Botanical Studies (2009) 50: 159-170.

Molecular cloning and expression of a sweet potato cysteine protease *SPCP1* from senescent leaves

Hsien-Jung CHEN[1,*], Guan-Jhong HUANG[2,6], Wei-Shan CHEN[3,6], Cheng-Ting SU[3], Wen-Chi HOU[4], and Yaw-Huei LIN[5,*]

[1]*Department of Biological Sciences, National Sun Yat-sen University, 804 Kaohsiung, Taiwan*
[2]*Graduate Institute of Chinese Pharmaceutical Sciences, China Medical University, 404 Taichung, Taiwan*
[3]*Graduate Institute of Biotechnology, Chinese Culture University, 111 Taipei, Taiwan*
[4]*Graduate Institute of Pharmacognosy Science, Taipei Medical University, 110 Taipei, Taiwan*
[5]*Institute of Plant and Microbial Biology, Academia Sinica, Nankang, 115 Taipei, Taiwan*

(Received August 18, 2008; Accepted November 19, 2008)

ABSTRACT. In this report a full-length cDNA, *SPCP1*, was isolated from senescent leaves of sweet potato (*Ipomoea batatas* (L.) Lam). *SPCP1* contained 1020 nucleotides (339 amino acids) in the open reading frame, and exhibited high amino acid sequence homologies (ca. 58% to 74%) with papain-like cysteine proteases of *Alnus glutinosa*, *Arabidopsis thaliana*, *Astragalus sinicus*, *Brassica napus*, *Daucus carota*, *Gossypium hirsutum*, *Hordeum vulgare*, *Iris hollandica*, *Medicago truncatula*, *Nicotiana tabacum*, *Oryza sativa*, *Ricinus communis*, *Trifolium repens*. Semi-quantitative RT-PCR and Western blot hybridization showed that *SPCP1* gene expression was enhanced significantly in natural senescent leaves and in dark-, ethephon-, and ABA-induced senescent leaves, whereas, was almost not detected in mature green leaves, stems, and roots. Initiation of chlorophyll degradation is earlier than the *SPCP1* gene expression during leaf senescence. *SPCP1* expression was also induced in sweet potato suspension cells treated with 1 mM ethephon. Evan blue staining showed that suspension cells were not significantly affected by ethephon treatment up to 2 mM, however, most of the cells died when treated with 10 mM ethephon. In conclusion, sweet potato *SPCP1* is likely a functional, senescence-associated gene and its expression levels were significantly enhanced at mRNA and protein levels in natural and induced senescent leaves and suspension cells. The physiological role and function of *SPCP1* were likely not in association with initiation of chlorophyll degradation and cell death during senescence.

Keywords: Cysteine protease; Ethephon; Leaf senescence; SPCP1; Sweet potato.

INTRODUCTION

Sweet potato (*Ipomoea batatas* (L.)) is a gamopetalous dicot and belongs to the order of Polemoniales and the family Convolvulaceae (Sihachakr et al., 1997). It is an important food crop in the tropics, and has been imported into Taiwan since the 17th century. Storage roots and leaves are the edible portions, and its nutritive constituents are mainly starch, lipid and protein. It also contains plenty of vitamin B complex, vitamin C, β-carotenoids, multiple minerals and high calcium (Yang et al., 1975; Hattori et al., 1985). Several medicative effects of sweet potato have been reported previously, including accelerated excretion of toxins and carcinogens, trypsin inhibitor (Hou et al., 2001) and antioxidant activity (Huang et al., 2007a and 2007b), inhibition of angiotensin converting enzyme activity (Hou et al., 2003; Huang et al., 2006), reduction of hypertension in diabetic mice, and growth inhibition and induction of apoptosis in NB4 promyelocytic leukemia cells (Huang et al., 2007c).

Leaf is the main place of photosynthesis and acts as a source of carbohydrate for sink nutrients in plants. Its longevity and senescence thus affect the photosynthesis efficiency and crop yield. Leaf senescence is influenced by endogenous and exogenous factors, including plant growth regulators, starvation, wound, and environmentsl stresses (Yoshida, 2003; Lim et al., 2007). Leaf senescence is the final stage of development and has been considered as a type of programmed cell death (Lim et al., 2007). During senescence, macromolecules are not only simply degraded, but also recycled. The released small molecules can be translocated from the senescent cells to young leaves, developing seeds, or storage tissues (Buchanan-

[6]Equal contribution to this work.

*Corresponding author: E-mail: boyhlin@gate.sinica.edu.tw, Phone: 886-2-27871172, Fax: 886-2-27827954 (Yaw-Huei Lin); E-mail: hjchen@faculty.nsysu.edu.tw, Phone: 886-7-5252000 ext. 3630, Fax: 886-7-5253630 (Hsien-Jung Chen).

Wollaston, 1997; Quirino et al., 2000). Leaf cells undergo highly coordinated changes in structure, metabolism, and gene expression during senescence in a defined order. Breakdown of chloroplast is the earliest and most significant change in cell structure (Makino and Osmond, 1991). The carbon assimilation is metabolically replaced by catabolism of chlorophyll and macromolecules such as proteins, membrane lipids, and RNA (Lim et al., 2007).

During senescence, breakdown of leaf proteins by proteases provides a large cellular nitrogen pool for recycling (Makino and Osmond, 1991). In plants, it is assumed that vacuole is the site involved in bulk protein degradation by virtue of its resident proteases. Different types of vacuoles have been reported in plants, including storage vacuole, lytic central vacuole (Vierstra, 1996; Marty, 1999), and small senescence-associated vacuoles (Otegui et al., 2005). Protein storage vacuoles are often found in seed tissues and accumulate storage proteins that are remobilized and utilized as the main nutrient resource for germination (Senyuk et al., 1998; Schlereth et al., 2001). Most cells in vegetative tissues have a large lytic central vacuole and many small senescence-associated vacuoles, which contain a wide range of proteases in an acidic environment (Otegui et al., 2005). Substrate proteins must be transported and sequestered into this vacuole for degradation.

Recently, a group of papain-like cysteine proteases have been isolated from various senescent tissues of different species. In *Arabidopsis* and *Brassica napus*, the orthologous genes, SAG12 and BnSAG12, were demonstrated for their conservation across species from gene structural comparisons and expression/regulation studies (Noh and Amasino, 1999). In the nitrogen-fixing symbiosis interaction of *Alnus glutinosa* and actiomycete *Frankia*, a nodule-specific papain-like cysteine protease AgNOD-CP1 with unknown function was identified and isolated (Goetting-Minesky and Mullin, 1994). Gene expression of the nodule-specific papain-like cysteine proteases, such as AsNOD32 of Chinese milk vetch (*Astragalus sinicus*) (Naito et al., 2000) and Tr-cp of white clover (*Trifolium repens*) (Asp et al., 2006) were detected in the senescing zone and correlated with the onset of nodule senescence. In *Arabidopsis*, papain-like cysteine protease *SAG12* was mainly localized in senescence-associated vacuoles (SAVs) of mesophyll and guard cells of senescing leaves, and was not required either for SAV formation or for progression of visual symptoms of senescence (Otegui et al., 2005). *SAG12* was expressed in sugar-induced and nitrogen deprivation-induced senescence (Gombert et al., 2006; Pourtau et al., 2006), and correlated at the whole plant level with the sink/source transition for nitrogen during both developmental and nutrient sress-induced leaf senescence (Gombert et al., 2006). In castor bean (*Ricinus communis* L.), a papain-like cysteine protease with a C-terminal KDEL was immunolocalized specifically in ricinosome, which was organelles co-purified with glyoxysomes from germinating *Ricinus* endosperm, a senescing tissue (Schmid et al., 1998). The papain-like cysteine endoprotease has the capacity to process the glyoxysomal malate dehydrogenase precursor protein into the mature subunit *in vitro* (Gietl et al., 1997). In tobacco, the senescence-associated vacuoles contain soluble chloroplast stromal proteins, including Rubisco and glutamine synthase, but lack the thylakoid proteins, such as D1 protein, LHCII of the PSII reaction center and PSII antenna. The Rubisco levels decrease steadily in SAVs incubated at 30°C, and was completely abolished by addition of protease inhibitors (Martinez et al., 2008). These results indicate that SAVs are involved in the degradation of the soluble photosynthetic proteins of the chloroplast stroma during senescence of leaves. Therefore, a possible role for these papain-like cysteine proteases in association with the clevage and maturation of proproteinases that are in turn involved in macromolecule degradation and re-mobilization during leaf senescence was suggested.

We have previously isolated several senescence-associated cysteine proteases from sweet potato senescent leaves in our laboratory (Chen et al., 2004; 2006; 2008). We report here the cloning and characterization of another cysteine protease, *SPCP1*, which exhibited high amino acid sequence identities with plant vacuolar papain-like cysteine proteases, including *Alnus glutinosa* AgNOD-CP1, *Astragalus sinicus* AsNOD32, *Trifolium repens* Tr-cp, *Arabidopsis thaliana* and *Brassica napus* SAG12.

MATERIALS AND METHODS

Plant materials

The storage roots of sweet potato (*Ipomoea batatas* (L.) Lam) were grown in a growth chamber, and plantlets from the storage roots were used as materials. Mature green leaves near the top of stems were detached for experiments of induced senescence by different treatment such as dark, ethephon, and ABA. Leaves with differential level of senescence were also used for experiments of tissue-specific expression.

PCR-based subtractive hybridization and RACE PCR

Molecular cloning of senescence-associated genes basically followed the previous report of Chen et al. (2000). Total RNAs were isolated individually from the mature green leaves and senescing leaves of sweet potato basically according to the method of Sambrook et al. (1989). The mRNAs were purified with a purification kit (Promega) and used for the differentially-expressed first strand cDNA synthesis with a PCR-based subtractive hybridization kit (Clontech) following the protocols supplied by the manufacturer. The double-strand cDNAs of senescing leaves were subtracted by that of mature green leaves, then ligated to pGEM-T vector for *E. coli* DH5α competent cell transformation. Recombinant plasmids were isolated for DNA sequencing using an ABI PRIZM 337 DNA Sequencer. Nucleotide sequence data

were analyzed using the Genetics Computer Group (GCG) programs. The RACE PCR method with the Marathon cDNA amplification kit (Clontech) was used to isolate the 5′ and 3′ ends of the interested cDNAs according to the protocols provided by the manufacturer.

Southern blot hybridization

Young (not expanded) leaves of sweet potato were harvested and ground in liquid N_2. The powder was transferred to a centrifuge tube, mixed gently and thoroughly with cetyltrimethylammonium bromide (CTAB) buffer (2% CTAB, 1.4 mM NaCl, 20 mM ethyle nediaminetetraacetate (EDTA), 0.2% β-mercaptoethanol, and 100 mM Tris-HCl pH 8.0) in a 20:1 (v:w) ratio, and kept at 60°C in a water bath for genomic DNA extraction according to the method of Chen et al. (2000). The total nucleic acid after precipitation with an equal volume of isopropanol was re-dissolved in sterile water, digested with restriction enzymes of *Eco*RI, *Hind*III or *Xba*I and separated on a 0.8% agarose gel. After electrophoresis, the DNA was transferred onto a Hybond-N^+ nylon membrane (Amersham) following the protocol of Molecular Cloning (Sambrook et al., 1989) for Southern blot hybridization.

Measurement of pigments

For quantitative analysis of pigment contents, the mature green leaves (S0) and senescing leaves (S1 and S2) of sweet potato were collected separately and extracted with 80% acetone (pH 7.8) buffered with 2.5 mM sodium phosphate according to the method of Chen et al. (2000). The absorbance of extracts was measured at wavelengths of 663.8 nm, 646.8 nm and 470 nm, respectively. Quantitative values of pigments for aqueous 80% acetone extracts were calculated from the absorbance data according to the report of Lichtenthaler (1987). For dark-induced senescence, detached mature green leaves were placed on a wet paper towel containing 3 mM 2-(N-mor pholino)ethanesulphonic acid (MES) buffer pH 7.0, then kept at room temperature in the dark for 0, 3, 6, 9 and 12 days. For 1 mM ethephon or 100 μM ABA treatments, the detached mature green leaves were also placed on a wet paper towel containing 3 mM MES (pH 7.0) plus different plant growth regulator, then kept in the dark for 0, 1, 2 and 3 (for ethephon) or 5 (for ABA) days. Leaves were individually collected for quantitative analysis of pigment contents and also for semi-quantitative RT-PCR.

Semi-quantitative RT-PCR

Total RNA was isolated from (a) different tissues including roots, stems, mature green leaves and senescing leaves, and (b) dark-treated, 1 mM ethephon-treated, or 100 μM ABA-treated mature green leaves of sweet potato. The primer pairs (Y136-5′: CACTTTACGGTTGTAAA CATTTTACA and Y136-3′: GAGATAATACACACCA ATTAATGGAT) were used to amplify the full-length *SPCP1* cDNAs for semi-quantitative RT-PCR analysis according to the method of Jonson et al. (2000). The full-length *SPCP1* cDNA was also labelled with digoxigenin-11-dUTP nucleotides as the probe for semi-quantitative RT-PCR product detection using Southern blot hybridization and CSPD substrate (Boehringer Mannheim) as described previously.

Production of polyclonal antibody against SPCP1

The full-length *SPCP1* cDNA was used as templates to amplify the PCR products encoding the putative mature SPCP1 protein with primers (Y136-5′M: GGTATTGA GGGTCGCGTTCCTACTACCGTGGACTGG and Y136-3′M: AGAGGAGAGTTA GAGCCCCAAGCAGAGGG ATATGAT). The amplified PCR products were purified first and then cloned directly into PET32Xa/LIC vector (Novagen) according to the protocols provided by the supplier. After induction with 1 mM IPTG, the expressed fusion proteins were extracted from cells with 8 M urea, and purified with His-tag affinity column according to the protocols from Novagen. The purified fusion protein was digested with protease Xa factor to release the expressed SPCP1 mature protein for N-terminal amino acid sequencing and as an antigen for polyclonal antibody production in rabbits. For N-terminal amino acid sequence determination, the purified fusion proteins were first digested with Xa factor, and then mixed with SDS sample extraction buffer and boiled in a 100°C water bath for 5 min. The samples were subjected to protein SDS-PAGE in 12.5% gels, then, transferred onto Millipore PVDF membranes. The band with molecular weight corresponding to the expressed SPCP1 mature protein was cut from PVDF membrane and used for N-terminal amino acid determination. For polyclonal antibody production, the purified fusion protein was first dgested with Xa factor, and then performed a 12.5% SDS-PAGE. The band with molecular weight corresponding to the expressed SPCP1 mature protein was cut from the 12.5% polyacylamide gel, then, mixed with appropriate amount of pH 7.5 phosphate buffer saline (PBS) containing 0.1% SDS. The eluted proteins in PBS containing 0.1% SDS were precipitated with acetone containing 10% trichloroacetic acid (TCA) at -20°C for 2 h. After centrifugation at 13,000 *xg* for 20 min, the pellet was washed with acetone twice, then, dried at room temperature. The acetone powder was re-dissolved in a small amount of PBS buffer containing 0.1% SDS and used as antigens for subcutaneous injections (Taiwan Bio-Pharm Inc.).

Western blot hybridization and activity staining

Polyclonal antibody obtained from rabbit antiserum was utilized for Western blot hybridization to study the gene expression of *SPCP1* in different tissues, leaves with various levels of senescence, and with dark or ethephon treatments as described previously. For enzymatic activity analysis, basically it follows the method reported by Lee and Lin (1995). The crude protein extract from S2 leaves was analyzed for protease activity in the

presence or absence of inhibitor L-3-carboxy-2,3-trans-epoxypropionyl-leucyl-amino (4-guanidino) butane (E-64) in gelatin-containing polyacrylamide gel. About 0.5 g S2 senescent leaves were ground with mortar and pestle in liquid N_2 and the powder was extracted with extraction buffer containing 10 mM Tris-HCl and 1 mM EDTA pH 6.8. The mixture was centrifuged at 13,000 *xg*, 4°C for 10 min, then, the supernatant was transferred to a new centrifuge tube. The crude extract was mixed with equal volume of 2x SDS buffer (4.6%) and stayed at 4°C for overnight. After incubation, sample buffer without SDS was added into the crude extract in the presence or absence of E-64 inhibitor, and perform electrophoresis on a 12.5% SDS-PAGE containing 0.4 to 1% gelatin. After electrophoresis, the gel was washed twice with isopropanol to replace SDS from gels, then, incubated in 100 mM Tris-HCl buffer containing β-mercaptoethanol with pH 6.0 at 37°C for 30 min to 1 h. After incubation, the gel was stained with Coomassie Blue R250 staining dye to detect the protease activity bands on gels.

Induction of SPCP1 in sweet potato cell suspension culture

Fully-expanded mature green leaves of sweet potato were collected and disinfected in 2% sodium hypochlorite for about 10 min, then washed with sterile water for three times. After disinfection, the leaves were cut into strips and incubated on solid MS medium supplemented with 3% sucrose, 1 ppm 2,4-D, and B5 vitamins for callus induction in a day/night cycle with 28°C day and 23°C night, 16 h day and 8 h night. The induced calli were routinely maintained and subcultured monthly on the same medium. For cell suspension culture establishment, calli about one month after subculture were transferred into the same liquid medium and shaked on an orbital shaker with 100 rpm. The cell suspension culture were routinely maintained once a week in a 1:2 (v:v) ratio of cell suspension to fresh medium at room temperature in the dark. For growth curve determination, total about 30 mL of diluted cell suspension as described above was filtered with Whatmann No. 4 filter paper at the intervals of day 0, 3, 6, 9, 12, 15 and 18, respectively, and the cells retained on the filter membrane were collected for fresh weight determination. Ethephon, an ethylene-releasing compound, can be catalyzed to release ethylene, HCl, and H_3PO_4 in a 1:1:1 ratio after uptake into cells. Therefore, treatment of 1 mM HCl plus 1 mM H_3PO_4 was used as additional control. For ethephon treatment, about 10 mL of diluted cell suspension was treated with 1 mM ethephon or 1 mM HCL plus 1 mM phosphate, and harvested at intervals (a) of day 0, 3 and 6, respectively, for fresh weight measurement, and (b) of day 1, and 3, respectively, for the induction of *SPCP1* gene expression. The effect of ethephon on cell viability was also assayed with evan blue staining dye. Suspension cells were treated with 0, 1, 2, 10 mM ethephon, respectively, for three days. After treatment, the cells were collected and stained with 0.25% evan blue staining dye in PBS buffer at 25°C for 30 min, then washed with PBS for 10 min before microscopic observation.

RESULTS

Nucleotide and amino acid sequences of SPCP1

With PCR-based subtractive hybridization and RACE PCR techniques, a full-length cDNA, *SPCP1* (GenBank accession no. AF242372), was cloned from senescent leaves. There were 1020 nucleotides (339 amino acids) in its open reading frame (Figure 1). GCG/fasta comparison showed that *SPCP1* exhibited high amino acid sequence homologies (58% to 74%) with papain-like cysteine proteases of *Alnus glutinosa* (AAA50755; 71%), *Arabidopsis thaliana* (AAK43946 and AAC49135; 58%), *Astragalus sinicus* (BAB13759; 71%), *Brassica napus* (AAD53011; 59%), *Daucus carota* (BAD29955; 73%), *Gossypium hirsutum* (AAT34987; 74%), *Hordeum vulgare* (CAB09697; 59%), *Iris hollandica* (AAR92154; 70%), *Medicago truncatula* (AAQ63885; 72%), *Nicotiana tabacum* (AAW78660; 60%), *Oryza sativa* (CAD40112; 67%), *Ricinus communis* (AAC62396; 60%), *Trifolium repens* (AAP32196; 73%).

From the alignment of *SPCP1* putative catalytic domain with other plant cysteine proteases, a conservation of the catalytic residues within the domains was observed. The

```
CACTTTACGGTTGTAAACATTTTACAACCATTTTTAATTACTATGGCTTCCAATAGCTTG
                                          M  A  S  N  S  L      6
AAGCTCCTCGTTGCTCTTGCACTAGTGTTTGCAACGTCGGCTTATTTAGCCACGTCTCGA
K  L  L  V  A  L  A  L  V  F  A  T  S  A  Y  L  A  T  S  R     26
ACATTGTCTGATTCATTGATGGTGGTGAGGCACGAGCAGTGGATGGCTCAGTATGGACGC
T  L  S  D  S  L  M  V  V  R  H  E  Q  W  M  A  Q  Y  G  R     46
GTTTACAAAACTGAAGCCGAAAAAACAAAACGATTTAACATTTTCAAAGAGAATGTAGAA
V  Y  K  T  E  A  E  K  T  K  R  F  N  I  F  K  E  N  V  E     66
TACATTGAGTCTTTTAATAAGGCGGGAACCAAGCCTTATAAGCTTGGCATCAATGCATTT
Y  I  E  S  F  N  K  A  G  T  K  P  Y  K  L  G  I  N  A  F     86
GCCGATTTGACTAACCAGGAATTCAAAGCATCTCGGAATGGATACAAATTGCCTCACGAC
A  D  L  T  N  Q  E  F  K  A  S  R  N  G  Y  K  L  P  H  D    106
TGCTCCTCCAATACCCCATTTAGGTATGAAAATGTGAGTTCAGTTCCTACTACCGTGGAC
C  S  S  N  T  P  F  R  Y  E  N  V  S  S ↑V  P  T  T  V  D    126
TGGAGGACGAAGGGAGCCGTTACTCCCGTTAAAGATCAAGGCCAATGTGGATGTTGCTGG
W  R  T  K  G  A  V  T  P  V  K  D  Q  G  Q  C  G  C  W      146
GCATTTTCTGCTGTTGCCGCCATGGAAGGAATTACGAAGCTCTCAACCGGCAATCTGATT
A  F  S  A  V  A  A  M  E  G  I  T  K  L  S  T  G  N  L  I    166
TCACTGTCAGAGCAAGAGCTAGTAGATTGCGACGTAAAAGGCACAGACCAAGGCTGCGAA
S  L  S  E  Q  E  L  V  D  C  D  V  K  G  T  D  Q  G  C  E    186
GGAGGCCTAATGGACGATGCATTCAGCTTCATAATAAACAACAAAGGCCTCACAACAGAA
G  G  L  M  D  D  A  F  S  F  I  I  N  N  K  G  L  T  T  E    206
TCCAACTACCCATACCAAGGAACCGACGGCTCCTGCAAGAAAAGCAAGTCATCCAACAGC
S  N  Y  P  Y  Q  G  T  D  G  S  C  K  K  S  K  S  S  N  S    226
GCAGCAAAGATCAGCGGCTACGAAGACGTCCCCGCTAACAGCGAGTCCGCCTTGGAGAAG
A  A  K  I  S  G  Y  E  D  V  P  A  N  S  E  S  A  L  E  K    246
GCCGTGGCCAACCAGCCTGTATCCGTAGCCATAGACGCCGGCGGATCGGATTTCCAGTTC
A  V  A  N  Q  P  V  S  V  A  I  D  A  G  G  S  D  F  Q  F    266
TACTCGAGCGGGGTTTTCACGGGAGAGTGCGGGACGGAGTTGGATCACGGCGTGACGGCT
Y  S  S  G  V  F  T  G  E  C  G  T  E  L  D  H  G  V  T  A    286
GTTGGGTATGGGATTGCCGAGGATGGGAGTAAGTATTGGTTGGTGAAGAATTCATGGGGA
V  G  Y  G  I  A  E  D  G  S  K  Y  W  L  V  K  N  S  W  G    306
ACAAGCTGGGGTGAGAAAGGGTACATTAGGATGCAGAAAGACATTGAGGCTAAGGAAGGG
T  S  W  G  E  K  G  Y  I  R  M  Q  K  D  I  E  A  K  E  G    326
CTTTGCGGTATTGCTATGCAATCATCATATCCCTCTGCTTGATAAATATATGTTTTTATT
L  C  G  I  A  M  Q  S  S  Y  P  S  A  *                      339
TGATGAGGCTTTGTATCAATTATTCCTAAAACGCCTCTGATCTGTAATTAATGTATCCAT
TAATTGGTGTGTATTATCTC
```

Figure 1. Nucleotide and amino acid sequences of sweet potato putative cysteine protease *SPCP1* isolated from senescent leaves. ATG (underlined) and TGA (underlined) represent the initiation and stop codons, respectively. The Gln (Q), Cys (C), His (H) and Asn (N) printed in white on black represent the conserved catalytic amino acid residues. The arrow (↑) represents the possible cleavage site of N-terminus of the *SPCP1*-encoded protein precursor.

Q139, C145, H282, and N303 amino acids of *SPCP1* labelled with asterisks and printed in white on black were identified as conserved catalytic residues (Figure 1). Southern blot hybridization showed that there was about six, six, and three bands detected for digestion with *Eco*RI, *Hind*III, and *Xba*I, respectively, with *SPCP1* probe (Figure 2). These data suggest that *SPCP1* encodes a putative cysteine protease, which may comprise multiple copy number in sweet potato genome.

Gene expression of SPCP1 is enhanced in naturally senescent leaves

SPCP1 gene expression was significantly enhanced during natural leaf senescence. The stages of leaf senescence was divided into S0, S1 and S2 according to the contents of cellular chlorophylls (a + b). S0 is the mature green leaf and its chlorophylls (a + b) is assigned as 100%. S1 and S2 are senescent leaves with 40% and 10% chlorophylls (a + b), respectively. The amplified PCR products were remarkably increased at S1 and S2 senescent leaves, however, was almost not detected in S0 mature green leaves, root, and stem. A metallothionein-like protein gene *G14*, which was isolated previously from sweet potato leaves and exhibited constitutive expression pattern in all tissues assayed (Chen et al., 2003), was used as a control. No significant variation of *G14* gene expression level was found among tissues and stages analyzed (Figure 3A). These data suggest that *SPCP1* is likely a senescence-associated gene and exhibits an enhanced expression pattern during natural leaf senescence.

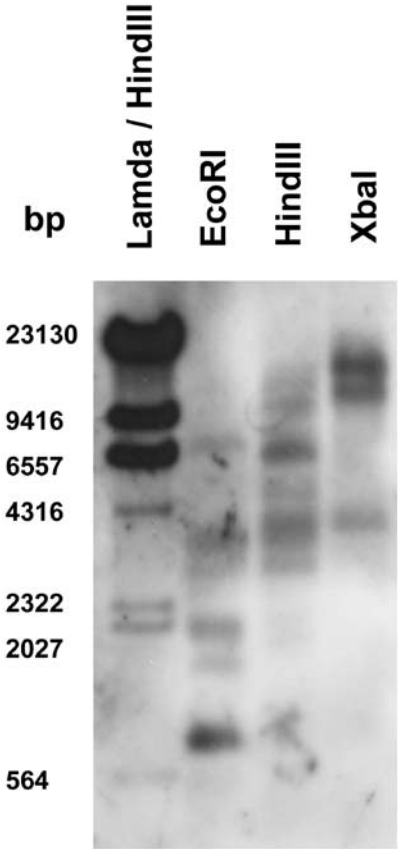

Figure 2. Southern blot hybridization of sweet potato *SPCP1*. The genomic DNA of sweet potato was digested with restriction enzymes (*Eco*RI, *Hind*III, or *Xba*I) and detected with the digoxigenin-labelled *SPCP1* probe.

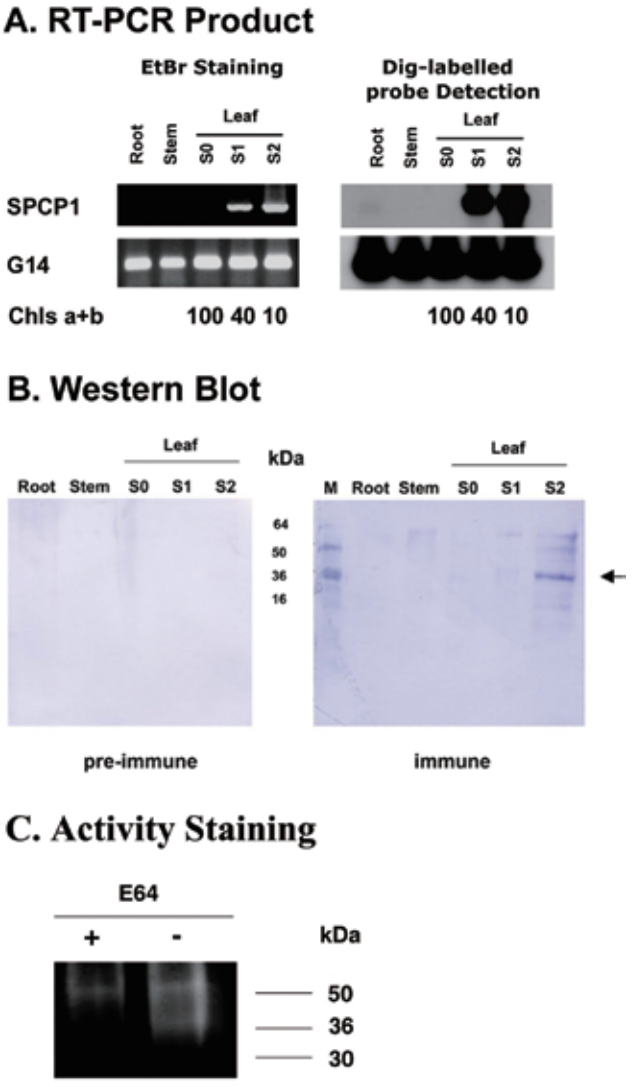

Figure 3. Temporal and spatial gene expression of sweet potato *SPCP1* detected with semi-quantitative RT-PCR and Western blot. (A) RT-PCR products. The amplified *SPCP1* RT-PCR products were detected with ethidium bromide staining (EtBr staining) and non-radioactive digoxigenin-labelled *SPCP1* cDNA probe. (B) Western blot. Western blot was performed with the polyclonal antibody raised against SPCP1. S0, S1, and S2 represented different senescent stages (100%, 40%, and 10% chlorophylls (a + b), respectively) of leaves. *G14*, which encoded a metallothionein-like protein and exhibited a constitutive expression pattern, was used as a control. The experiments were performed three times and a representative one was shown. (C) Activity staining. The crude protein extract from S2 leaves was analyzed for protease activity in the presence or absence of inhibitor E-64 in gelatin-containing polyacrylamide gel.

Western blot hybridization with polyclonal antibody raised against SPCP1 showed that a band with a molecular mass around 36 kDa was detected and its amount increased remarkably at S2 stage (Figure 3B). The data are consistent with the semi-quantitative RT-PCR results (Figure 3A) and provide further evidence to support *SPCP1* as a functional gene. Protease activity staining with S2 senescent leaves in gelatin-containing polyacrylamide gel showed that an activity band with a molecular mass about 50 kDa was detected and insensitive to inhibitor E-64 inhibition. However, the activity band with a molecular mass around 36 kDa exhibited sensitivity to inhibitor E-64 inhibition (Figure 3C). Cysteine proteases are in general sensitive to E-64 protease inhibitor (Mitsuhashi et al., 2004), however, proteases such as asparaginyl endopeptidase (an atypical cysteine endopeptidase) has been reported with insensitivity to inhibitor E-64 (Okamoto and Minamikawa, 1999). These data suggest the existence of cysteine proteases with a molecular mass ca. 36 kDa.

SPCP1 gene expression is enhanced by dark, ethephon, and ABA

Induction of *SPCP1* gene expression was studied with detached mature green leaves. For dark treatment, contents of chlorophylls (a + b) decreased gradually and

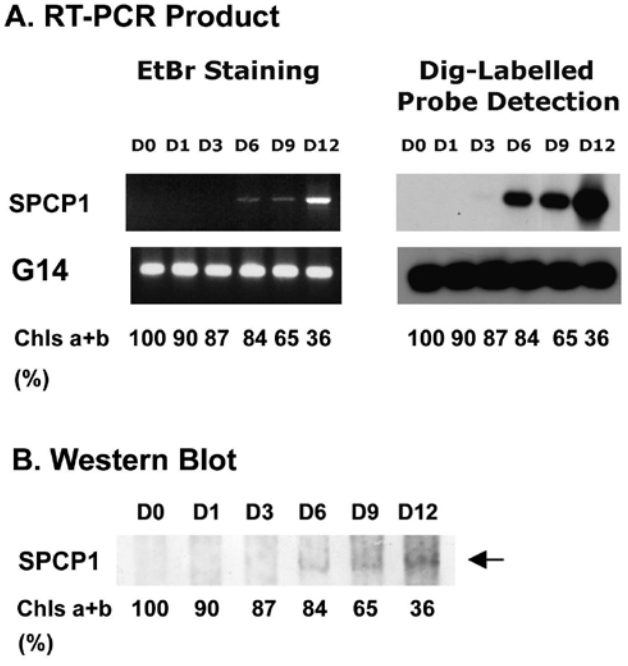

Figure 4. Effects of dark treatment on *SPCP1* gene expression in detached mature green leaves within a 12-day period. (A) RT-PCR products. Changes of *SPCP1* RT-PCR products in dark-treated mature green leaves were detected with ethidium bromide (EtBr) staining and non-radioactive digoxigenin-labelled *SPCP1* probe. *G14*, which encoded a metallothionein-like protein and exhibited a constitutive expression pattern, was used as a control. D denotes dark treatment. (B) Western blot. Western blot was performed with the polyclonal antibody raised against SPCP1. The experiments were performed three times and a representative one was shown.

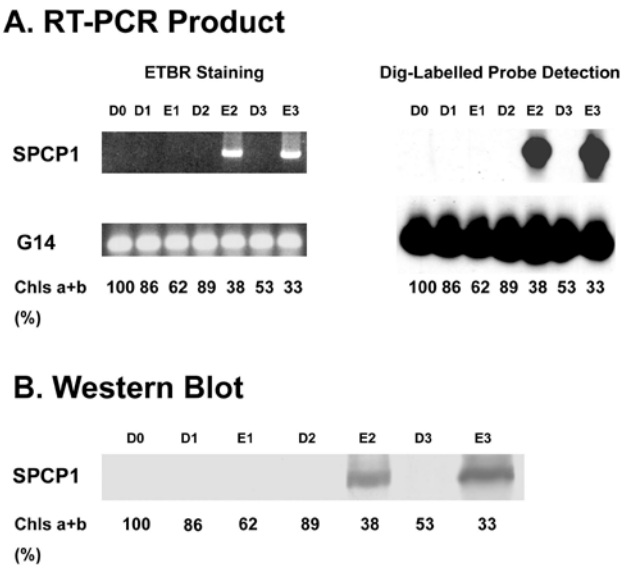

Figure 5. Effect of ethephon, an ethylene-releasing compound, on *SPCP1* gene expression in detached mature green leaves within a 3-day period. (A) RT-PCR products. Changes of *SPCP1* RT-PCR products in 1 mM ethephon-treated and dark-treated mature green leaves were detected with ethidium bromide (EtBr) staining and non-radioactive digoxigenin-labelled *SPCP1* probe. *G14*, which encoded a metallothionein-like protein and exhibited a constitutive expression pattern, was used as a control. E and D denote ethephon and dark treatments, respectively. (B) Western blot. Western blot was performed with the polyclonal antibody raised against SPCP1. The experiments were performed three times and a representative one was shown.

the amount at day 12 was about one third that of day 0. Gene expression of *SPCP1* was not significantly increased from day 0 to day 3; however, was remarkably enhanced from day 6 till day 12. For *G14*, no significant variation was found in dark treatment (Figure 4A). Western blot hybridization also detected a band with molecular weight around 36 kDa, which gradually increased from day 6 till day 12 (Figure 4B).

For 1 mM ethephon treatment, loss of contents of chlorophylls (a + b) from treated mature green leaves were much faster than that of the untreated dark control and the amount at day 3 was about one third that of day 0. The semi-quantitative RT-PCR products of *SPCP1* increased from day 2 to day 3 in ethephon-treated samples, whereas, no significant change of *SPCP1* from the untreated dark controls was observed. The increased amounts in ethephon treated samples were much higher and faster than that of the untreated dark controls from day 2 to day 3, and were correlated with the rate of leaf senescence using changes of pigment contents as a senescent indicator. For *G14*, no significant variation was found in ethephon treatment (Figure 5A). Western blot hybridization detected a band with molecular mass around 36 kDa from day 2 till day 3 after ethephon treatment (Figure 5B), which is consistent with the semi-quantitative RT-PCR results (Figure 5A).

For 100 µM ABA treatment, loss of chlorophylls (a + b) from treated mature green leaves were also faster than that of the untreated dark control and the amount at day 5 was about 22% that of day 0. *SPCP1* gene expression significantly increased from day 1 to day 5 in ABA-treated samples compared to that of untreated dark controls, and were correlated with leaf senescence. For *G14*, no significant variation was found in ABA treatment (Figure 6A). Western blot hybridization detected a band with molecular mass around 36 kDa at day 3 after ABA treatment (Figure 6B), which is consistent with the semi-quantitative RT-PCR results (Figure 6A). These data, thus, support *SPCP1* as a functional, senescence-associated gene, and its gene expression was enhanced in dark-, ethephon-, ABA-induced senescent leaves.

SPCP1 was induced by ethephon in suspension cells

Sweet potato cell suspension were used to study ethephon effects on cell growth and *SPCP3* gene expression. The growth curve of sweet potato suspension cells was shown in Figure 7A. It took ca. 9 to 12 days after subculture for suspension cells to reach the stationary phase. For 1 mM ethephon treatment, cell growth was repressed ca. 18% that of untreated control at day 3, however, was not significantly different at day 6. For

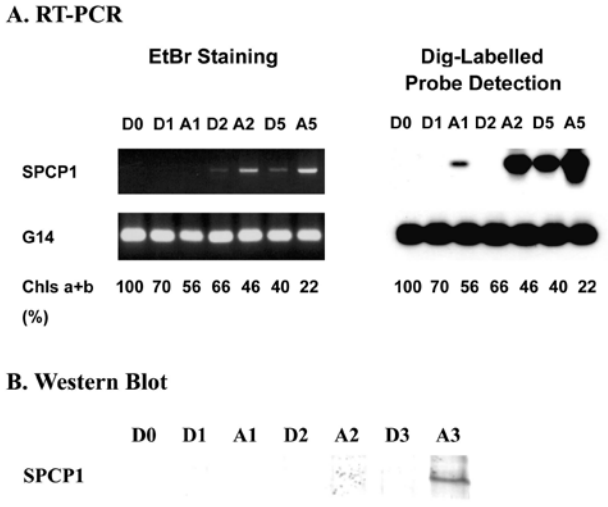

Figure 6. Effect of ABA on *SPCP1* gene expression in detached mature green leaves within a 5-day period. (A) RT-PCR products. Changes of *SPCP1* RT-PCR products in 100 µM ABA-treated and dark-treated mature green leaves were detected with ethidium bromide (EtBr) staining and non-radioactive digoxigenin-labelled *SPCP1* probe. *G14*, which encoded a metallothionein-like protein and exhibited a constitutive expression pattern, was used as a control. A and D denote ABA and dark treatments, respectively; (B) Western blot. Western blot was performed with the poly-clonal antibody raised against SPCP1. The experiments were performed three times and a representative one was shown.

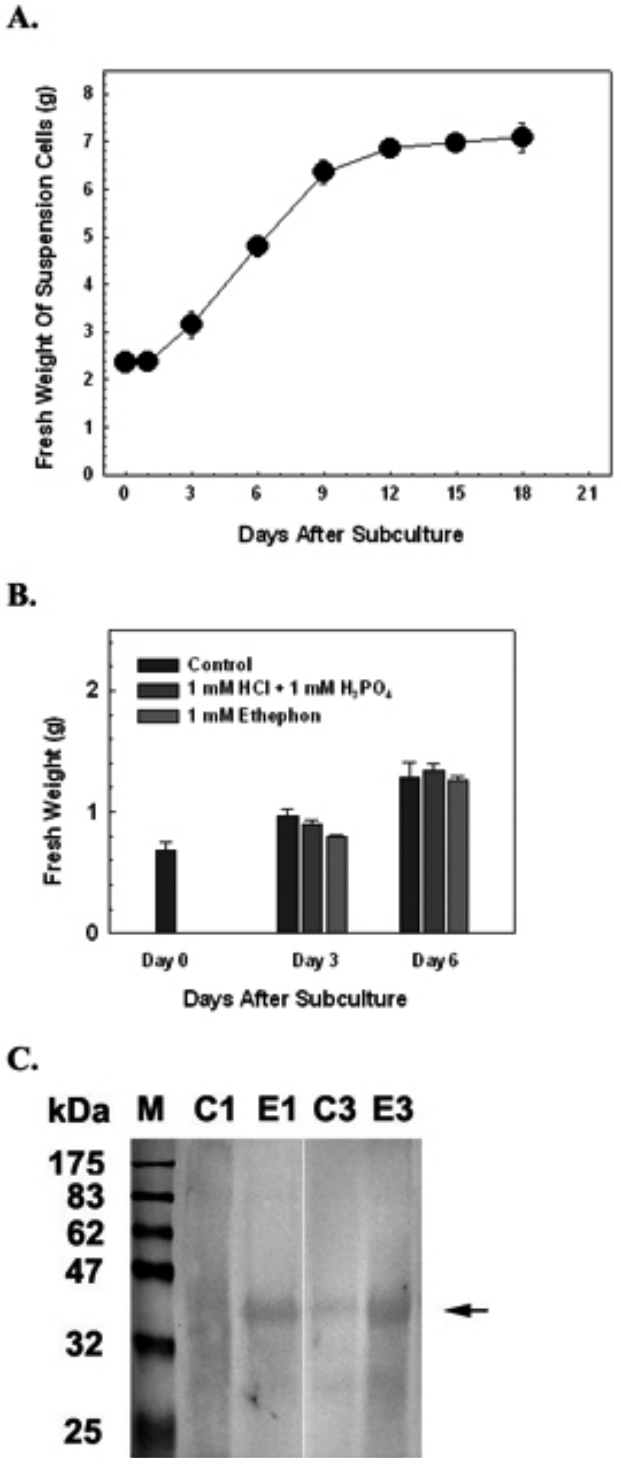

Figure 7. The effects of 1 mM ethephon on cell growth and induction of SPCP1 in suspension culture. (A) The growth curve of sweet potato suspension cells was determined within a 18-day period of culture, and the fresh weight of cells were measured at day 0, 1, 3, 6, 9, 12, 15 and 18 after subculture. (B) The effect of 1 mM ethephon on cell growth in suspension culture. Control and 1 mM HCl + 1 mM H_3PO_4 denote untreated control and control treated with 1 mM HCl + 1 mM H_3PO_4, respectively. (C) The induction of SPCP1 by 1 mM ethephon in suspension cells. C and E denote the control treated with 1 mM HCl + 1 mM H_3PO_4 and 1 mM ethephon-treated sample, respectively.

control treated with 1 mM HCl and 1 mM H_3PO_4, cell growth was also slightly repressed ca. 8% that of untreated control at day 3, and was almost the same at day 6 (Figure 7B). Western blot hybridization showed that SPCP1 was significantly induced and enhanced to express by 1 mM ethephon compared to control treated with 1 mM HCl and 1 mM H_3PO_4 at day 1 and day 3 (Figure 7C). Evan blue staining showed that the nuclei of most cells were not stained and was viable in untreated control, and treatments with 1 mM or 2 mM ethephon. However, the nuclei of most cells were stained blue and died in 10 mM ethephon treatment. These results conclude that 1 mM ethephon do not drastically cause cell death in cell suspension, however, can induce *SPCP1* gene expression in cell suspension culture.

DISCUSSION

Sweet potato *SPCP1* encoded a putative papain-like cysteine protease, and its ORF contained 339 amino acids (Figure 1). The predicted molecular mass was ca. 34 kDa. Western blot hybridization and protease activity assay detected a band with molecular mass near 36 kDa (Figure 3). The protease activity band was also sensitive to inhibitor E-64. In carrot cell suspemsion culture, different cysteine proteases with sequential development of protease activities during somatic embryogenesis were observed and exhibited sensitivity to E-64 protease inhibitor (Mitsuhashi et al., 2004). These data provide evidence to support the existence of a functional cysteine protease with a molecular mass near 36 kDa for *SPCP1* in sweet potato senescent leaves.

Sweet potato *SPCP1* gene expression was significantly enhanced at mRNA and protein levels in natural and induced senescent leaves (Figures 4, 5 and 6). Gene expression of nodule-specific papain-like cysteine proteases, such as AsNOD32 of Chinese milk vetch (*Astragalus sinicus*) (Naito et al., 2000) and Tr-cp of white clover (*Trifolium repens*) (Asp et al., 2006) were detected in the senescing zone and correlated with the onset of nodule senescence. In *Arabidopsis*, papain-like cysteine protease *SAG12* was detected mainly localized in senescence-associated vacuoles of mesophyll and guard cells of senescing leaves (Otegui et al., 2005). Our results agree with these reports and support sweet potato *SPCP1* a senescence-associated gene. In sweet potato, *SPCP1*

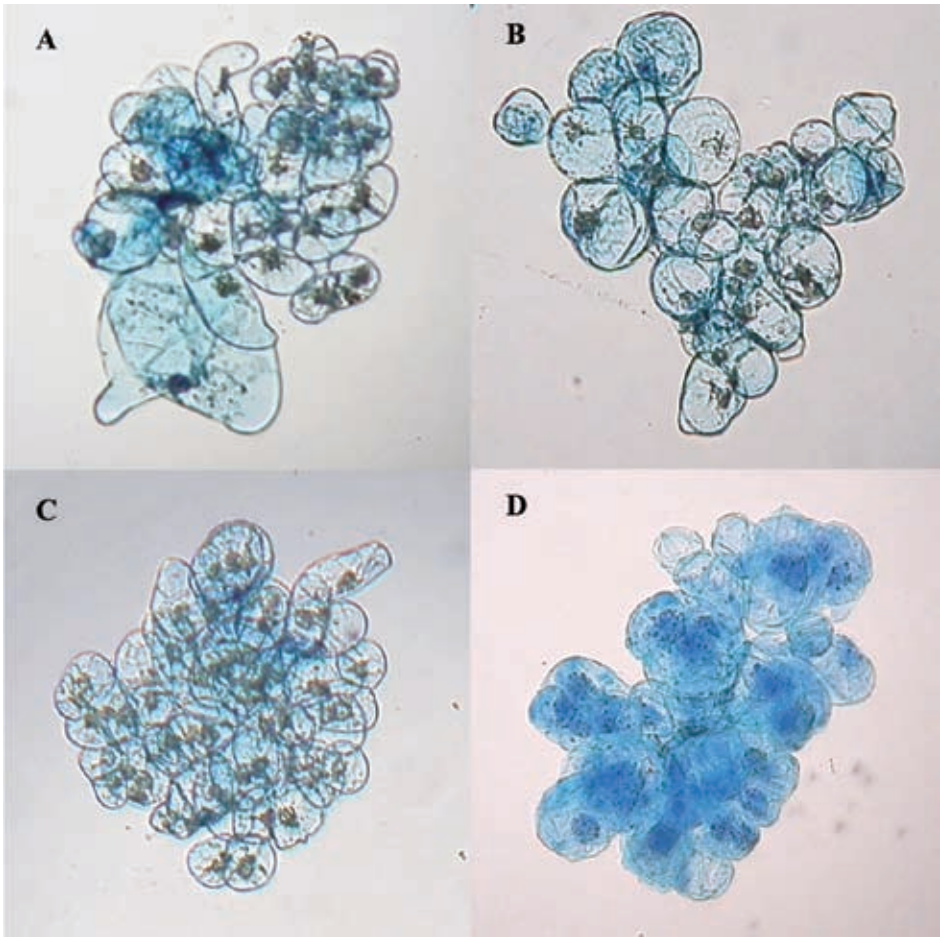

Figure 8. Evan blue staining of ethephon-treated sweet potato suspension cells. The suspension cells were treated with 0, 1, 2, and 10 mM ethephon, respectively, for 3 days, then were harvested and stained with Evan blue staining dye. (A), (B), (C), and (D) denote 0, 1, 2, and 10 mM ethephon-treated samples, respectively.

gene expression level could be induced by dark, ethephon, and ABA (Figures 4, 5, and 6). Buchanan-Wollaston et al. (2005) analyzed gene expression patterns and signal transduction pathways of senescence in *Arabidopsis* induced by different factors, including dark, starvation, ethylene, ABA, salicylic acid, and jasmonic acid (JA). Transcriptome analysis revealed that pathways such as dark, ethylene, and JA are all required for expression of many genes during developmental senescence. Genes associated with essential metabolic processes such as nitrogen degradation and mobilization can utilize alternative pathways for induction (Buchanan-Wallaston et al., 2005). These data provide a possible explanation for the induction of sweet potato *SPCP1* gene expression by different factors such as development, dark, ABA, and ethephon possibly due to or in association with multiple signal transduction pathways.

In natural and induced senescent leaves, the chlorophyll contents were used as a marker to indicate the senescence levels. During senescence, cysteine protease *SPCP1* expression was induced and enhanced. However, the time of *SPCP1* expression was later than the initiation time of chlorophyll degradation and decrease of chlorophyll content (Figures 4, 5 and 6). Therefore, the possible function of *SPCP1* may not be directly in association with the initiation of chlorophyll degradation. In *Arabidopsis*, transgenic plants expressing SAG12-GFP fusion protein under the control of *SAG12* promoter demonstrated that SAG12 was mainly localized in the senescence-associated vacuoles of chloroplast-containing mesophyll and guard cells in senescing leaves (Otegui et al., 2005). In tobacco, the senescence-associated vacuoles contain soluble chloroplast stromal proteins, including Rubisco and glutamine synthase, but lack the thylakoid proteins, such as D1 protein, LHCII of the PSII reaction center and PSII antenna. The Rubisco levels decreased steadily in senescence-associated vacuoles incubated at 30°C, and was completely abolished by addition of protease inhibitors (Martinez et al., 2008). Our results agree with these reports and suggest that sweet potato *SPCP1* may not be directly in association with chlorophyll degradation during leaf senescence.

In sweet potato, *SPCP1* gene expression level is higher in ethephon-induced senescent leaves (Figures 5 and 7C). The effect of ethephon as a senescence accelerator is indirect. It is first decomposed into HCl, phosphate and ethylene in a 1:1:1 ratio before the ethylene action. In order to prevent the side effects caused by HCl and phosphate, final concentrations of 1 mM HCl and 1 mM phosphate were added to the medium as a control when 1 mM ethephon was used for treatment. No significant effects of HCl and phosphate at the concentration applied on *SPCP1* gene induction were concluded (Figures 5 and 7C). Leaf senescence is considered as a type of programmed cell death (Lim et al., 2007). Leaf senescence was induced by 1 mM ethephon treatment (Figure 5). However, 1 mM ethephon treatment did repress cell growth at the beginning, but did not cause significant cell death in suspension cells (Figures 7 and 8). *SPCP1* gene expression was induced and enhanced drastically in both 1 mM ethephon-treated mature green leaves and suspension cells. These data suggest that *SPCP1* gene expression may not be directly in association with programmed cell death. In tobacco, different markers, such as senescence marker (*SAG12*) and HR cell death markers (*HIN1* and *HSR203J*), were used to study the programmed cell death (PCD) caused by incompatible pathogen interaction and senescence. The *HSR203J* is upregulated during HR but not during leaf senescence. However, *Arabidopsis SAG12* gene expression is associated with leaf senescence but is not detected in the HR PCD (Pontier et al., 1999). With T-DNA insertion mutagenesis, a sag12-2 *Arabidopsis* mutant was generated and used to study the correlation of phenotypic effect and gene expression with wild type. *SAG12* gene expression was detected in wild type, but not in sag12-2 mutant. However, absence of altered senescence phenotype could be observed between wild type and sag12-2 mutant (Otegui et al., 2005). These results clearly demonstrated that *SAG12* is not required for the visual progression of leaf senescence, and support that sweet potato *SPCP1* may not be directly in association with chlorophyll degradation and cell death during leaf senescence. The dose of 1 mM ethephon treatment, which caused leaf yellowing and final cell death in treated leaves, however, did not induced significant cell death in treated suspension cells (Figures 5, 7 and 8). The reason is not clear. However, the variation of constituents between (a) the medium for cell suspension culture (MS with 3% sucrose, B5 vitamin, and 1 ppm 2,4-D) and (b) the buffer for detached leaves (0.3 mM MES, pH 7.0) may partly respond for the difference.

The possible physiological role and function of *SPCP1* in sweet potato senescent leaves are not clear. In *Arabidopsis*, *SAG12* was expressed in nitrogen deprivation-induced senescence, and correlated at the whole plant level with the sink/source transition for nitrogen during both developmental and nutrient sress-induced leaf senescence (Gombert et al., 2006). Senescence-associated vacuoles were involved in the degradation of soluble chloroplast stroma proteins in tobacco leaves (Martinez et al., 2008). In castor bean (*Ricinus communis* L.), a papain-like cysteine protease with a C-terminal KDEL was immunolocalized specifically in ricinosome, which was organelles co-purified with glyoxysomes from germinating *Ricinus* endosperm, a senescing tissue (Schmid et al., 1998). The papain-like cysteine endoprotease has the capacity to process the glyoxysomal malate dehydrogenase precursor protein into the mature subunit *in vitro* (Gietl e al., 1997). These data suggest a possible role for these papain-like cysteine proteases in association with the clevage and maturation of proproteinases that are involved in macromolecule degradation and re-mobilization during leaf senescence. Whether sweet potato *SPCP1* play a role in association with macromolecule degradation and remobilization as mentioned above await for further

investigation. We conclude that sweet potato *SPCP1* is a functional senescence-associated gene with cysteine protease activity. It is not directly in association with chlorophyll degradation and programmed cell death during leaf senescence.

Acknowledgment. The authors thank the financial support (NSC95-2313-B-110-006-MY2) from the National Science Council, Taiwan.

LITERATURE CITED

Asp, T., S. Bowra, S. Borg, and P.B. Holm. 2004. Cloning and characterization of three groups of cysteine protease genes expressed in the senescing zones of white clover (*Trifolium repens*) nodules. Plant Sci. **167:** 825-837.

Buchanan-Wollaston V. 1997. The molecular biology of leaf senescence. J. Exp. Bot. **48:** 181-199.

Buchanan-Wollaston, V., T. Page, E. Harrison, E. Breeze, and P.O. Lim. 2005. Comparative transcriptome analysis reveals significanct differences in gene expression and signaling pathways between developmental and dark/starvation-induced senescence in *Arabidopsis*. Plant J. **42:** 567-585.

Chen, H.J., W.C. Hou, W.N. Jane, and Y.H. Lin. 2000. Isolation and characterization of an isocitrate lyase gene from senescent leaves of sweet potato (*Ipomoea batatas* cv. Tainong 57). J. Plant Physiol. **157:** 669-676.

Chen, H.J., W.C. Hou, J.S. Liu, C.Y. Yang, D.J. Huang, and Y.H. Lin. 2004. Molecular cloning and characterization of a cDNA encoding asparaginyl endopeptidase from sweet potato (*Ipomoea batatas* (L.) Lam) senescent leaves. J. Exp. Bot. **55:** 825-835.

Chen, H.J., W.C. Hou, C.Y. Yang, D.J. Huang, J.S. Liu, and Y.H. Lin. 2003. Molecular cloning of two metallothionein-like protein genes with differential expression patterns from sweet potato (*Ipomoea batatas* (L.) Lam.) leaves. J. Plant Physiol. **160:** 547-555.

Chen, H.J., G.J. Huang, W.C. Hou, J.S. Liu, and Y.H. Lin. 2006. Molecular cloning and characterization of a granulin-containing cysteine protease SPCP3 from sweet potato (*Ipomoea batatas*) senescent leaves. J. Plant Physiol. **163:** 863-876.

Chen, H.J., I.C. Wen, G.J. Huang, W.C. Hou, and Y.H. Lin. 2008. Expression of sweet potato asparaginyl endopeptidase caused altered phenotypic characteristics in transgenic *Arabidopsis*. Bot. Stud. **49:** 109-117.

Gietl, C., B. Wimmer, J. Adamec, and F. Kalousek. 1997. A cysteine endopeptidase isolated from castor bean endosperm microbodies processes the glyoxysomal malate dehydrogenase precursor protein. Plant Physiol. **113:** 863-871.

Goetting-Minesky, M.P. and B.C. Mullin. 1994. Differential gene expression in an actinorhizal symbiosis: Evidence for a nodule-specific cysteine proteinase. Proc. Natl. Acad. Sci. USA **91:** 9891-9895.

Gombert, J., P. Etienne, A. Ourry, and F.L. Dily. 2006. The expression patterns of SAG12/Cab genes reveal the spatial and temporal progression of leaf senescence in *Brassica napus* L. with sensitivity to the environment. J. Exp. Bot. **57:** 1949-1956.

Hattori, T., T. Nakagawa, M. Maeshima, K. Nakamura, and T. Asahi. 1985. Molecular cloning and nucleotide sequence of cDNA for sporamin, the major soluble protein of sweet potato tuberous roots. Plant Mol. Biol. **5:** 313-320.

Hou, W.C., Y.C. Chen, H.J. Chen, Y. H. Lin, L.L. Yang, and M. H. Lee. 2001. Antioxidant activities of trypsin inhibitor, a 33 kDa root storage protein of sweet potato (*Ipomoea batatas* (L.) Lam cv. Tainong 57). J. Agri. Food Chem. **49:** 2978-2981.

Hou, W.C., H.J. Chen, and Y.H. Lin. 2003. Antioxidant peptides with angiotensin converting enzyme inhibitory activities and applications for angiotensin converting enzyme purification. J. Agri. Food Chem. **51:** 1706-1709.

Huang, D.J., W.C. Hou, H.J. Chen, and Y.H. Lin. 2006. Sweet potato (*Ipomoea batatas* [L.] Lam 'Tainong 57') storage root mucilage exhibited angiotensin converting enzyme inhibitory activity in vitro. Bot. Stud. **47:** 397-402.

Huang, G.J., H.J. Chen, Y.S. Chang, M.J. Shue, and Y.H. Lin. 2007a. Recombinant sporamin and its synthesized peptides with antioxidant activities in vitro. Bot. Stud. **48:** 133-140.

Huang, G.J., M.J. Sheu, H.J. Chen, Y.S. Chang, and Y.H. Lin. 2007b. Inhibition of reactive nitrogen species in vitro and ex vivo by trypsin inhibitor from sweet potato 'Tainong 57' storage roots. J. Agri. Food Chem. **55:** 6000-6006.

Huang, G.J., M.J. Sheu, H.J. Chen, Y.S. Chang, and Y.H. Lin. 2007c. Growth inhibition and induction of apoptosis in NB4 promyelocytic leukemia cells by trypsin inhibitor from sweet potato storage roots. J. Agri. Food Chem. **55:** 2548-2553.

Jonson, T., E.H. Mahlamaki, R. Karhu, L. Gorunova, B. Johansson, and M. Huglund. 2000. Characterization of genomically amplified segments using PCR: Optimizing relative-PCR for reliable and simple gene expression and gene copy analyses. Gen. Chrom. Cancer **29:** 192-199.

Lee, T.M. and Y.H. Lin. 1995. Trypsin inhibitor and trypsin-like protease activity in air- or submergence-grown rice (*Oryza sativa* L.) coleoptiles. Plant Sci. **106:** 43-54.

Lichtenthaler, H.K. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Meth. Enzym. **148:** 350-382.

Lim, P.O., H.J. Kim, and H.G. Nam. 2007. Leaf senescence. Annu. Rev. Plant Biol. **58:** 115-136.

Makino, A. and B. Osmond. 1991. Effects of nitrogen nutrition on nitrogen partitioning between chloroplasts and mitochondria in pea and wheat. Plant Physiol. **96:** 355-362.

Martinez, D.E., M.L. Costa, F.M. Gomez, M.S. Otegui, and J.J. Guiamet. 2008. "Senescence-associated vacuoles" are involved in the degradation of chloroplast proteins in tobacco leaves. Plant J. (Published Online 28 June, 2008).

Marty, F. 1999. Plant vacuoles. Plant Cell **11:** 587-599.

Mitsuhashi, W., T. Yamashita, T. Toyomasu, Y. Kashiwagi, and T. Konnai. 2004. Sequential development of cysteine proteinase activities and gene expression during somatic embryogenesis in carrot. Biosci. Biotechnol. Biochem. **68:** 705-713.

Naito, Y., M. Fujie, S. Usami, Y. Murooka, and T. Yamada. 2000. The involvement of a cysteine proteinase in the nodule development in Chinese milk vetch infected with *Mesorhizobium huakuii* subsp. Rengei. Plant Physiol. **124:** 1087-1095.

Noh, Y.S. and R.M. Amasino. 1999. Regulation of developmental senescence is conserved between *Arabidopsis* and *Brassica napus*. Plant Mol. Biol. **41:** 195-206.

Okamoto, T. and T. Minamikawa. 1999. Molecular cloning and characterization of *Vigna mungo* processing enzyme 1 (VmPE-1), an asparaginyl endopeptidase possibly involved in post-translational processing of a vacuolar cysteine endopeptidase (SH-EP). Plant Mol. Biol. **39:** 63-73.

Otegui, M.S., Y.S. Noh, D.E.Martinez, M.G. Vila Petroff, L.A. Staehelin, R.M. Amasino, and J.J. Guiamet. 2005. Senescence-associated vacuoles with intense proteolytic activity develop in leaves of *Arabidopsis* and soybean. Plant J. **41:** 831-844.

Pontier, D., S. Gan, R.M. Amasino, D. Roby, and E. Lam. 1999. Markers for hypersensitive response and sequence show distinct patterns of expression. Plant Mol. Biol. **39:** 1243-1255.

Pourtau, N., R. Jennings, E. Pelzer, J. Pallas, and A. Wingler. 2006. Effect of sugar-induced senescence on gene expression and implications for the regulation of senescence in *Arabidopsis*. Planta **224:** 556-568.

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

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press.

Schlereth, A, D. Standhardt, H.P. Mock, and K. Muntz. 2001. Stored cysteine proteinases start globulin mobilization in protein bodies of embryonic axes and cotyledons during vetch (*Vicia sativa* L.) seed germination. Planta **212:** 718-727.

Schmid, M., D. Simpson, F. Kalousek, and C. Gietl. 1998. A cysteine endopeptidase with a C-terminal KDEL motif isolated from castor bean endosperm is a marker enzyme for the ricinosome, a putative lytic compartment. Planta **206:** 466-475.

Sihachakr, D., R. Haïcour, J.M. Cavalcante Alves, I. Umboh, D. Nzoghé, A. Servaes, and G. Ducreux. 1997. Plant regeneration in sweet potato (*Ipomoea batatas* L., Convolvulaceae). Euphytica **96:** 143-152.

Vierstra, R.D. 1996. Proteolysis in plants: mechanisms and functions. Plant Mol. Biol. **32:** 275-302.

Vincent, J.L., M.R.Knox, T.H. Ellis, P. Kalo, G.B. Kiss, and N.J. Brewin. 2000. Nodule-expressed Cyp15a cysteine protease genes map to syntenic genome regions in *Pisum* and *Medicago* spp. Mol. Plant Microbe. Int. **13:** 715-723.

Yang, T.H., Y.C. Tsai, C.T. Hseu, H.S. Ko, S.W. Chen, and R.Q. Blackwell. 1975. Protein content and its amino acid distribution of locally produced rice and sweet potato in Taiwan. J. Chin. Agri. Chem. Soc. **13:** 132-138.

Yoshida, S. 2003. Molecular regulation of leaf senescence. Curr. Opin. Plant Biol. **6:** 79-84.

從甘藷老化葉片分子選殖及表現半胱胺酸蛋白分解酶

陳顯榮[1] 黃冠中[2] 陳巍珊[3] 蘇正庭[3] 侯文琪[4] 林耀輝[5]

[1] 中山大學 生物科學系
[2] 中國醫藥大學 中國藥學研究所
[3] 中國文化大學 生物科技研究所
[4] 台北醫學大學 生藥學研究所
[5] 中央研究院 植物暨微生物研究所

本研究報告從甘藷老化葉片分離出一條全長的 cDNA 稱之 *SPCP1*，*SPCP1* 其 ORF 含有 1020 個核苷酸（339 氨基酸）且與不同物種包括赤楊、阿拉伯芥、紫雲英、油菜、胡蘿蔔、棉花、大麥、鳶尾、苜蓿、菸草、水稻、蓖麻、及白花三葉草等類似木瓜酶的半胱胺酸蛋白分解酶胺基酸序列具有高相似性（約 58% 至 74%)。半定量 RT-PCR 及西方墨點雜交結果顯示 *SPCP1* 在自然老化及黑暗、ethephon、及 ABA 誘導的老化葉片中會被活化及增加表現量，然而於成熟的綠色葉片、莖、及根中 *SPCP1* 並無顯著增加。葉片老化過程中葉綠素開始降解的時間比 *SPCP1* 基因表現早。1 mM ethephon 處理對於甘藷懸浮培養細胞亦會誘導 *SPCP1* 的表現，Evan blue 染色結果顯示 ethephon 處理濃度高至 2 mM 時對懸浮培養細胞亦無顯著影響，然而 10 mM 濃度時大部份的細胞會死亡。依據這些實驗結果我們結論甘藷 *SPCP1* 為有功能的老化相關基因，其 mRNA 及蛋白質的表現於天然的或誘導的老化葉片及懸浮培養細胞皆顯著增加，*SPCP1* 的功能與角色可能與老化過程中葉綠素開始降解及細胞死亡無關。

關鍵詞：半胱胺酸蛋白分解酶 ；Ethephon； 葉片老化 ；*SPCP1*； 甘藷。