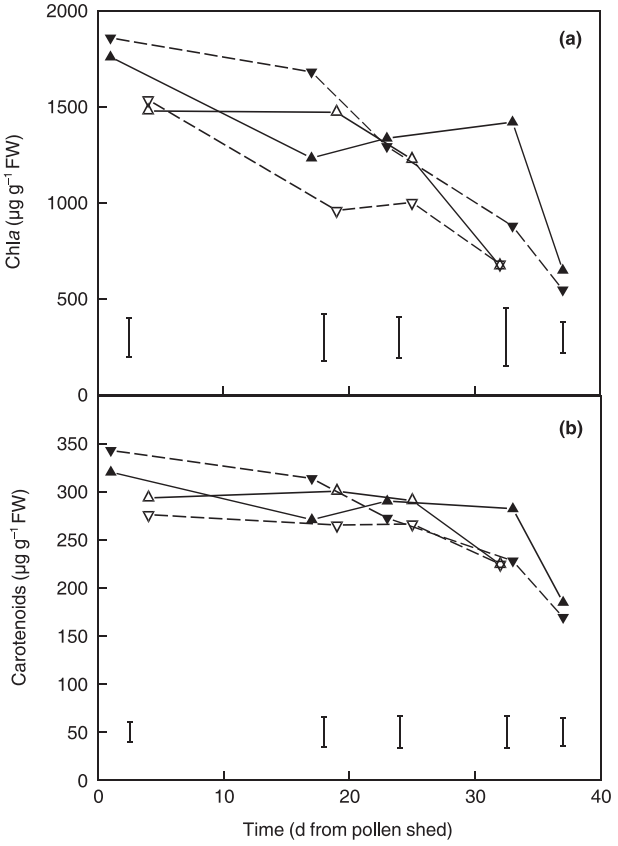


**Fig. 8** Time course of Chl*a* (a) and carotenoid (b) content of third leaf below flag for wild-type (open symbols) and mutant (closed symbols) maize (*Zea mays*) grown at high (△, ▲) or low nitrogen (▽, ▼). Vertical bars are standard errors of difference.

In Figs 7–10, the final time point shown for wild-type plants (32 daps) represents the last stage at which leaves could be harvested and subjected to meaningful analyses for chlorophyll, carotenoid and other components. Beyond this point the leaves were brown and desiccated, and began to crumble. For mutant plants it was possible to harvest leaves with some turgor and chlorophyll content up to 37 daps, and this is thus the final data point presented for these plants.

## Leaf chlorophyll and carotenoid content and chlorophyll to protein ratios

The pattern of changes in Chl*a* (Fig. 8) and Chl*b* (not shown, see later in this section) content with time paralleled changes in leaf N. Again, in wild-type plants, high-N treatment retarded the decline in chlorophyll content over the first 20 daps, but the decline thereafter was rapid and by 32 daps (the last point at which harvesting of viable leaves was possible), the leaves had similar chlorophyll contents regardless of earlier N treatment. In contrast, the greatest effect of N treatment upon mutant leaf chlorophyll content was seen at 33 daps, at which point high-N-treated mutant plants had a leaf Chl*a* content > 50% greater than that of low-N-treated mutant plants and almost twice that of wild-type plants. Towards the end of the life of the leaf (32 daps for wild-type, 37 daps for mutant), Chl*a* and Chl*b* contents in both genotypes and both treatments were similar, at approx. 600 and 100 µg $g^{-1}$ FW, respectively. The Chl*a* : Chl*b* ratio did not change significantly throughout the period of the experiment, remaining at approx. 6.6 : 1 (SEM 0.13) for both genotypes and both N treatments, so the data are not presented and the amounts of Chl*b* are not presented separately.

Total carotenoid content expressed on a FW basis was initially slightly higher in mutant than in wild-type leaves, but it was otherwise similar in both genotypes at both N amounts, except that mutant plants treated with high N retained carotenoids for longer than low-N mutant plants, eventually declining to *c.* 50% of initial values by the stage of the final harvest (Fig. 8).

Chlorophyll to protein ratios did not differ between nitrogen amounts within a genotype ($P > 0.43$), so the averages for genotypes are presented in Fig. 9, which demonstrates the

 

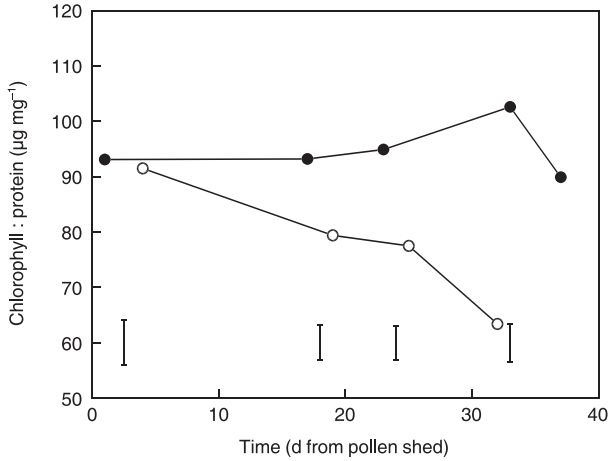


**Fig. 9** Time course of ratio of total chlorophyll to protein for third leaf below flag averaged over both nitrogen treatments for wild-type (open circles) and mutant (closed circles) maize (*Zea mays*). Vertical bars are standard errors of difference.

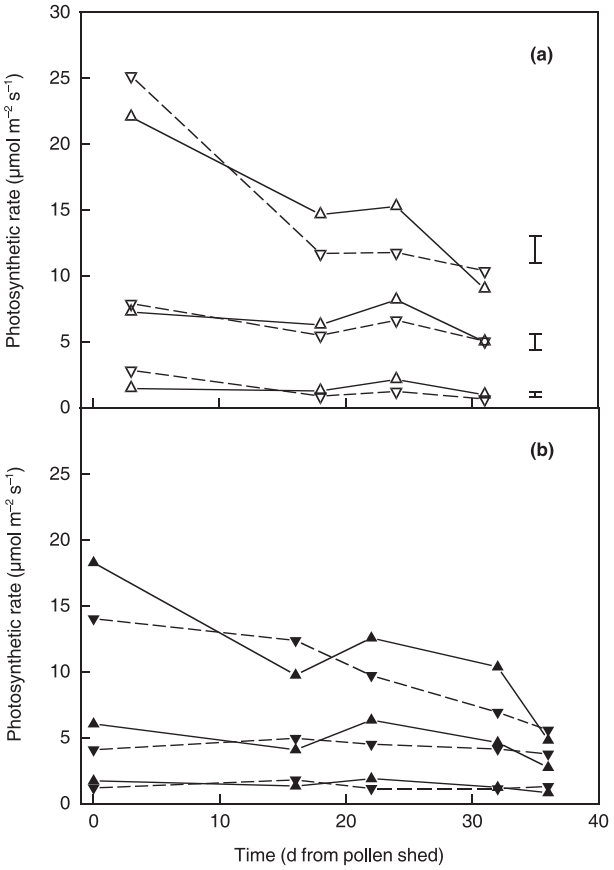


**Fig. 10** Time course of photosynthetic rate for average of third and fifth leaves below flag (except first measurement, average of flag and five leaves below it) for wild-type (open symbols) (a) and mutant (closed symbols) (b) maize (*Zea mays*) grown at high (△, ▲) or low nitrogen (▽, ▼). Top, middle and bottom pairs of lines measured at a PPFD of 1156, 155 and 52 µmol m$^{-2}$ s$^{-1}$, respectively. Vertical bars are standard errors of difference, which apply to both panels.

relative stability in the ratio for the mutant compared with a continual decline in the wild-type.

### Photosynthesis

Photosynthetic rates at the higher light intensity showed the expected decline with time ($P < 0.001$, Fig. 10) in parallel with the decline of leaf N content with age (Fig. 7). Except at the lowest light intensity reported, there was also a lower ($P < 0.001$) rate of photosynthesis in the mutant. Significantly higher photosynthetic rates were not seen in response to increased N applied within the wild-type or the mutant, as expected from the largely similar leaf N contents across N treatments. A supplementary investigation (data not shown) concluded that the amount of mineralization occurring in the soil-based medium was high and there was also some nitrogen available in the loam-based growing medium. Thus, the effects of increasing N applied to the growing medium were not sufficient to change leaf N content and would not be expected to change photosynthesis. As reported earlier in this paper, there was a delay in chlorophyll breakdown in the mutant, and as a result photosynthetic measurements were continued for longer. No enhancement in photosynthetic rate was seen in the mutant in the latter stages of senescence, apart from a very low rate at the final measurement after the leaves on the control had died. Overall, no major photosynthetic rate advantages were seen in the mutant, although the greater area of the upper leaves would probably enhance net assimilation during grain filling.

## Discussion

Sequence comparisons indicate that the two *See2* genes of maize encode cysteine proteases of the legumain/haemoglobinase group. This class of proteases includes vacuolar processing enzymes (VPEs) expressed in maturing seeds of castor bean (Hara-Nishimura *et al.*, 1991, 1993), soybean (Shimada *et al.*, 1994) and *Arabidopsis thaliana* (Kinoshita *et al.*, 1995); germinating seedlings of castor bean, soybean and pumpkin (Hiraiwa *et al.*, 1993) and vetch (Becker *et al.*, 1995); maturing and germinating seeds of rice (Kato *et al.*, 2003); ripening fruit of *Citrus* (Alonso & Granell, 1995); nucellus of barley grains (Linnestad *et al.*, 1998); and haemoglobinase in human (Tanaka *et al.*, 1996) and human parasites (Klinkert *et al.*, 1989; el Meanawy *et al.*, 1990; Merckelbach *et al.*, 1994).

From the cDNA sequence, the predicted amino acid sequence of *See2* is of a similar length to other legumains. Both *See2*$\alpha$ and *See2*$\beta$ have a putative signal sequence and one of two potential glycosylation sites described in Citvac (Alonso & Granell, 1995) is also conserved in both *See2*s. The two *See2*s map to homoeologous maize chromosomes and,

given that the two maize genome ancestors diverged some 20 million yr ago (Gaut & Doebley, 1997), the predicted protein sequences of the two copies of *See2* are remarkably well conserved, suggesting functionality is maintained in both copies. The genomic DNA clones of *See2α* and *See2β* were sequenced and compared (Table 1). Intron 1, which is the most highly diverged intron, also contains a TATA motif 55 bp upstream of the start of exon 2 in *See2α* and 200 bp upstream of the start of exon 2 in *See2β*. Moreover, cDNAs were cloned which lacked exon 1 and contained sequence from the 3′ of intron 1 at the 5′ end but lacked any other intron sequences (Fig. 3c). Both *See2α* and *See2β* contain a methionine which is coded 15 bp from the start of exon 2; this is unique to maize and rice legumains. In other words, it appears that alternative RNA and also protein could be transcribed and translated, respectively, from both *See2* genes in maize. However, *See2* exon 1 contains the cysteine active site of maize legumain and therefore any alternative gene product with a transcription start site within intron 1 could not function as a cysteine protease. If such transcripts were translated, a truncated protein would lack both the signal peptide sequence and cysteine active site, and therefore would not be targeted to any organelle or be capable of functioning as a legumain. However, such a truncated protein might have the effect of diluting any regulatory factor which binds to full-length SEE2.

A palindromic 212 bp fragment within intron 2 of *See2α* (but not *See2β*), the perfect terminal inverted repeats, and its occurrence in other diverse maize genes all suggest that the DNA fragment or a related sequence is mobile. However, a genomic Southern analysis, under conditions of moderate stringency, using the 212 bp fragment as probe, did not indicate that this sequence was highly abundant in the maize genome (data not shown). The additional DNA fragment in *See2α* comprises a long inverted repeat with potential for a stable secondary structure to occur close to the intron 2/exon 3 splice site. One *See2α* cDNA was identified which contained a partial deletion of exon 3: it is possible that the generation of a secondary structure within intron 2 perturbed normal RNA splicing. In a previous study in maize, transposable element insertions near the splice junctions were found to have a more severe effect on splicing than those in the centre of the intron (Luehrsen & Walbot, 1992). However, the insertion into the *See2α* intron 2 is presumably not highly disruptive since full-length cDNAs were identified more often than those containing a deletion. Nevertheless, a short (102 bp) inverted repeat identified in the *See2α* exon 1 may have been another MITE which inserted into the 5′ coding region of the gene. This region is the most divergent between *See2* and other legumain genes and codes for the signal peptide and the N-terminal pro-region. The insertion could possibly have been tolerated because it was not in the region coding for the mature protein. The occurrence of retrotransposon sequence outside the gene sequence and a putative MITE within an intron is in agreement with the large-scale sequence data from maize of Tikhonov *et al.* (1999).

The 40 kDa antigen detected by antibody to a recombinant *Vicia sativa* legumain is smaller than the predicted size of 53 kDa based on the deduced amino acid sequence of the two *See2* cDNAs (Fig. 4). The predicted precursor proteins of other legumains are of a similar size: 53 kDa in jack bean (Takeda *et al.*, 1994), 54 kDa in *Citrus* (Alonso & Granell, 1995), 54 kDa in *Arabidopsis thaliana* (Kinoshita *et al.* (1995) and 55 kDa in soybean (Shimada *et al.*, 1994). However, the size of the *See2* protein on the basis of SDS-PAGE and western blotting compares favourably to other legumains, including vetch (38 kDa) (Shutov *et al.*, 1982), soybean (33 or 39 kDa) (Muramatsu & Fukazawa, 1993; Shimada *et al.*, 1994), jack bean (37 kDa) (Abe *et al.*, 1993), castor bean (37 kDa) (Hara-Nishimura *et al.*, 1993; Hiraiwa *et al.*, 1999) and *Vicia sativa* (35–40 kDa) (Becker *et al.*, 1995), which indicates that the protein normally detectable in maize and these other species has already been processed. The 40 kDa protein is scarce in young leaves, and increases in concentration through senescence (Fig. 4).

*See2* was cloned, from both a differential screen (Smart *et al.*, 1995) and subsequently a cDNA subtraction, as a leaf senescence up-regulated message, and this senescence-enhanced expression was confirmed by northern analysis (Fig. 1a). However, plant legumains have primarily been associated with maturing and germinating seeds, although a similarity between leaf senescence and seed germination has already been reported for another maize protease, *See1* (Griffiths *et al.*, 1997). Since salvage of resources, particularly protein, is characteristic of both stages of development, germination and senescence-related gene expression may be expected to have common components. However, *See2* and other legumains are probably not directly involved in bulk remobilization of protein N but rather in the post-translational modification of other proteins targeted to the vacuole. For example, castor bean legumain is capable of processing a broad range of molecular structures into mature protein (Hara-Nishimura *et al.*, 1991, 1995). Studies of the bond specificities of isolated legumains show cleavage on the C-terminal side of an exposed asparagine residue in the substrate (Hara-Nishimura *et al.*, 1993, 1995). There is also a report that the legumain of vetch may act on aspartate in addition to asparagine (Becker *et al.*, 1995). The seed-specific asparaginyl endopeptidase REP-2 of rice has been shown to act as an activator of REP-1, the other seed-specific cysteine endopeptidase which is responsible for digestion of rice seed storage protein (Kato *et al.*, 2003). On the basis of DNA and amino acid homology, it is predicted that *See2* has a role in maize leaf senescence through the post-translational modification and activation of proteins, including other degradative enzymes, stored in the vacuole. In animal systems, programmed cell death is characterized by a complex of proteases that form part of a cascade in which each protease activates the next protease in the sequence by

 

enzymatic cleavage of a zymogen (Solary *et al.*, 1998). A comparable process might be predicted to occur at the end of leaf senescence (Thomas & Donnison, 2000). A subset of legumain-like proteases, including the senescence-enhanced *See2* of maize (Smart *et al.*, 1995), the ripening up-regulated Citvac of *Citrus* (Alonso & Granell, 1995), the root nodule-specific and senescence-associated *GmCysP1* of soybean (Oh *et al.*, 2004) and the VPEs of *Arabidopsis* (Hara-Nishimura, 1998), have been identified in senescing plant tissue and might perform such a role.

A *Mu* insertion was identified in the first intron of *See2β* using a reverse genetics screen based on PCR. After backcrossing and selfing, plants of this putative mutant genotype were characterized both developmentally and physiologically. Insertion of *Mu* into introns of Adh (Rowland & Strommer, 1985) and $GS_{1\text{-}4}$ (K.J. Edwards & S. Haines, unpublished) have previously been described which give rise to identifiable phenotypes. Since *See2β* expression appears down-regulated in the mutant plant, and given the large number of plants characterized, there is good reason to believe that the phenotype we have observed is directly attributable to the insertion in *See2β*. On one occasion we have observed a double *See2α* and *See2β* mutant plant which exhibited near wild-type amounts of *See2* transcript (data not shown); however, we attribute this to silencing of one of the *Mu* elements that had inserted in the *See2α* intron and the *See2β* intron. This phenomenon has been identified previously and possible mechanisms discussed by other authors (Walbot & Stapleton, 1998; Lisch *et al.*, 2002; Slotkin *et al.*, 2003). Given the existence of *See2α* and *See2β* genes on homoeologous chromosomes, it might be expected that *See2α* would complement the *See2β* mutation. However, RT-PCR results indicate a different pattern of expression of the two genes during senescence. Moreover, a *Mu* insertion in the *See2α* gene did not appear to result in a visible phenotype and double mutants did not appear different from *See2β* mutant plants (I. S. Donnison *et al.*, unpublished).

The senescence of the mutant leaves followed a similar pattern to the senescence of wild-type leaves. At the latest sampling points, however, mutant plants contained more chlorophyll than wild-type plants and there was an extension, albeit at a low rate, in photosynthesis beyond the time at which wild-type leaves died. Thus the mutant might be described as exhibiting a delayed senescence phenotype. This view was reinforced by the much more constant chlorophyll to protein ratio in the leaves of the mutant when compared with the wild-type (Fig. 9), which suggests that in the mutant, a type of stay-green in which protein and chlorophyll degradation proceed much more in parallel than in the wild-type has been produced. Although chlorophyll catabolism can be regarded as a visible symptom of protein mobilization (Thomas *et al.*, 2002), it normally proceeds proportionately more rapidly. There were additional similarities to other classes of delayed senescence plants; for example, the reduced stem weight seen in the mutant plants was analogous to the reduced stem height of transgenic *See1* promoter-ipt plants (Robson *et al.*, 2004), in which overexpression of a gene encoding a cytokinin biosynthesis enzyme resulted in retardation of leaf senescence. The earlier tassel emergence in the mutant suggests an earlier switch to reproductive growth and also explains the lower plant weights of mutant plants. An earlier-flowering and delayed-senescence phenotype, including reduced stem height, has also been observed in T0 hybrid maize plants transformed with an *See2* short-sense construct (I. S. Donnison & H. Thomas, unpublished). In maize, stem is an important sink for plant resources. It is possible that the increased allocation of nutrient to cobs in the mutant may be due not only to the delay in leaf senescence and N mobilization, but also to the reduced sink force exercised by the stem. The effect of *See2* and other mutations on stems warrants further study, and it should be noted that the relationship between stay-green and stem source–sink behaviour is complex, since in contrast to the examples above, Pommel *et al.* (2006) found that depletion of stem assimilates during late grain fill occurred more rapidly in a normally senescing maize variety than in a stay-green variety.

A delayed senescence or stay-green genotype, in which N reallocation from older senescing leaves is compromised, might be expected to adjust its internal N economy to compensate, especially under N-limiting conditions. In our study we observed that there was an altered allocation of resources within leaves to produce greater area for a given weight and N content per unit weight in the mutant under a given N treatment, although the N content per unit area was similar in wild-type and mutant. This increased leaf area was at the expense of a lower maximum rate of photosynthesis per unit leaf area in high light, resulting in little difference in photosynthetic capacity per leaf. He *et al.* (2003) also reported a lower photon-saturated photosynthetic rate in a stay-green maize line in comparison with a wild-type line, and concluded that high yield was attained by longer green area duration and not by a high maximum rate of photosynthesis. The success of this strategy can be seen under N-limited conditions, where the wild-type plants had lower yields. Although the cob weight was slightly lower in the mutant under the high-N treatment, it was scarcely reduced under N limitation, whereas cob weight in the wild-type fell to *c.* 25% of the high-N value (Table 2). Similarly, cob N content was much less affected in the mutant than in the wild-type by N limitation, to the extent that under low N the cob represented almost half the total DW and over 65% of the total N in the above-ground plant parts, whereas the wild-type cob under the same conditions made up only *c.* 10% of the DW and 20% of the total N. These effects occurred in spite of the relatively small differences between N additions used here in the context of the relatively large contributions to total N uptake from the soil and mineralization. It may indicate behaviour that is less clearly visible in the more usual experimentation carried out

over a wide range of N treatments and it would be interesting to test this in further experimentation. The yield advantages of this type of stay-green may have considerable agronomic value when maize is grown under conditions of reduced N availability, including that caused by drought. It may, like some other stay-green maize types (Ma & Dwyer, 1998), also have the advantage of reduced fertilizer requirements by virtue of its higher N use efficiency. In other words, although this mutation may be defined as a stay-green phenotype, the most dramatic effects were on flowering time and N distribution/allocation. Earlier flowering might be predicted to engage the senescence programme earlier in mutant plants, whereas these plants exhibited delayed senescence. Therefore, senescence in these plants probably started sooner and finished later and may explain the improved N reallocation to the cob. This increase in N use efficiency could make a contribution to an increase in sustainability under marginal environmental conditions.

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