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Cloning and characterization of tomato leaf senescence-related cDNAs

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Abstract

Senescence-related cDNA clones designated SENU1, 4, 5 (senescence up-regulated) and SEND32, 33, 34, 35 and 36 (senescence down-regulated) isolated from a tomato leaf cDNA library [9] were characterized. Southern analysis showed that SEND32 is encoded by a single-copy gene while SEND33, 34, 35, 36 and SENU1 and SENU5 are members of small gene families. DNA and protein database searches revealed that SEND32, SEND35, SENU1 and SENU5 are novel cDNAs of unknown function. SEND33 encodes ferredoxin, SEND34 encodes a photosystem II 10 kDa polypeptide and SEND36 encodes catalase. The SENU4 sequence is identical to the P6 tomato protein previously reported to be pathogenesis-related [46]. The mRNA levels of SENU1, 4 and 5 increased during leaf senescence and SENU1 and SENU5 were also expressed at high levels during leaf development and in other plant organs. The SENU4 mRNA was associated more specifically with leaf senescence, although low expression was also detected in green fruit. The mRNAs for all SEND clones decreased during tomato leaf development and senescence and all except SEND32 were expressed at low levels in other plant organs. The accumulation of mRNA homologous to SENU4 and the decrease in abundance of SEND32 provide good molecular markers for leaf senescence.

Introduction

Leaf senescence is the terminal developmental phase in the life of a plant and leads to the death of the leaves [33, 45]. Leaf yellowing caused by chlorophyll loss is the most widely used marker to define the onset of senescence, although a series of biochemical and physiological changes are associated with the process. These include degradation of proteins, nucleic acids, membranes, a decline in photosynthetic capability, disintegration of organelles and export of metabolites into the young growing regions and storage organs of the plant [33]. In tomato and some other species leaf senescence is accompanied by an increase in ethylene biosynthesis [22]. Membrane integrity and the subcellular compartmentation are maintained until the latest phase, allowing a proper cooperation between various organelles in the catabolism of leaf constituents. This dynamic, tightly regulated process is probably controlled by the nucleus [33].

The initiation of leaf senescence can be induced by a number of environmental factors such as shading, high light, temperature extremes, water stress, nutrient deficiency and pathogenic infection [39, 45]. Some developmental processes such as fruit ripening and seed development can also induce leaf senescence [34]. In the absence of induction by specific environmental and developmental factors, leaf senescence is initiated in an age-related manner [20]. Despite extensive characterization of the physiological and biochemical

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers Z75523 (SENU1), Z75524 (SENU5), Z75519 (SEND32), Z75520 (SEND33), Z75521 (SEND34), and Z75522 (SEND35)

changes associated with leaf senescence, the molecular events that control this process are still not well defined.

It has been shown that changes in transcription and translation are associated with the process of leaf senescence [4, 6, 10, 23, 24, 32, 44, 49] and levels of specific mRNAs for photosynthesis-associated genes decline during leaf senescence [1, 20, 21, 22]. Recently, senescence-related cDNA genes have been isolated and characterized from *Arabidopsis* [20, 29, 35, 43], *Brassica napus* [4, 19], carnation [26, 36], cucumber [18], maize [40], rose [16] and tomato [9]. These senescence-related genes encode proteases, nucleases, and other enzymes which are presumed to be involved in this degradative process. Very few mRNAs have been shown to be expressed specifically during senescence and there is a lack of understanding of how the process is initiated.

In this paper we report the characterization and analysis of senescence-related cDNA clones for mRNAs differentially expressed during tomato leaf senescence. These encode three up-regulated mRNAs distinct from those encoding proteases described previously [9] plus five mRNAs that are down-regulated during senescence. The expression of one specific mRNA in each class appears to provide molecular markers suitable for studying the onset of senescence.

Materials and methods

Growth of plants

Wild-type tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) plants were grown in compost in 7 cm pots without the addition of nutrient feed, under warm white fluorescent light (125 μmol m^{-2} s^{-1}) for 16 h at 24 °C and 8 h dark at 18 °C. Leaves at different stages of senescence were harvested as described previously [22] and frozen immediately in liquid nitrogen. Under these standard conditions, the senescence of the first true leaves began about 8 weeks after germination.

Induction of leaf senescence using different environmental conditions

Leaf senescence was induced in wild type plants by water stress, increased light and temperature conditions. For increased light conditions plants were grown under control conditions except that the growth room light was increased from 125 to 250 μmol m^{-2} s^{-1}. For water stress and increased temperature conditions plants were grown under control conditions for 5 weeks and then either were not watered till harvesting or the growth room temperature was increased to a day cycle of 30 °C for 16 h light and 24 °C for 8 h dark.

Construction and screening of a tomato leaf senescence cDNA library

A cDNA library was constructed from poly(A)$^+$ RNA isolated from senescent tomato leaves as described previously [9]. The cDNA library was differentially screened using radiolabelled single stranded cDNA probes synthesized from poly(A)$^+$ RNA from green and senescing tomato leaves [9]. Clones isolated after the first round of screening which exhibited differential expression were put through second and third round screens using similar probes until plaque-pure clones were isolated [9].

RNA isolation and gel blot analysis

The total RNA isolation procedure described by Wadsworth *et al.* [48] was scaled up to extract RNA from leaves, stems and roots [22]. Stages of leaf development were as described previously [9, 22]. Stem and root tissue was harvested from 8-week old plants. RNA samples were checked for integrity and equal loading on gels by ethidium bromide staining (Fig. 1D). RNA gel blot analysis was carried out as described [22].

Radiolabelled probes

Plasmid DNA isolation and modification, DNA fragments for random priming, subcloning, and agarose gel electrophoresis were performed using standard techniques [37], or by following the manufacturers' instructions (Bio 101, La Jolla, CA; Promega; Stratagene; Qiagen). All DNA probes were labelled using random primers according to the procedure described by Feinberg and Vogelstein [14].

DNA sequence analysis

The cDNA clones were sequenced according to the dideoxy-nucleotide chain termination method [38]. The nucleotide sequence was determined using a Sequenase kit (USB), or a Taq Track Deaza Sequencing kit (Promega) according to the manufacturers' instructions. Sequencing reactions were resolved on 5–7% Hydrolink Long Ranger (AT Biochem) polyac-

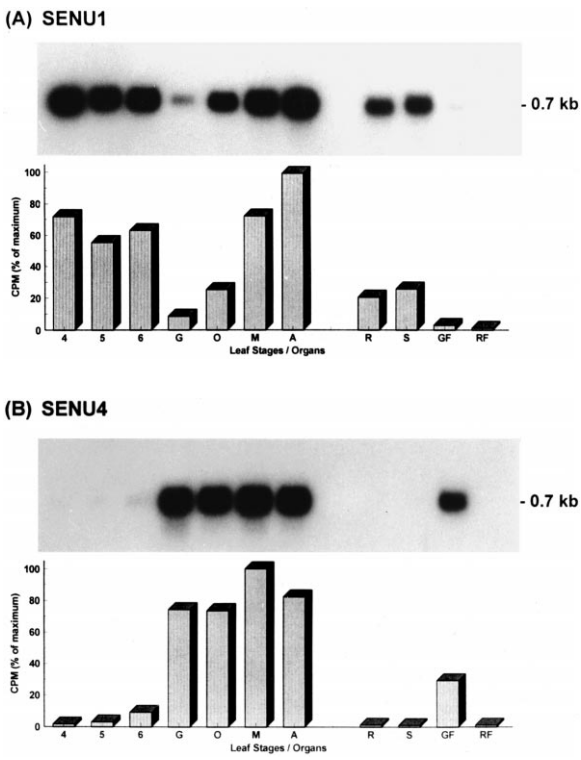

Figure 1. Expression of SENU1, 4 and 5 mRNAs during tomato leaf development, senescence and in different organs. Total RNA (10 μg) from second true tomato leaves 4-, 5-, and 6-week-old, different stages of senescence and from various organs was isolated for northern analysis (D). The blots were probed with: (A) SENU1; (B) SENU4; and (C) SENU5. The RNA samples were: 4, 4-week-old fully expanded leaves; 5, five-week-old leaves; 6, six-week-old leaves; G, 8-week-old upper green leaves; O, onset of senescence; M, mid senescence; A, advanced senescence; R, root; S, stem; GF, green fruit; RF, ripe fruit.

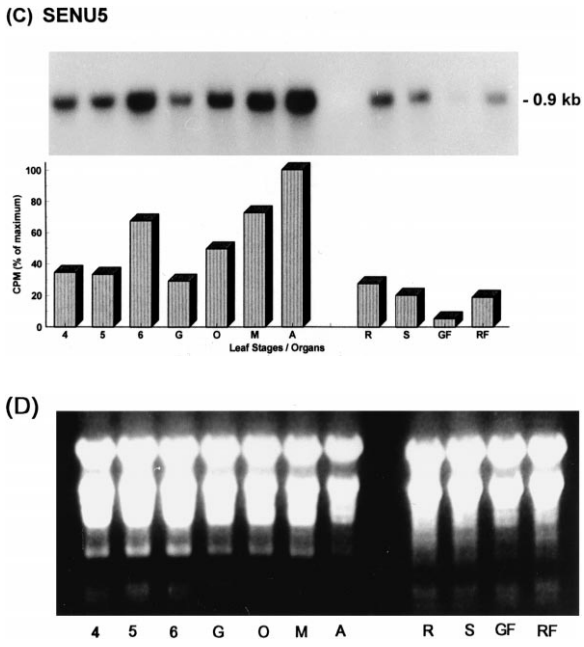

Figure 1. Continued.

rylamide gels. Nucleotide sequencing was also carried out using Applied Biosystems Model 373A DNA sequencer, version 1.2.0. The DNA sequence data were analysed with the University of Wisconson Genetics Computer Group (UWGCG) package [22].

Genomic Southern blot analysis

Genomic DNA was isolated from tomato leaves according to the method of Bernatzky *et al.* [2]. About 10 μg genomic DNA was digested, separated on a 0.8% agarose gel and transferred onto GeneScreen Plus membrane (Du Pont) by capillary blotting. Hybridization was carried out following the same procedure as described for RNA gel blot analysis [22].

Transcriptional run-on assays

Nuclei were isolated using the method described by Cox and Goldberg [5], and stored at –70 °C. Yield of genomic DNA was determined by incubating aliquots of nuclei in 0.05 M Tris-HCl pH 8.0, 25 mM EDTA, and 500 mM NaCl, with 2.5 μg/ml proteinase K at 37 °C for 1 h. The solution was phenol extracted, and genomic DNA precipitated by addition of 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. The pellet was resuspended in 50 μl H_2O and DNA concentration determined by measuring absorbance at 260 nm.

Transcription was re-initiated in aliquots of nuclei containing 20 μg DNA, by incubation in transcription buffer containing 50 mM Tris-HCl, pH 8.0, 75 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM ATP, 0.5 mM GTP, 0.5 mM CTP and 0.01 mM UTP, to which 8 Mbq ^{32}P-UTP and 200 units of RNase inhibitor (RNaseguard, Pharmacia) were added. The reaction was incubated at room temperature for 1 h. To terminate transcription, $CaCl_2$, RNase-Free DNase (Boerhinger) and proteinase K were added to final concentrations of 2 mM, 0.01 unit/μl and 0.1μg/μl respectively, and the solution was incubated at 37 °C for 30 min. An equal volume of 0.1 M Tris-HCl pH 8.0, 0.05 M EDTA, 1 M NaCl, 2% sodium sarcosine and

0.2 μg/μl proteinase K was added, and incubation continued for a further 30 min at 37 °C. The solution was then phenol/chloroform-extracted and transcripts were precipitated by addition of 2 μg of carrier RNA. Transcripts were finally resuspended in 100 μl H_2O and added to hybridization solution.

Linearized plasmid probes (10 μg) were transferred to a nylon membrane (GeneScreen Plus, Du Pont) using a Hybri-Slot manifold (BRL Life Technologies). Hybridization was carried out in 5× SSPE, 5× Denhardt's solution, 1% SDS and 200 μg/ml of salmon sperm DNA, at 65 °C for 16 h. The membrane was washed in 2× SSPE, 0.5× SSPE and 0.2× SSPE successively, at 65 °C for 30 min.

Results

Senescence up-regulated clones

In this paper we describe three cDNA clones (SENU1, 4 and 5) for senescence up-regulated mRNAs. The SENU1 cDNA clone is 660 nucleotides in length. The corresponding mRNA to SENU1 is 0.7 kb in size. The clone contains a poly(A) tail at the 3′ end but lacks ca. 40 bases at the 5′ end. An open reading frame of 161 amino acids starts from the nucleotide at position 2 and finishes at nucleotide position 481. A search of DNA and protein sequence databases revealed no homology with either the nucleotide or predicted polypeptide sequence of SENU1.

The complete nucleotide sequence of SENU4 (525 bp) has been determined. This clone is identical to the cDNA encoding the tomato pathogenesis related (PR) protein P6 previously isolated from *Cladosporium fulvum*-infected tomato leaves [46]. P6 is one of the two isoforms of the extracelluar basic tomato PR protein also known as P14 [31, 46]. SENU4 is not full-length, based on the P6 sequence. It lacks 160 bp at the 5′ end and displays an alternative polyadenylation site (at base 685 on the P6 sequence).

The SENU5 is a novel cDNA clone of 846 bp. The SENU5 mRNA is 0.9 kb in length and the cDNA clone is missing ca. 50 bases at the 5′ end. An open reading frame of 272 amino acids starts from nucleotide 10 and finishes at 825. The search of DNA and protein sequences presently deposited in the databases revealed no homology with SENU5.

Expression of senescence up-regulated mRNAs during tomato leaf development, senescence and in different organs

Northern blot analysis was used to determine the expression of senescence-related mRNAs in tomato leaf development and senescence. Samples were taken of 4-, 5- and 6-week-old second true leaves after germination. Approximately 8–10 weeks post-germination a series of leaf senescence stages from mature green to advanced senescence could be obtained as previously described by John *et al.* [22]. Northern blot analysis indicated that SENU1 is expressed at high levels in 4-, 5- and 6-week-old green leaves (Fig. 1A). The mRNA levels decreased in 8-week-old green leaves just before the onset of senescence, and then increased during subsequent stages of senescence (Fig. 1A). The SENU1 mRNA was present in root and stem but accumulation was extremely low in green and ripe fruit (Fig. 1A). The SENU4 mRNA abundance was very low in 4-, 5- and 6-week-old green leaves (Fig. 1B) but mRNA levels increased in 8-week-old green leaves and mRNAs levels were maintained at onset, mid and advanced stages of senescence (Fig. 1B). The levels of SENU4 mRNA in roots, stems and ripe fruit were extremely low but there was significant expression in green fruit. The SENU5 mRNA accumulation continued to increase during leaf development and peaked at the advanced stage of senescence (Fig. 1C). The expression of SENU5 mRNA was detected in roots but was also found in other organs used in this study (Fig. 1C).

Senescence down-regulated clones

Five cDNA clones (SEND32, 33, 34, 35 and 36) for mRNAs which decreased in accumulation during tomato leaf senescence were isolated [9]. Two of these clones (SEND32 and 35) encode previously unreported sequences. The SEND32 clone is 679 bp in length. The corresponding mRNA is 1.5 kb in size which indicated that SEND32 is missing ca. 800 bp at the 5′ end. There is an open reading frame from nucleotide position 3 to 491 but no sequence homology could be found by database search. The SEND35 cDNA clone is 532 bp in length and the mRNA is 1.4 kb, indicating that the clone is incomplete. There is a poly(A) tail at the 3′ end, thus the 5′ end is truncated. Nucleotide and protein database searches revealed no homology with the SEND35 sequence. The SEND33 clone is 574 bp long and has significant homology (60%) at the DNA level

(A)

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Tomato       MASIS---GTMISTSFLPRKPAVTSLKAISNVG-EALFGL 36
Spinach      MAATTTTMMGM-ATTFVPKPQAPPMMAALPSNTGRSLFGL 39
Pea          MATTPALYGTAVSTSFLRTQPMPMSVTTTKAFS-NGFLGL 39
Wheat        MAAALSLRA-----PFSLRAVAPPAPRVALAPAALSLAAA 35
Arabidopsis  MAST-ALSSAIVGTSFIRRSPAPISLRSLPSANTQSLFGL 39

Tomato       KSG--RNGRITCMASYKVKLITPEGPIEFECPDDVYILDQ 74
Spinach      KTG-SRGGRMT-MAAYKVTLVTPTGNVEFQCPDDVYILDA 77
Pea          KTSLKRGDLAVAMASYKVKLVTPDGTQEFECPSDVYILDH 79
Wheat        KQV--RGARLRAQATYKVKLVTPEGEVELEVPDDVYILDQ 73
Arabidopsis  KSGTARGGRVTAMATYKVKFITPEGELEVECDDDVYVLDA 79

Tomato       AEEEGHDLPYSCRAGSCSSCAGKVTAGSVDQSDGNFLDED 114
Spinach      AEEEGIDLPYSCRAGSCSSCAGKLKTGSLNQDDQSFLDDD 117
Pea          AEEVGIDLPYSCRAGSCSSCAGKVVGGEVDQSDGSFLDDE 119
Wheat        AEEEGIDLPYSCRAGSCSSCAGKLVSGEIDQSDQSFLDDD 113
Arabidopsis  AERAGIDLPYSCRAGSCSSCAGKVVSGSVDQSDQSFLDDE 119

Tomato       QEAAGFVLTCVAYPKGDVTIETHKEEELTA 144
Spinach      QIDEGWVLTCAAYPVSDVTIETHKEEELTA 147
Pea          QIEAGFVLTCVAYPTSDVVIETHKEEDLTA 149
Wheat        QMEAGWVLTCHAYPKSDIVIETHKEEELTA 143
Arabidopsis  QIGEGFVLTCAAYPTSDVTIETHKEEDIV  148
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(B)

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                .         .         .         .         .
Potato  MASTVMSSLSLKPTFTLEKTSVKGLPSLARSSSSFKVVASGVKKLKTDKP 50
                |||||||||||.|||||||||.||||||||||.||||||||||
Tomato  ........LSLKPTFTLEKISVKGLPSLTRSSSSFKVVARGVKKLKTDKP 42
                .         .         .         .         .
Potato  YGINGSMALRDGVDASGRKPKGKGVYQYVDKYGANVDGYSPIYNTDEWSP 100
        ||||||||||||||||||||||||||||||||||||||||||||||||||
Tomato  YGINGSMALRDGVDASGRKPKGKGVYQYVDKYGANVDGYSPIYNTDEWSP 92
                .         .         .
Potato  SGDVYVGGTTGLAIWAVTLVGILAGGALLVYNTSALAQ* 138
        |||||||||||||||||||:||||||||||||||||||
Tomato  SGDVYVGGTTGLAIWAVTLLGILAGGALLVYNTSALAQ* 130
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Figure 2. Alignment of tomato ferredoxin with previously identified ferredoxins and tomato photosystem II 10 kDa polypeptide with potato. A. The ferredoxin deduced amino acid sequence is aligned with protein sequence of spinach accession number P00221 [50], pea accession number p09911 [13], wheat accession number P00228 [3] and *Arabidopsis* accession number p16972 [41]. The alignment was generated using a Clustal method with PAM 250 residue weight table on DNA star Program. Dark shading indicates identical residues. B. The SEND34 predicted amino acid sequence is aligned with photosystem II 10 kDa polypeptide of potato accession number p06183 [12] for comparison using GAP program from the UWGCG package. The identical amino acids are shown by | and conserved subsititutions are shown by :.

with ferredoxin-1 of pea and spinach [13, 50]. The corresponding mRNA is 0.8 kb. The cDNA contains a complete ferredoxin coding sequence with homology to spinach, pea, wheat and *Arabidopsis* but lacks the 5′ untranslated sequence (Fig. 2A). SEND34 is 558 bp in size and the mRNA is ca. 0.6 kb. The sequence, which lacks the first 8 codons, has 97% identity with the potato photosystem II 10 kDa polypeptide (Fig. 2B) at both the nucleotide and predicted amino acid level [12] and ca. 80% with that from spinach and *Arabidopsis* at the amino acid level [17, 25]. SEND36 is 474 bp long and revealed 100% homology with tomato catalase [11] at the 3′ end.

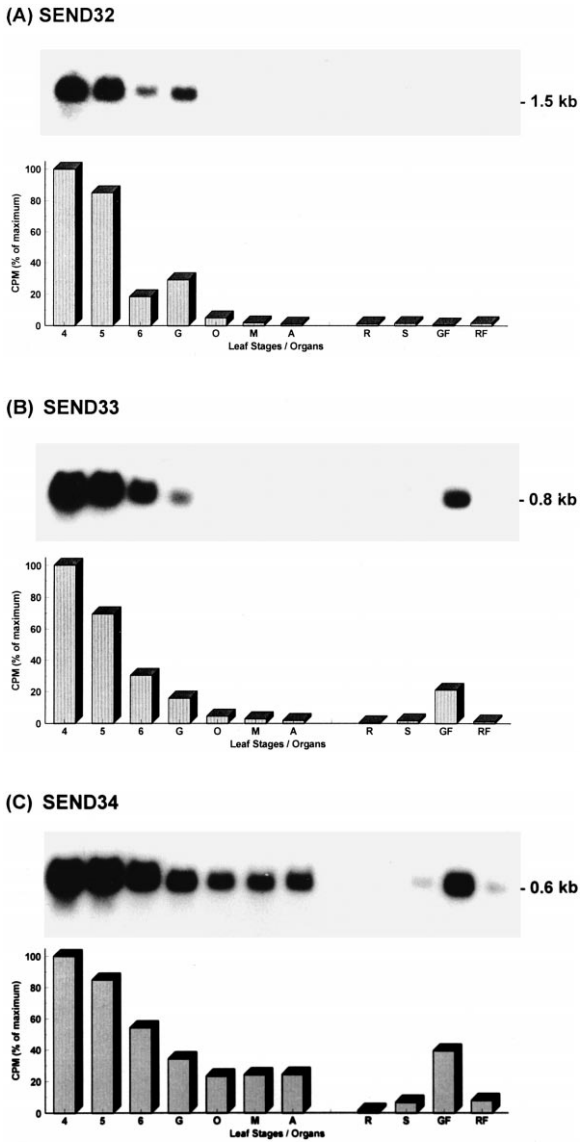

Figure 3. Expression of SEND mRNAs duing tomato leaf development, senescence and in different plant organs. The RNA samples were the same as described in the legend to Fig. 1. The blots were probed with: (A) SEND32; (B) SEND33; (C) SEND33; (D) SEND34; (E) SEND35 and (F) SEND36.

Figure 3. Continued.

Expression of SEND mRNAs during tomato leaf development, senescence and in various organs

The expression of senescence down-regulated (SEND) mRNAs was determined using northern blot analysis. The SEND32 mRNA levels were high in leaf development (Fig. 3A) but below the limit of detection after the onset of senescence and in all other plant organs investigated. The concentration of SEND33, 34, 35 and 36 mRNAs were also high during leaf development and generally declined in older leaves and during senescence (Fig. 3B–E). All mRNAs except SEND32 showed significant expression in other organs and were more abundant in green fruit than in root, stem and ripe fruit (Fig. 3B–E). Levels of SEND32 mRNA were inversely correlated with senescence and showed extremely low expression in other organs.

Southern analysis of senescence-related clones

Tomato genomic DNA was isolated from young leaves and digested with restriction enzymes *Bam*HI, *Eco*RI and *Hin*dIII, and the DNA gel blots were probed with all the radiolabelled cDNA inserts. Each probe hybridized to one, or a small number of DNA fragments, with the exception of SEND33 which gave multiple bands (Fig. 4). The approximate sizes of the fragments were 4 kb plus a faint band for SENU1 cut with *Eco*RI; 7 kb for SENU5 cut with *Hin*dIII; 8 kb for SEND32 cut with *Bam*HI; 3 kb and 10 kb for SEND34 cut with *Hin*dIII; 4 kb for SEND35 cut with *Eco*RI; and 1.5 kb and 3.5 kb for SEND36 cut with *Hin*dIII. SEND33 produced two hybridizing fragments when cut with

*Bam*HI, the largest of these being over 12 kb, the other was 4 kb (the spot to the right of Fig. 4C is an artefact outside the sample lane).

Transcriptional activity of SEN genes

Nuclear run-on assays were used to examine the transcriptional activity of SENU genes during three stages of leaf development. Transcripts were labelled in nuclei from the top two terminal leaves, from 8-week-old green leaves, and from leaves at the onset of senescence (G and O stages respectively in Fig. 1). Labelled transcripts were then used to detect SENU plasmid probes applied to nylon membranes (Fig. 5). Other probes included in the study were *ACO*1, encoding 1-aminocyclopropane 1-carboxylic acid oxidase, which catalyses the final step in ethylene production, *ubi*3, encoding ubiquitin, and the cDNA SEND32.

Transcription of all SENU sequences was detected in nuclei from mature green leaves and those at onset of senescence, although SENU1 appeared to be transcribed at a very low level compared to the other SENU clones, and produced only a faint signal on the origional autoradiograph. Transcription of SENU5 and the cysteine protease encoding cDNAs, SENU2 and SENU3 [9], was also detected in immature leaves, although SENU5 was at a very low level. Transcription of SEND32, the only down-regulated clone tested, was extremely low but detectable in all three leaf stages examined. *ACO*1 transcription was detected in nuclei from mature green leaves and at the onset of senescence, while ubiquitin transcription was observed in all three leaf stages, though more abundantly in mature green leaves.

Expression of senescence-related mRNAs during tomato leaf senescence induced by different environmental factors

Leaf senescence was induced by water stress, increased light and higher temperature as described in Materials and methods. Onset of leaf senescence was approximately one week earlier in water stressed plants and those treated with increased light, compared to control plants. The effect of environmental factors on the expression pattern of senescence-related mRNAs was determined using northern blot analysis. All SENU mRNAs continued to be expressed in senescing leaves, irrespective of the conditions under which senescence was induced (data not shown).

Discussion

We have isolated three cDNAs for up-regulated mRNAs (SENU1, 4 and 5) and five cDNAs for down-regulated mRNAs (SEND32, 33, 34, 35 and 36) during tomato leaf senescence. Two cDNA clones, SENU1 and SENU5, encode polypeptides of unknown functions. SENU4 is identical to a sequence encoding a tomato protein P6, previously reported as being related to pathogenesis [46]. The expression of all three mRNAs was up-regulated during senescence but there were differences during tomato leaf development. The SENU1 and SENU5 mRNAs were quite abundant during leaf development and increased in concentration during senescence while SENU4 mRNA levels were extremely low during development and rose dramatically in mature green leaves just prior to visible senescence and remained at high levels even in fully yellow leaves (Fig. 1). Although the conditions under which plants were grown may have resulted in activation of sress-related genes, a comparison of northern data from this study with that of plants grown under non-stressed conditions, as described previously [9], has shown that the SENU genes are not related to stress.

The SENU4 sequence is identical to the tomato pathogenesis-related protein P6 and has 57% similarity at the amino acid level with the LSC94 cDNA clone encoding a pathogenesis-related protein also shown to be expressed during leaf senescence in *Brassica napus* [19]. The PR proteins are divided into many classes (reviewed by Linthorst [28]) and thought to protect the plant against pathogen attack. However, expression of PR genes in the absence of pathogen invasion, has been shown to be developmentally regulated in many plant organs, including early embryo development of carrot [7], in the aleurone layer of developing barley seeds [27], in the sepals of tobacco flowers [30], in leaves of healthy flowering tobacco plants [15], and during *Brassica napus* leaf senescence [19]. Our results show that the transcripts of the PR protein P6 were expressed specifically during tomato leaf senescence, suggesting that these proteins are also developmentally regulated during tomato leaf senescence. Genomic Southern analysis of SENU1 and SENU5 showed that (Fig. 4) both are members of small gene families. There are less than five copies of the P6 gene in the tomato genome [46].

The first round of leaf senescence cDNA library screening yielded more clones encoding down-regulated than up-regulated mRNAs [9]. It was obvious from previous studies that mRNAs for photosynthesis-associated genes decline in abundance during leaf

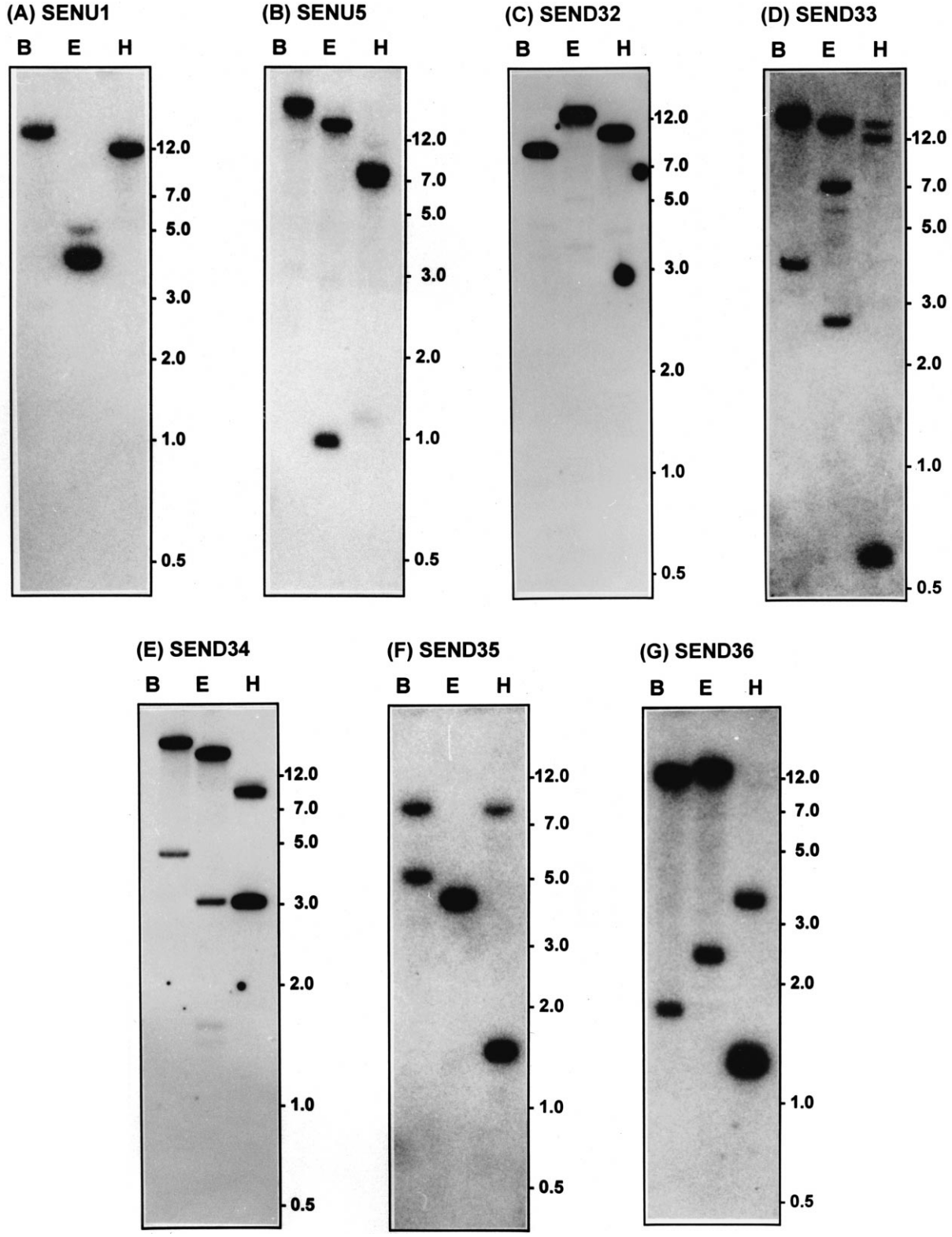

Figure 4. Southern analysis of SEN clones. Tomato genomic DNA was digested with *Bam*H1, *Eco*RI, *Hin*dIII, resolved on 0.8% agarose gel and blotted onto GeneScreen Plus membranes. The membranes were probed with: (A) SENU1, (B) SENU5, (C) SEND32, (D) SEND33, (E) SEND34, (F) SEND35 and (G) SEND36.

senescence [2, 19, 21, 22]. We obtained two photosynthesis-associated cDNA clones, SEND33 and SEND34, encoding ferredoxin and photosystem II 10 kDa polypeptides, respectively. The mRNAs for both of these clones were expressed at very high levels during early leaf development and declined with leaf age as expected (Fig. 2). Both mRNAs accumulated in green fruits but expression in other organs was minimal. From the expression pattern and high sequence similarity it is concluded that the ferredoxin encoded by SEND33 functions in photosynthesis and is not the nonphotosynthetic type implicated in the chlorophyll degradation pathway [47].

The general expression patterns of mRNAs corresponding to SEND35 and 36 clones were similar to those found for the photosynthesis-related mRNAs. The SEND36 nucleotide sequence has 100% homology with catalase [11] from tomato. Catalase is a ubiquitous heme protein that catalyzes the conversion of hydrogen peroxide formed by superoxide dismutase into water and molecular oxygen, a process that is important for responses to stress but also occurs during aging. Catalase mRNA levels were ca. 25% of maximum even at advanced stages of senescence.

The SEND32 mRNA encodes a polypeptide of unknown function and its accumulation was maximum during early leaf development. The expression levels decreased with age and were below the limit of detection at the onset of leaf senescence. The SEND32 mRNA was not detected in any other plant organs under our northern blot conditions. These results clearly indicated that SEND32 mRNA which is probably encoded by a single-copy gene, was leaf specific and turned off abruptly at the onset of leaf senescence. Thus SEND32 is a very good molecular marker to determine the onset of senscence in tomato leaves.

Transcriptional run-on assays were used to determine whether the SENU clones described here, in addition to two previously described cysteine protease-encoding cDNAs [9], were actively transcribed during senescence. The results showed that all five SENU clones were transcribed in mature green leaves and in leaves at the visible onset of senescence. With the possible exception of SENU2, no increase in transcript abundance was observed in leaves at the onset of senescence compared to mature green leaves. Only SENU4 appears to be truly senescence-related. It was not detected by either run-on or northern analysis in younger leaves, but was actively transcribed in mature green leaves and during onset of senescence. SENU5 and the cysteine protease genes SENU2 and SENU3 were

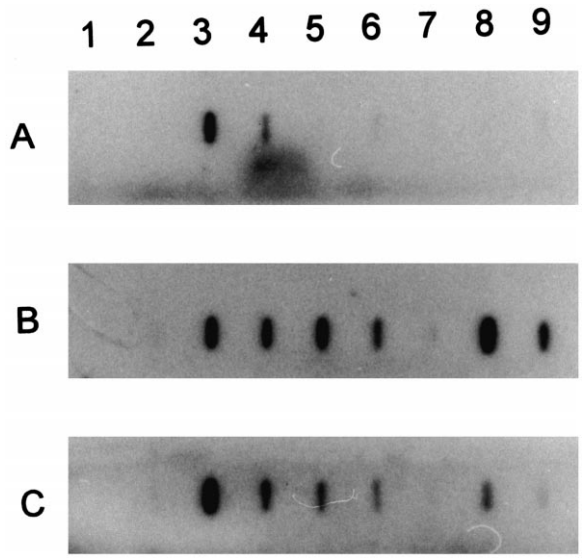

Figure 5. Transcriptional run-on assays for the SEN clones. Transcripts in nuclei from: (A) top terminal leaves, (B) mature green leaves and (C) leaves at onset of senescence were labelled with ^{32}P and hybridized to filter-bound probes for: 1, pBluescript; 2, SENU1; 3, SENU2; 4, SENU3; 5, SENU4; 6, SENU5; 7, SEND32; 8, ACO1; 9, Ubiquitin.

detected by run-on analysis in immature leaves, and northern analysis showed that SENU1 was expressed in developing leaves, as well as those undergoing senescence.

None of the genes so far isolated and characterized appears to be involved in the initiation of leaf senescence but rather, they are involved in the execution of the process probably acting downstream of a controlling gene. Experiments in *Drosophila melanogaster* have shown that the 'reaper' gene, which controls cell death is induced by many different signals [42, 51]. The reaper gene appears to function as a regulator, and is not part of the basic cell death effector machinery. Genes involved in the execution of the death programme, such as proteases, act down stream of reaper. An important goal for the future is to identify the gene(s) which cause the leaf senescence syndrome and the genes involved in the signal transduction pathway that switches on the senescence program. Since changes in the regulation of the SENU4 and SEND32 mRNAs occur before the visible onset of senescence, these may prove useful molecular markers for the initiation of the senescence process.

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