Plant Molecular Biology **34:** 815–821, 1997.

Short communication

Sequencing, expression pattern and RFLP mapping of a senescence-enhanced cDNA from *Zea mays* with high homology to oryzain γ and aleurain

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Received 29 July 1996; accepted in revised form 22 April 1997

Key words: cysteine proteinase, gene expression, leaf senescence, *Zea mays*

Abstract

Sequence analysis of a 1.4 kb clone from a cDNA library of senescing *Zea mays* leaves reveals an open reading frame for a 360 amino acid protein. Both the DNA and deduced amino acid sequences are highly homologous to the cysteine proteinases oryzain γ and aleurain. Northern analysis demonstrates that the corresponding RNA level increases during natural leaf senescence, seedling germination and in chilling of tolerant maize lines, but decreases in a sensitive line. The mRNA level also decreases in regreening leaves, in dark-induced senescence and in nutrient or water stress. Southern and RFLP analysis provide evidence that the gene has two copies, on chromosomes 2 and 7.

Leaf senescence is the final stage of development, during which genetically controlled degradation and remobilisation of cell components takes place. Senescence-enhanced cDNAs have been isolated from a range of species including tomato [5, 7], radish [1, 14, 21], cucumber [8], *Arabidopsis* [11, 15, 17, 19], *Brassica* [3, 10], *Petunia* [20], barley [2, 12] and maize [18]. The identity of the cloned sequences may be inferred from their similarity to known sequences in some cases. For example, cysteine proteinases have been found in *Arabidopsis* (SAG2 and SAG12, [11, 15]), *Petunia* (P21 [20]), maize (*See1* and *See2*, [18]) and tomato (SENU2 and SENU3, [7]). This paper presents further characterisation of *See1*, including detailed sequence analysis, mRNA expression pattern in natural senescence, germination, chilling, regreening, dark-induced senescence and nutrient or water stress, gene copy number and chromosome localisation by RFLP mapping.

The senescence-enhanced cDNA *See1* was isolated by differential screening of a λgt10 cDNA library from early senescing maize leaves 12–20 days after pollen shed (DAPS) with probes made from cDNA of 0 DAPS and 16 DAPS [18]. Complete DNA sequencing of See1 revealed an open reading frame capable of encoding a polypeptide of 360 amino acids, with a molecular mass of 39 kDa (Figure 1). Both the DNA sequence and the deduced amino acid sequence show high levels of homology to cysteine proteinases of the oryzain γ/aleurain family, but much less similarity to other rice or maize cysteine proteinases, such as oryzains α and β, and CCP1 (Figure 1, Table 1 and Figure 2). Other proteinase cDNAs are included in the sequence comparison of Table 1 and the graphical representation in Figure 2 because they have also been shown to be upregulated during leaf senescence. There are a large number of families of cysteine proteinases. Table 1

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X99936 (ZMSEE1).

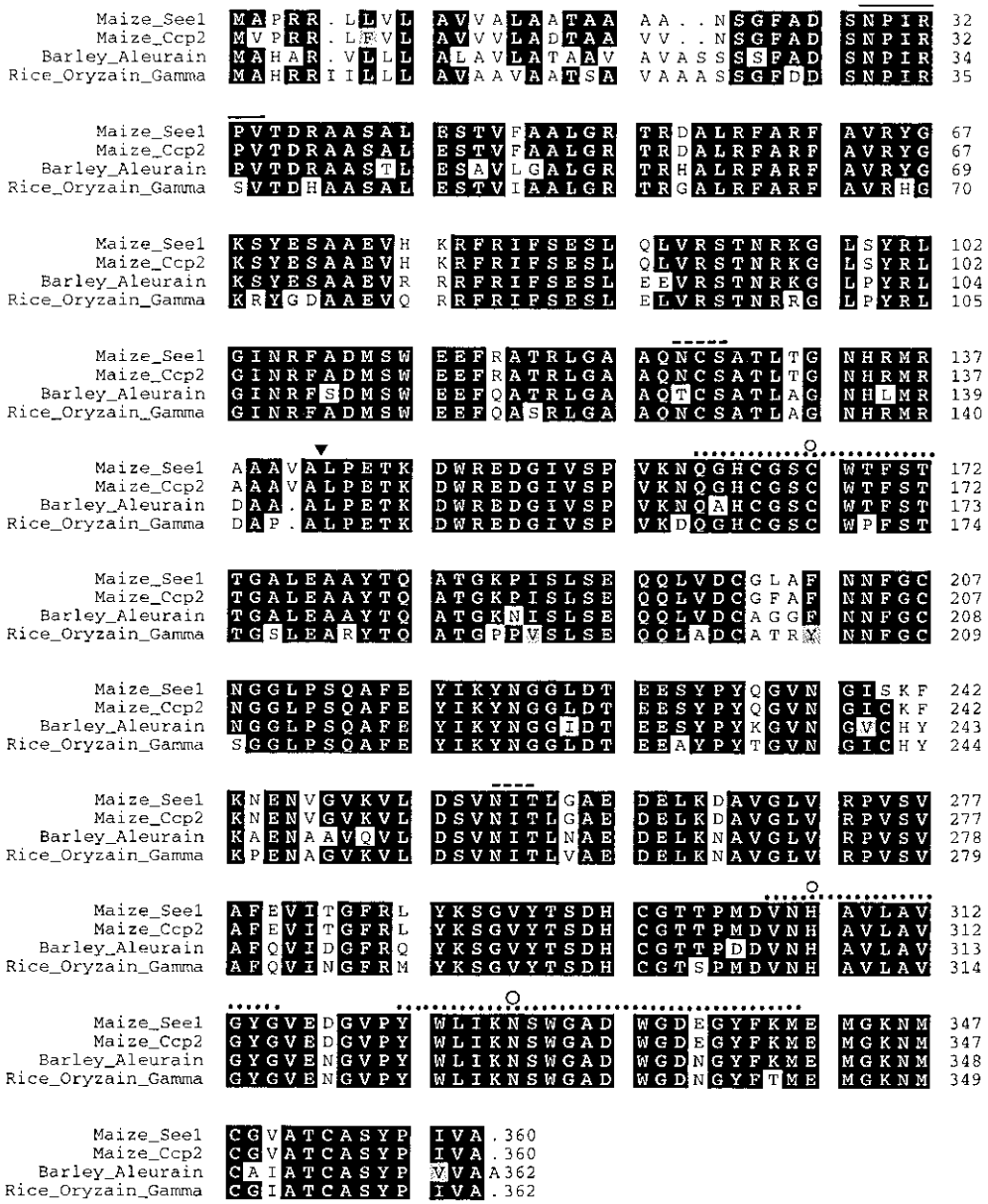

Figure 1. Sequence alignment of the predicted See1 polypeptide with other sequences of the same family of cysteine proteinases. There is a solid black line above the putative vacuole-sorting signal sequence and a dashed line above two potential glycosylation sites. The solid triangle indicates the likely site of cleavage to generate the mature protein. Conserved residues around the cysteine, histidine and asparagine triad (open circles) of this family of cysteine proteinases are shown by the dotted lines. The proteins were aligned using the PILEUP program of the Wisconsin Genetics Computer Group software package and are displayed using PRETTYBOX. Black shading shows identical amino acids while grey shading indicates conservative substitutions.

and Figure 2 show clearly that the senescence-related tomato and *Petunia* sequences belong to the same family as *See1*, and are thus more similar to *See1* than other monocot cysteine proteinases, such as oryzain α and β. Senescence-related proteinases more similar to oryzains α and β do exist, as the position of the *Arabidopsis* SAG12 sequence in Figure 2 illustrates. The maize CCP1 proteinase, which is upregulated during germination, is a member of yet another cysteine proteinase family.

Table 1. DNA and protein sequence homologies between cysteine proteinases expressed in leaf senescence and/or germinating seeds. The figures in parenthesis show the length of DNA sequence homology in base pairs or the length of protein sequence homology in amino acids. The relevant references are in square brackets.

Protein sequence homology (%)	DNA sequence homology (%)									
	Maize See1	Maize CCP2	Barley aleurain	Rice oryzain γ	Tomato cyp-3	Petunia P21	Rice oryzain α	Rice oryzain β	*Arabidopsis* SAG12	Maize CCP1
Maize See1 [18]		99.3 (1379)	83.4 (1075)	78.8 (1417)	69.0 (1082)	69.0 (1058)	57.5 (1114)	56.4 (1114)	57.6 (688)	52.9 (978)
Maize CCP2 [6]	97.8 (360)		83.5 (1086)	80.0 (1360)	69.3 (1083)	68.8 (1076)	57.5 (1111)	56.6 (1104)	57.7 (688)	52.8 (976)
Barley aleurain [24]	85.6 (355)	84.0 (362)		84.3 (1085)	66.0 (1053)	66.5 (1066)	57.5 (1070)	58.0 (1114)	54.8 (673)	55.3 (595)
Rice oryzain γ [22]	83.7 (363)	82.6 (363)	82.9 (362)		67.4 (1061)	67.1 (1084)	56.9 (1112)	57.4 (1111)	56.3 (679)	52.3 (970)
Tomato cyp-3[7]	70.3 (360)	70.0 (360)	66.8 (358)	65.9 (358)		80.3 (1358)	55.3 (900)	54.9 (882)	56.8 (678)	63.2 (185)
Petunia P21 [20]	71.1 (360)	70.6 (360)	70.7 (358)	67.3 (358)	83.8 (358)		55.5 (681)	57.4 (665)	56.9 (677)	63.8 (185)
Rice oryzain α [22]	40.1 (332)	40.4 (332)	42.8 (311)	41.8 (311)	42.8 (311)	42.1 (311)		68.5 (1140)	56.3 (924)	56.7 (134)
Rice oryzain β [22]	41.9 (332)	42.2 (332)	43.1 (332)	42.4 (340)	43.8 (292)	43.5 (292)	62.4 (460)		61.6 (656)	56.3 (606)
Arabidopsis SAG12 [15]	37.2 (317)	37.5 (317)	37.5 (317)	36.9 (317)	40.6 (310)	41.3 (310)	47.6 (347)	49.7 (310)		57.4 (223)
Maize CCP1 [6]	38.1 (318)	38.4 (318)	37.1 (310)	35.2 (318)	35.9 (370)	36.1 (368)	37.0 (324)	36.7 (365)	37.7 (326)	

The *See1* DNA and protein sequences are very similar to a cysteine proteinase designated CCP2, which was isolated from germinating maize seeds [6]. There is an obvious similarity between events in seed germination and leaf senescence, as both developmental stages include remobilisation of stored reserves on a large scale.

Detailed comparison of See1 with the maize cDNA CCP2 [6] and the rice cDNA for oryzain γ [22] reveals that the deduced amino acid sequences share features such as a putative vacuole-sorting signal sequence ^{29}NPIRPV ([4], solid line above sequence in Figure 1), two potential glycosylation sites, ^{125}NCS and ^{256}NIT (dashed lines above sequence in Figure 1) and the catalytic cysteine-histidine-asparagine triad, ^{167}C-^{307}H-^{327}N (open circles), with the surrounding conserved regions (dotted lines above sequence in Figure 1). Further evidence for the identity of the N-terminal portion of the protein sequence comes from the observation that oryzains α, β and γ share with *See1* the characteristics of a signal sequence. These have been defined by Watson [23], using the example of zein, as a charged residue within the first five amino acids (^{4}R and ^{5}R in *See1*), followed by a core of at least nine hydrophobic residues (residues 6–16 in *See1*). In addition, the start of the mature protein is predicted to be at ^{145}L in oryzain γ, by sequence similarity to the known amino terminus of mature oryzain α, and this corresponds to ^{143}L in the two maize sequences (see closed triangle in Figure 1).

It is interesting to note that there are nine nucleotide differences within the open reading frame between

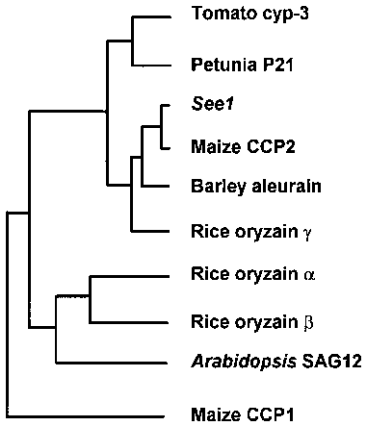

Figure 2. Dendrogram illustrating the degree of sequence similarity between different cysteine proteinases expressed in leaf senescence and/or germinating seeds (for references, see Table 1).

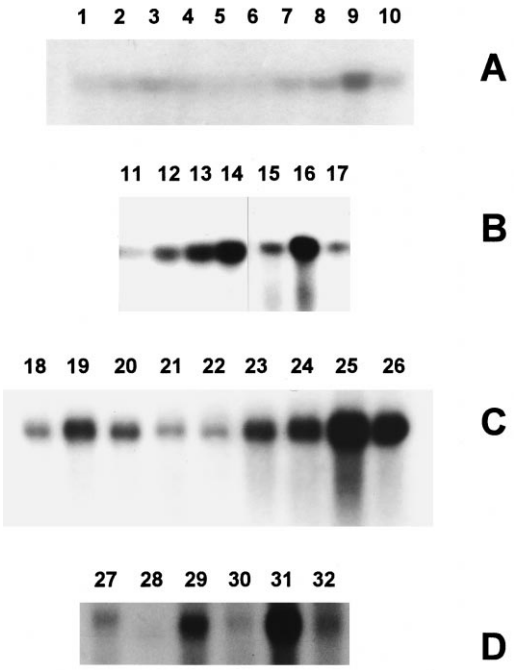

Figure 3. Northern analysis using RNA from maize leaves and germinating seeds. RNA was extracted using a standard phenol extraction/LiCl precipitation method [16] and each lane contained 10 μg of total RNA which was hybridised at high stringency to the cDNA probe *See1*. A. Natural senescence of mature leaves. Lanes 1–10 contain RNA extracted at 5-day intervals from the ear leaves of mature LS plants -5 to $+40$ days after pollen shed. B. Natural seedling senescence and germinating seeds. Lanes 11–14: RNA extracted from LS seedling leaves 6, 5, 4 and 3 at a single date of harvesting, when leaf 3 was visibly yellowing while leaf 6 was still green. Lanes 15–17: RNA extracted from LS maize seeds 1, 3 and 5 days after germination. C. Chilling of mature and seedling leaves and regreening. Lanes 18–19: RNA from the ear leaf of mature CT plants before and after 13 days of severe chilling. Lanes 20–21: RNA from the ear leaf of mature CS plants before and after 13 days of severe chilling. Lanes 22–24: RNA from the third leaf of LS seedlings before chilling, after 8 days of mild chilling and 12 days of severe chilling. Lanes 25–26: RNA from leaf 7 (yellow) and leaf 8 (regreening) of a mature maize plant after pollen shed. D. Dark-induced senescence and more seedling stress treatments. Lanes 27–28: RNA from a single fully-expanded leaf of a mature maize plant before and 5 days after detaching, cutting into 1 cm squares and incubating the leaf material on moist filter paper at 30 °C in the dark. Lanes 29–32: RNA from leaf 3 of LS seedlings grown for 20 days at 25 °C day/22 °C night (16 h day), in a cabinet with a high light level ($>$ 900 μmol m^{-2} s^{-1}). The growth media and daily watering solutions were sand/full-strength Hoagland's solution (lane 29), sand/deionised water (nutrient stress, lane 30), soil/tap water (lane 31) and soil/no water for last 8 days of experiment (lane 32).

See1 and CCP2, eight of which result in amino acid changes but only two of which are not conservative (A or D at position 16, S or C at position 240). Intriguingly, six of the nine amino acid changes are within the signal sequence region. It is difficult to predict whether such differences are due to variation between genotypes, developmental stages (germination versus senescence), alleles of the same gene on different chromosomes or sequencing. Inaccuracies of sequencing are the least likely explanation as each position was sequenced on average six times in each direction. The fact that five of the six changes in the signal sequence are conservative strongly suggests that there is no difference in function between the corresponding regions of *See1* and CCP2. It is striking that greater variation occurs between all the sequences compared in Figure 1 within the signal sequence than anywhere else throughout the sequence.

The transcript level of *See1* in maize has been studied using total RNA extracted from leaves of several genotypes under a variety of conditions. Naturally-senescing leaves of mature LS (later senescence) plants have been studied at a range of times from -5 to $+40$ DAPS [18]. A peak in the level of the 1.4 kb message is obvious at 35 DAPS (Figure 3A), ca. 10 days after the start of visible senescence as measured by a major drop in relative chlorophyll content. More *See1* mRNA is also found in older seedling leaves. Figure 3B shows the level of *See1* mRNA in leaves 3–6 of LS seedlings at a single date of harvesting, when leaf 3 was visibly yellow while leaf 6 was still completely green.

The sequencing information presented earlier showed that See1 had a high degree of similarity to cysteine proteinases expressed in germinating seeds (maize CCP2, [6]; rice oryzain γ, [22]). Therefore, the pattern of expression of See1 was checked using RNA extracted from LS seedlings 1, 3 and 5 days after ger-

mination (Figure 3B, lanes 15–17). The message level peaks at 3 days after germination (lane 16), a result which agrees with that reported by Domoto *et al.* [6].

The pattern of mRNA accumulation has also been studied in senescence of the third leaf of LS seedlings during mild and more severe chilling (Figure 3C, lanes 22–24). Such conditions occur naturally in the spring (for seedlings) and autumn (for mature plants) when maize is grown in Northern Europe. The mild 8-day chilling treatment of 13-day-old LS seedlings at 14–16 °C day/6 °C night (12 h day, PAR 1000 μmol m^{-2} s^{-1}) causes the normal senescence symptom of yellowing. These conditions produce an increase in See1 message level (Figure 3C, lanes 22 and 23), similar to that seen in natural senescence of both mature and seedling leaves.

In the severe chilling experiment, 20-day-old LS seedlings were transferred to a temperature regime of 10 °C day/5 °C night (14 h day, PAR $>$ 750 μmol m^{-2} s^{-1}) for 12 days. The effect of this more severe chilling treatment was to slow the growth of the seedlings compared with warm-grown control seedlings and to cause some photodamage and drying, particularly along the margins of the leaves. Nevertheless, the LS genotype is classified as being fairly tolerant of severe chilling and the level of See1 mRNA is as high under these cold conditions as in the mild chilling treatment (Figure 3C, lanes 23–24).

The level of *See1* mRNA was also investigated in mature maize plants of two additional genotypes, CT (chilling-tolerant) and CS (chilling-sensitive), which are more and less chilling-tolerant, respectively, than LS. At 15 days after pollen shed, mature CT and CS plants were subjected to the same severe chilling regime as the LS seedlings. After 13 days of cold, an increase in *See1* mRNA was detectable in the CT line, while a decrease was observed in the CS line (Figure 3C, lanes 18–21). It appears that in the severe chilling treatment, an increase in *See1* mRNA level is correlated with chilling tolerance.

The pronounced greyish-white lesions and brown patches of chilling-sensitive plants such as CS appear to resemble visually the hypersensitive response to pathogen attack (e.g. [9]), more than natural senescence. The hypersensitive response is a form of plant programmed cell death [13]. The data presented on *See1* mRNA levels in the maize line CS provide evidence that the *See1* gene, which is up-regulated in natural senescence, is down-regulated when more sudden cell death occurs.

To investigate what would happen to *See1* mRNA expression when a mature maize leaf regreened, a mature maize plant was selected one week after pollen shed and the lowest living yellow leaf (leaf 7) was harvested for RNA extraction. The stem of the plant was then severed above leaf 9 and the plant was left for 14 days. By this time, leaves 8 and 9 of other plants of identical age next to the detopped plant had died completely, but leaf 8 of the detopped plant had a relative chlorophyll content 37% higher than at the start of the experiment. Leaf 8 was harvested and total RNA was extracted from leaves 7 and 8. It can be seen from Figure 3C (lanes 25–26) that the level of *See1* mRNA was lower in the leaf which had regreened than in the yellowing leaf, despite the fact that the greener leaf was older.

To answer the question of how *See1* would be expressed in detached, dark-induced leaf senescence, mature green leaves were cut into 1 cm squares and incubated on damp filter paper at 30 °C in the dark for 5 days. After this treatment, the leaf segments were visibly yellowing, especially at the cut margins. RNA was extracted from the leaves before and after the incubation and lanes 27–28 of Figure 3D show that the level of *See1* was considerably lower in the leaf sample induced to senesce artificially than in the green leaf. This experiment provides further evidence that gene expression during dark-induced senescence differs from that in the natural process (see [2]).

The level of *See1* mRNA was also studied in LS seedlings subjected to nutrient or water stress under high light conditions (Figure 3D, lanes 29–32). Six seeds per 15 cm diameter pot were germinated, grown for 20 days at a temperature of 25 °C day/22 °C night (16 h day, PAR $>$ 900 μmol m^{-2} s^{-1}) in sand or soil and watered daily with full-strength Hoagland's solution, deionised water (nutrient stress), tap water or no water for the last 8 days (water stress). Leaves of the sand-grown seedlings given Hoagland's solution were broad and green while leaves of seedlings watered with deionised water were thinner, shorter and less green (relative chlorophyll contents for leaf 3 were 22.8 and 17.6, respectively). Soil-grown seedlings given tap water were predominantly green, with older leaves starting to turn yellow at the tip, while water-stressed seedlings had limp leaves of a grey-green colour (Relative chlorophyll contents for leaf 3 were 25.1 and 13.1, respectively). For each treatment, leaf 3 was generally the oldest living leaf of a four- or five-leaf seedling. The level of *See1* mRNA in leaf 3 of the seedlings given different treatments was much higher in

naturally-senescing seedlings (sand/Hoagland's, lane 29; soil/tap water, lane 31, Figure 3D) than in stressed seedlings (sand/deionised water, lane 30; soil/drought, lane 32, Figure 3D).

The northern analysis presented in this paper may be summarised as follows. Leaves of naturally senescing seedlings or mature maize plants contain more *See1* mRNA than younger leaves. There is also a transient peak in *See1* mRNA during seedling germination. Seedlings and mature plants of chilling-tolerant maize lines contain more *See1* mRNA in their leaves during chilling treatments than is found in chilling-sensitive maize. Regreening, detachment and dark incubation, nutrient stress and drought all result in a decrease in *See1* mRNA. Of course, it is not possible to distinguish between a decrease in *See1* mRNA level and a significant increase in other mRNAs in the population using this technique, with equal amounts of total RNA loaded in each track. However, other techniques we use, such as differential display, highlight the fact that there is a remarkable degree of similarity overall between different samples of leaf RNA. This observation leads us to be more confident that significant changes in band intensity reflect the level of *See1* mRNA, rather than major fluctuations in the rest of the mRNA population.

All stresses which have a severe effect on the plant (chilling of a chilling-sensitive line, detachment and dark treatment, extreme nutrient or water stress) cause a decrease in *See1* mRNA level, while natural senescence or tolerated stress cause an increase in *See1* mRNA. These results support the theory that a plant must be healthy to carry out the full senescence programme. A major environmental stress reduces a plant's fitness, causing an altered pattern of senescence. One difference we have found is a reduction in the amount of *See1* mRNA. If the message is translated into active protein, then less *See1* mRNA will result in less protease activity, causing reduced protein recycling and less export of metabolites to the rest of the plant. Thus the pattern of expression of genes such as *See1* may be critical in determining the tolerance of plants to sub-optimal conditions.

Southern analysis of *Zea mays* genomic DNA using *See1* as a probe and *Eco*RI or *Dra*I as restriction enzymes reveals the presence of two major bands (data not shown). RFLP mapping with *Eco*RI identifies two loci for the *See1* gene. These were mapped using 46 individuals in the T232 by CM37 recombinant inbred mapping population (supplied by Ben Burr, Brookhaven National Laboratory, USA). One locus is very tightly linked to umc5a on chromosome 2 and the other is located between npi112 and bnl15.21 on chromosome 7. The two loci are therefore designated see1a and see1b respectively.

Acknowledgements

We would like to thank Keith Edwards for providing the expertise in RFLP mapping and Ann Thomas for assistance with the northern analysis. This project has been supported by both the Biotechnology and Biological Sciences Research Council and Zeneca Seeds, under the BBSRC CWIS initiative.

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