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RESEARCH ARTICLE

Application of rice (*Oryza sativa* L.) suspension culture in studying senescence *in vitro* (I). Single strand preferring nuclease activity

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Single Strand-Preferring Nucleases (SSPNs) have been implicated in the triggering and progress of cell death pathways, including senescence in higher plants, though the biological roles of such enzymes are still obscure. In the present study, heterotrophic cell suspension cultures of *Oryza sativa* L. (rice) *cv* Taipei 309 were used to investigate changes in Single Strand-Preferring Nuclease activity associated with cell death *in vitro*. An acid nuclease activity (pH 5.5) was found which was strongly stimulated in the presence of 10 mM Zn^{2+} and inhibited by 10 mM EDTA and EGTA. An increase in SSPN activity was concomitant with a loss of cell viability, total protein and the onset of stationary phase of growth in the cell cultures. Using DNA-SDS-PAGE two major SSPNs were detected with the molecular weights of 26 KDa and 53.5 KDa. There are possibilities that these two enzymes represent monomeric and dimeric forms of the same nuclease or they are two different enzymes. The 26 KDa nuclease was partially purified using heparin-Sepharose column chromatography. The results confirm the value of plant cell suspension cultures for the investigation of the molecular process underlying plant cell ageing, senescence processes and programmed cell death (PCD). Possible senescence-associated roles of SSPNs are discussed.

Enzymes capable of degrading double and or single-stranded nucleic acids have been reported in a number of plant species including wheat leaves (Blank and McKeon, 1989; Blank and McKeon, 1991a; Blank and McKeon, 1991b), barley leaves (Wood et al. 1998), lentil seedlings (Kefalas and Yupsanis, 1995), mung bean (Kowalski et al. 1976), wheat chloroplasts (Kuligowska et al. 1988; Monoko et al. 1994), alfalfa seedlings (Yupsanis et al. 1996), sugarcane suspension cultures (Thom et al. 1982), pea chloroplasts (Kumar et al. 1995), cultured mesophyll cells of *Zinnia elegans* which have been induced to undergo xylogenesis (Thelen and Northcote, 1989) and tomato protoplasts (Abel and Glund, 1986). The biological roles of plant nucleases are not well elucidated, though it is likely that such enzymes participate in nucleic acid catabolism and play roles in DNA replication, repair and recombination as well as the control of gene expression (Hanson and Fairley, 1969; Kumar et al. 1995; Yupsanis et al. 1996).

Recent evidence suggests that nucleases may play a role in programmed cell death processes (PCD) in plants. PCD underlies several aspects of plant development, senescence and defence (Greenberg, 1996; Jones and Dangl, 1996; and Lam, 1997). Such processes may, perhaps, be mechanistically similar to animal cell apoptosis, a

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specialised form of PCD, in that affected plant cells exibit condensation and vacuolisation of the cytoplasm and cell shrinkage (Mittler and Lam, 1995a; Yen and Yang, 1998). In addition, some types of plant cell death are accompanied by endonucleotic DNA cleavage and degradation, a hallmark of apoptosis (Ryerson and Heath, 1996; Wang et al. 1996). Finally, a homologue of the gene, *dad1*, known to be involved in repressing apoptosis in animals (Nakashima et al. 1993; Sugimoto et al. 1995), has been recently isolated in *Arabidopsis* (Gallois et al. 1997) and rice plants (Tanaka et al. 1997), suggesting that some features of genetically programmed cell death are conserved between plants and animals.

In vitro cultures constitute useful and easily manipulated systems for identifying molecular factors associated with cell senescence and PCD in plants (McCabe et al. 1997). For example, *Arabidopsis* cell cultures were employed to demonstrate similarities and differences between molecular processes occurring during senescence of whole plants and cultured cells (Callard et al. 1996). De Jong et al. (2000) used tomato cell suspension cultures and found similarities between characteristic symptoms of apoptosis in animal cells and programmed cell death in tomato cell cultures. Additionally, cultured mesophyll cells of *Zinnia elegance* provide a model for the investigation of xylem differentiation, which is accompanied by cell death and DNA degradation (Mittler and Lam, 1995b).

The activity of many plant nucleases is pH and/or ion-dependent. Some nucleases show maximum activity at acid pHs, such as the SSPNs found in barley (pH 6; Brown and Ho, 1986) and *Zinnia elegans* (pH 5.5; Thelen and Northcote, 1989). Some SSPNs exhibit maximum activity at alkaline pHs, such as those found in scallion (pH 8.5-9; Uchida et al. 1993) and lentil (pH 8; Kefalas and Yupsanis, 1995). Thelen and Northcote, 1989 found a pH and Zn^{2+}-dependent SSPN in *Zinnia elegans* during xylogenesis. Kefalas and Yupsanis, 1995 reported a pH and Ca^{2+}-dependent SSPN in lentil. However, cell cultures have some disadvantages that should be taken into consideration, *i.e.* phosphate starvation could induce nuclease activity and also despite similarities, cell cultures are dedifferentiated and unorganised, compared to the organised leaf cells of a whole plant. Therefore, the results obtained from cell cultures should be compared with those of the whole plant in order to be able to expand and relate the results. In the case of rice, our results confirm what Sodmergen et al. 1991 found. They reported the existence of several ion-dependent DNases in senescing rice leaves, four of which being Zn^{2+}-dependent. They also related the breakdown of cpDNA in senescing rice leaves to the activity of zinc dependent nucleases. Furthermore, as a family member with rice, it has been shown in barley that SSPN activity is increased in dark-induced and natural leaf senescence (Wood et al. 1998).

In the present study, cell suspension cultures of *Oryza sativa* (L.) *cv* Taipei 309 were employed to demonstrate a correlation between increased SSPN activity with the onset of cell senescence *in vitro*. The potential functions of SSPNs are discussed in the context of plant cell senescence and PCD *in vivo* and *in vitro*. Here we also describe the biochemical characterisation of SSPN activity in senescing rice cells. The effect of different ions and chelating agents on the SSPN activity of rice cell extracts have been assessed.

Materials and Methods

Plant material

Taipei 309 cell suspension cultures were initiated from embryogenic calli induced from mature rice seed scutella (Finch, 1991). Cells were maintained in 100 ml conical flasks in modified AA2 medium, based on the formulation of Müller and Grafe, 1978 as modified by Abdullah et al. 1986. Suspensions were incubated on a rotary shaker (120 rpm) at 28 ± 2ºC in the dark. The suspension cultures were subcultured every 7 d. For the determination of growth kinetics, changes in SSPN activity and cell viability, suspension cultures were grown for four weeks post-subculture without transferring to the fresh medium. It should be noted that in Figure1, Figure 2, Figure 3 and Figure 4, error bars represent ± standard error for the corresponding replication.

Determination of growth kinetics of cell suspension cultures

The growth kinetics was measured by determining the changes in fresh weight (g/flask) with time. One ml of rice cells was inoculated into preweighed 100 ml conical flasks containing 34 ml of AA2 medium. The fresh weight gained (g/flask) was expressed as the difference between the initial and final weights.

Determination of cell viability (TTC assay)

In order to determine changes in cell viability during senescence of rice cells, TTC assay was carried out. Cell viability was assayed by the conversion of 2,3,5 triphenyltetrazolium salt (TTC) into red formazan by dehydrogenase activity of viable cells (Towill and Mazur, 1975).

Extraction of soluble protein and determination of protein concentration

Rice cells (1-, 2-, 3- and 4-week old cells) (3 replicates) were grown in 100 ml conical flasks for 4 weeks and collected every week by sieving through 30 µm-pore sieves. The cells (1-4 g f.wt) were air-dried, frozen in liquid

N_2 and ground to a fine powder in liquid N_2 with a pestle and mortar. Powdered cells were transferred into a 15 ml polypropylene screw-capped tube containing ice-cold extraction buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl and 1 mM N-ethylmaleimide;) in the ratio of 2 ml extraction buffer: 1g (f. wt). The suspension was vortexed for 30 sec and centrifuged (3600 x g) in a bench top swinging bucket centrifuge for 20 min at room temperature. The supernatant was dispensed in 1.5 ml Eppendorf tubes and used for nuclease activity assays or stored at -70°C for future use. Protein concentration of supernatants was determined using the Bio-Rad protein assay (Bio-Rad Laboratories http://www.bio-rad.com) according to the manufacturer's recommendation. Bovine serum albumin (BSA Fraction V) was added as a standard.

Determination of nuclease activity using an in vitro assay

Nuclease activity was determined by the release of acid-soluble nucleotides from single-stranded (ss) or double-strand (ds) calf thymus DNA, following the method of Blank and McKeon, 1989, with minor modifications. Double-stranded calf thymus DNA (Sigma) was dissolved (1mg/ml) in sterile distilled water (SDW), boiled for 10 min and placed on ice immediately to prevent the renaturation of the single-stranded DNA. The assay mixture (300 µl) contained 0.02% (w/v) DNA (ss or ds), 0.01% (w/v) BSA (Sigma), 25 µl cell extract and 1 or 10 mM of a divalent cation (Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+} or Zn^{2+}). Duplicate reactions were stopped after 0 and 20 min by the addition of 1 ml 3.5% (v/v) perchloric acid, then centrifuged (12000 x g) in a bench-top microcentrifuge for 5 min (room temperature) to precipitate undigested nucleic acid. The absorbance (A) of the supernatant was determined at 260 nm. Nucleotide release from ss-DNA substrates was proportional to the change in A_{260} from 0-20 min of the reaction. Enzyme activity was expressed as δA_{260} mg of $protein^{-1} min^{-1}$ (mg of protein is the amount of protein in 25 µl cell extract, which was calculated separately for each assay). In order to visualise the nucleases present in rice cell extracts and as a means of further biochemical analysis of nuclease activities, cell extracts were analysed by DNA-SDS-PAGE.

Separation of proteins in rice cell extracts was performed according to the method of Laemmli, 1970, using one dimensional discontinuous PAGE (Mighty Small II gel kit, Höffer Scientific, UK). Substrate ss-DNA was included in the gel matrix (Blank et al. 1983). Nuclease activity was detected by 'negative staining' of gels with toluidine blue (0.01 % (w/v); Sigma, Dorset, UK) for 20 min, followed by destaining in scrubbed reverse-osmosis water for 10 min and photographed (Blank et al. 1983). SSPNs were visualised as colourless bands against a dark blue background of stained (undigested) substrate. Molecular weights of rice SSPNs were determined by comparison of their mobilities with the mobilities of known prestained SDS-PAGE molecular weight markers, namely, (β-galactosidase (180 KDa), fructose-6 phosphate dehydrogenase (116 KDa), pyruvate kinase (84 KDa), fumarase (58 KDa) and triosephosphate isomerase (26.6) KDa; (Sigma)).

pHs used for the nuclease activity assay

Following that rice nucleases have the optimum activity at pH 5.5 (Sodmergen et al. 1991) and barley nucleases at pH range of 7-7.5 (Wood et al. 1998), only two pHs were used in all nuclease activity assays, using 0.1 M Tris-HCl, to make pH 7.4 and 0.1 M citrate buffer to make pH 5.5.

Heparin-Sepharose purification of SSPN from cell culture filtrate

The culture filtrate from 3-week-old Taipei 309 cell suspensions (100 ml) was treated with acetone to precipitate proteins. The proteins were redissolved in the extraction buffer. The buffer was substituted with 0.01 M citrate buffer, pH 5.5, 0.001 M N-ethylmaleimide, using Microsep Microconcentrator tubes (10 k cut off; Flowgen, UK) (see Discussion). Proteins were further concentrated by centrifugation at 7000 x *g* for 30 min at 10°C and transferred to fresh 1.5 ml Eppendorf tubes (fraction I). The protein concentration and SSPN activity of fraction I were determined. A heparin-Sepharose column (1 ml, Pharmacia Biotech, Uppsala, Sweden) was washed with 4 column volumes of the start buffer to remove the preservatives and equilibrated with 5 column volumes of 0.01 M citrate buffer (pH 5.5). One ml of fraction I was applied (0.3 ml/min) onto the column through a 1 ml syringe and the flow through, containing unbound proteins, was collected in a 1.5 ml Eppendorf tube. The column was washed (0.5 ml/min) with three column volumes of start buffer. Then the bound proteins were eluted (0.3 ml/min) with 1 column volume of the elution buffer (serial gradient of 50, 100, 150, 200, 400, 600, 800 and 1000 mM NaCl in the start buffer). Eluted fractions (250 µl) were collected and protein concentration and SSPN activity of each fraction were determined using a 10 µl sample of each. The fractions (eluted at 200, 400 and 600 mM NaCl) containing at least 0.01 units of enzyme activity (1 unit (U) is defined as the amount of enzyme yielding an δA_{260} of 0.01 min^{-1} in the standard *in vitro* assay) were pooled. The pooled fractions were transferred into two Microsep Microconcentrator tubes at a maximum volume of 1.5 ml, centrifuged at 7000 x *g* for 1 h at 10°C and transferred to a fresh 1.5 ml Eppendorf tube and stored at -20°C (fraction II). The amount of protein and enzyme activity in fraction II were determined.

Results

Growth kinetics, viability and protein content

Cell growth, measured by an increase in fresh weight (g/flask, displayed a sigmoidal-shaped growth curve, with exponential, a small decline in growth and finally, death phases. The increase in fresh weight continued for up to

two weeks, then followed by a slight decline phase between weeks 2 and 3 in which cell f.wt. fell by 15%. Finally a sharp fall in cell culture f.wt. after week 3 was observed (Figure 1). Cell viability measured by the TTC assay showed a drastic decrease from about 29 to about 7 A_{490} units/g.f.wt. from week 1 to week 2 and the decline continued in weeks 3 and 4 (Figure 2). Total protein content (mg/flask) of cells also showed decreases from 11.99 in week 1 to 8.62 in week 2, to 6.69 in week 3 and to 1.64 (mg/flask) in week 4 (Figure 3).

SSPN activity

Using extracts of 2-week-old Taipei 309 cells, as the source of nuclease activity and following the *in vitro* assay, an SSPN activity was found. This nuclease activity was further discovered to be zinc and acid pH dependent (see below). Subsequent analysis using cell extracts from 1-4 week old cells, revealed that the nuclease activity increased more than two folds from week 1 to week 2, remained relatively constant between weeks 2 and 3 and increased sharply in week 4 (Figure 4).

Ds nuclease activity

The ds nuclease activity of rice cell extract was very low compared to that of SSPN activity (33.8 and 18 fold less in the presence of 1 and 10 mM Zn^{2+}), indicating that the cell extracts did not contain any significant Ds nuclease activity (Table 1).

The effect of cations, EDTA and EGTA on SSPN activity

At pH 7.4, in the absence of added ions SSPN activity was 0.046, and in the presence of Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+} and Zn^{2+}, enzyme activity was 0.02, 0.01, 0.38, 0.06 and 0.02 δA_{260} mg of $protein^{-1}$ min^{-1}, respectively. These results compared to the enzyme activity at pH 5.5 and in the presence of zinc, clearly indicates that no significant SSPN activity is detectable at pH 7.4. The highest enzyme activity at pH 7.4 is observed with Cu^{2+}. However, gel activity assay revealed no Cu^{2+} dependent SSPNs (data not shown). At pH 5.5 and in the absence of added cations, the SSPN activity was 0.21 δA_{260} mg of $protein^{-1}$ min^{-1}. Addition of 1 mM Zn^{2+} caused an increase in SSPN activity to 2.12 and at 10 mM and it caused an increase to 1.6 δA_{260} mg of $protein^{-1}$ min^{-1} (Figure 5). Ca^{2+}, Mg^{2+}, Cu^{2+}, EDTA and EGTA had small inhibitory effects on the SSPN activity at 1 mM compared to the controls. Only EDTA and EGTA inhibited the enzyme activity at 10 mM while, at the same concentration, the cations had almost no effect on the nuclease activity compared to controls. Mn^{2+} did not show an inhibitory or stimulatory effect on the enzyme activity at either concentration. The most inhibitory effect was that of EDTA at 1 and 10 mM, where the SSPN activity was reduced to 0.02 and 0.03 δA_{260} mg of $protein^{-1}min^{-1}$, respectively (Figure 5). Inhibition of the SSPN activity by the chelating agents further indicates that the enzyme is cation-dependent.

DNA-SDS-PAGE analysis of nuclease activity in the presence of Zn^{2+}

Several Zn^{2+}-dependent SSPNs including 26 and 53.5 KDa enzymes were detected in extracts of senescing rice cells. Between the 26 and 53.5 KDa bands several diffuse bands were also observed. However, in comparison, the increase in the activity of 26 KDa nuclease was much more pronounced with age (Figure 6).

Partial purification of the 26 KDa nuclease

When nuclease activity assay was carried out on cell filtrates, the same 26 KDa and 53.5 KDa SSPNs were revealed on the gel (data not shown). Since cell filtrates had far less unwanted proteins, it proved much easier for enzyme purification, compared to the protein profiles from the cell extracts. The elution profile of SSPN from the heparin-Sepharose column is shown in Figure 7. A sample of the protein (5 µg) before (fraction I) and after purification (fraction II), were analysed by SDS-PAGE and DNA-SDS-PAGE (Figure 8). During heparin-Sepharose chromatography, the enzyme started to elute at 200mM NaCl and the elution continued up to 600 mM NaCl (Figure 7, shown as two vertical arrows). The dotted lines (--■--) show the amount of protein in each fraction. From fraction 6 onwards almost all proteins were eluted from the column. The enzyme activity was not present in the fractions 1-9 (▲). At fraction 9 the enzyme activity slightly increased and the highest enzyme activity was seen in fraction 11. Fractions 9, 10 and 11 correspond to 200, 400 and 600 mM NaCl ●). The enzyme was purified ca. 28 fold. Even though the heparin-Sepharose column removed a lot of unwanted proteins, the purification was not complete. For the purpose of further characterisation, purification to homogenity and sequencing the enzyme, another step of purification such as zinc chelating affinity column chromatography would have probably been beneficial.

Discussion

Cell cultures constitute an essential tool for developmental, biochemical and molecular biological investigations in the study of programmed cell death, in particular apoptosis in animal cells (Cotter and Al-Rubeai, 1995), but remain underexploited in such investigations in plant species. The omission is perhaps surprising given that cell cultures provide a more or less uniform population of synchronously developing plant cells. Furthermore, at least some aspects of gene expression during the programmed cell death pathway of leaf senescence also occur during senescence of plant cells *in vitro* (Callard et al. 1996; Tanaka et al. 1997).

Table 1. dsDNase activity of 2-week-old rice cell extract in the presence of cations, EDTA and EGTA (n = 3 ± standard error).

Ion/chelating agent	dsDNase activity(dA_{260} mg of protein^{-1} min^{-1})	
	1 mM	10 mM
Control	0.056 ± 0.02	0.056 ± 0.02
Ca	0.037 ± 0.01	0.026 ± 0.02
Mg	0.043 ± 0.00	0.058 ± 0.01
Mn	0.06 ± 0.02	0.024 ± 0.01
Cu	0.018 ± 0.01	0.027 ± 0.01
Zn	0.063 ± 0.00	0.089 ± 0.01
EDTA	0.019 ± 0.00	0.056 ± 0.01
EGTA	0.053 ±0.02	0.076 ± 0.02

One characteristic symptom of apoptosis in animal cells is nuclear DNA cleavage, brought about by nucleases that introduce double- and single-strand cuts in the DNA duplex (Bortner et al. 1995). Recently, a caspase (cysteine-containing aspartate-specific protease)-activated nuclease was purified and cloned from mouse cells undergoing apoptosis (Enari et al. 1998; Sakahira et al. 1998). Caspase like activities have been detected following the infection of tobacco plants with TMV and also in tomato cell cultures undergoing cell death by chemicals. (delPozo and Lam, 1998; De Jong et al. 2000). This has provided evidence for the presence of a caspase-like plant protease that participate in HR (delPozo and Lam, 1998) and chemically induced cell death. In plants, previous reports have suggested that nucleases may be involved in programmed cell death during xylogenesis (Thelen and Northcote, 1989; Mittler and Lam, 1995b; Fukuda, 1996, Fukuda et al. 1998), hypersensitive response (Mittler and Lam, 1995a; Ryerson and Heath, 1996; Wang et al. 1996) mechanical stress (Mittler and Lam, 1997), natural and induced leaf senescence (Blank and McKeon, 1989; Wood et al. 1998) and chemically induced senescence (De Jong et al. 2000). Conceptually these lines of evidence provide some information for the existence of (very) similar cell death pathways in both plant and animal systems. Stay green genotypes provide important sources to study senescence. Thomas and Howarth, 2000, Borrell et al. 2000 and Borrell et al. 2001, have discussed the status of stay green genotypes in studying senescence. Borrell et al. 2001 propose that at the whole plant level, the balance between nitrogen demand and supply, determines the greenness (or senescence) of the plant, and at the cell level, the retention of chloroplast proteins and consequently photosynthetic apparatus, plays the same role. This means that cellular components are the basic and crucial determining factors in the onset and progress of senescence. A thorough systematic understanding of senescence in plants has become a necessity as, for example, it has been calculated by Thomas and Howarth, 2000 that even delaying senescence in sorghum by 2d, will result in 11% increase in the total C contribution of a leaf. One way to better understanding of plant senescence is the use of cell cultures, as for example Callard et al. 1996 found similar gene expressions in cell suspension cultures undergoing senescence and organ senescence in *Arabidopsis*. We are also reporting the activity of similar zinc-dependent nucleases in senescing rice cell cultures with those found in senescing rice leaves under natural conditions (Sodmergen et al. 1991). In the present paper, therefore, we have employed heterotrophic suspension cultured cells of rice to preliminarily study the possible role of SSPN activity in the growth, senescence and death of plant cells.

The growth kinetics, as measured by change in g.f.wt. per flask with time, showed that the cells were in the rapid cell division phase between week 1 and 2 and reached the maximum growth at week 2. Thereafter, as expected, the cell f.wt. per flask began to decline, a trend which continued up to week 3. Then, the cell f.wt. per flask showed a sharp decline and reached a minimum at week 4.

Mosmann, 1983, suggested TTC test as an ideal colourimetric assay for measuring the viability of living cells as it utilise a colourless substrate and is converted to a coloured product by the living cells, but not by dead cells or tissue culture medium. Cell viability measured in this manner, showed a decrease throughout the culture period which was concomitant with a decrease in protein content. Changes in protein synthesis and degradation before or during senescence have been reported in many plant species (Camp et al. 1984; Cheng and Kao, 1984; Ford and Shibles, 1988; Klerk and VanLoon, 1997). It is possible that the decrease in protein content has affected the amount of dehydrogenase enzymes in the cells and in turn, has caused decreases observed in cell viability (see below). The TTC assay measures only the activity of one set of enzymes and there may be other alternatives or complementary assays required to better determine the status of cell viability.

The decrease in intracellular protein content in this experiment may in part be explained by a high rate of protein turnover, a process with critical importance to plant cells (Nooden and Leopold, 1988; Huffaker, 1990) and in part by leakage or secretion of proteins from senescing cells. High protein turnover rates are typical of cells that have entered stationary phase because the environmental conditions have changed, *i.e.* due to the exhaustion of nutrients. In this experiment, after 2 weeks, the cells had reached the maximum yield and already started the stationary phase of growth, thus, a high rate of protein turn over due to the exhaustion of nutrients and proteolytic activities could have caused the decrease in protein content. Recently De Jong et al. 2000 showed that in chemically induced cell death in tomato cell cultures, proteolysis played a critical role. However, despite the high rate of turn over in some proteins during senescence, some other proteins seem to be preferentially synthesized and/or protected against proteolytic enzymes (Klerk and VanLoon, 1997). Presence of zinc dependent SSPNs until late phases of cell growth in this research, may be one of such cases, which requires further investigations.

It was shown that there was an increase in SSPN activity in the cell cultures with the continuation of their incubation in the culture medium. Thus, increased SSPN activity was correlated with growth arrest, senescence and death of heterotrophic cultured cells. In some plants, RNase activity has been reported to increase during phosphate starvation (Bariola et al. 1994; Köck et al. 1995). RNases induced under phosphate starvation are thought to be a part of Pi starvation rescue proposed to exist in higher plants (Goldstein et al. 1988). One function of this system would be to liberate Pi from RNA to facilitate its remobilisation (Duff et al. 1991). Blank and McKeon, 1991a and Blank and McKeon, 1991b proposed a similar role for nucleases in senescing wheat leaves i.e nucleases play a major role in the economical use of nitrogen and its remobilisation from senescing organs to the filling grains. Even though cell cultures are unorganised clumps of cells and unlike whole plants remobilisation of elements to other organs or tissues does not mean much, but the trend of several events like increases in nuclease activity seems alike.

The results demonstrated that rice cells contain Zn^{2+} dependent SSPN activity. At least two SSPNs exist, 26 KDa and 53.5 KDa, with other possible activities of intermediate molecular weights. According to the Wilson, 1975, classification of plant nucleases, the 26 KDa nuclease shares some characteristics of plant nuclease I, such as: molecular weight, inhibition by EDTA and requirement for acid pH and a divalent cation for maximum activity or stability. A number of plant nucleases have been reported to be ion-dependent. Sodmergen et al. 1991 found several Ca^{2+}, Mn^{2+}, and Zn^{2+}-dependent nucleases in senescing rice leaves. The Zn^{2+}-dependent nucleases were sized 13, 15, 29 and 51 KDa. However, the Zn^{2+}-dependent nucleases were also active at pH 5.5. In *Zinnia* cells (Thelen and Northcote, 1989) undergoing xylogenesis a similar Zn^{2+} and acid pH-dependent SSPN with a molecular weight of 43 KDa was found.

Zinc, on one hand, has been found to be an essential requirement for the activity or stabilisation of several plant nucleases (Wilson, 1975; Blank and McKeon, 1989; Thelen and Northcote, 1989; Sodmergen et al. 1991; Uchida et al. 1993; Kumar et al. 1995; Yupsanis et al. 1996) and on the other hand it is an inhibitor of other plant nucleases (Monoko et al. 1994; Yupsanis et al. 1996). It is not clear whether Zn^{2+} is essential for the stabilisation of the proper tertiary or quaternary structure of the SSPN (Hanson and Fairley, 1969; Uchida et al. 1993) or in fact it influences the configuration of the substrate and thus affects the SSPN activity (Wilson, 1975).

In principle, SDS-PAGE should denature and separate the polypeptide subunits of multimeric enzymes. However, in the DNA-SDS-PAGE system employed in the present study, enzyme samples were not boiled in the denaturation buffer prior to electrophoresis. Therefore, one possibility is that the 26 and 53.5 KDa SSPN enzymes represent monomeric and dimeric forms of the same nuclease. Alternative possibilities are that the two enzymes are in fact two separate enzymes or the production of the 26 KDa enzyme is the result of proteolytic activity on the 53.5 KDa enzyme i.e the latter is a dimeric enzyme and is split into two monomers upon proteolytic activity. Dimeric SSPNs have been reported to be present in plants and both enzyme have been reported to be active (Kowalski et al. 1976).

Heparin column chromatography was employed to purify the 26 KDa nuclease from cell filtrates. The cell filtrates should have been enriched with the 26 KDa SSPN but it was observed that unexpectedly, fraction I was enriched

with the 53.5 KDa SSPN. This may have been due to the aggregation of the 26 KDa SSPNs after concentration by the 10 k cut-off Microconcentrator tubes. In the end of purification procedure, there was more of the 26 KDa SSPN than the 53.5 KDa SSPN. It would be advantageous if these two enzymes are purified to homogeneity and sequenced in order to discover whether there is only one dimeric enzyme with two monomers or there are two different enzymes.

The cell line used was normally maintained by weekly subculture; if cells are left in spent media for more than this period of time, they would most certainly be subject to stresses of nutrient depletion, low oxygen and possibly toxin accumulation. These are culture conditions that have been known to induce apoptosis in animal cells (Marvel et al. 1994; Cotter and Al-Rubeai, 1995) and recognised as circumstances predisposing PCD in plants Havel and Durzan, 1996a; Havel and Durzan, 1996b).

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APPENDIX

Figures

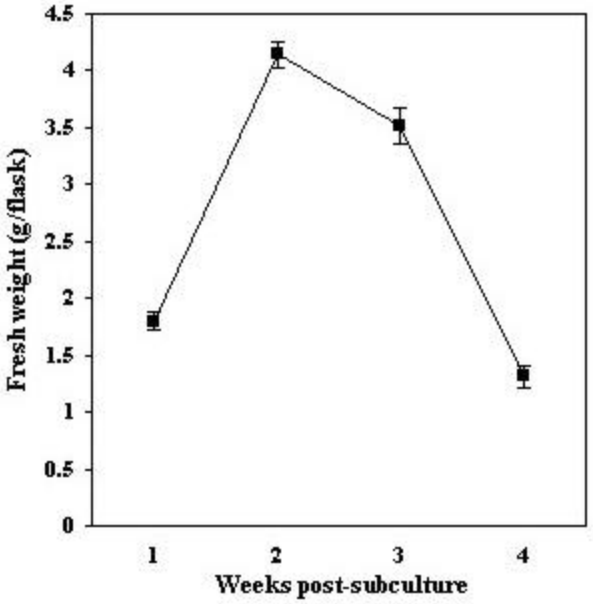

Figure 1. Growth kinetics of Taipei 309 cells (n=4, error bars represent standard error .

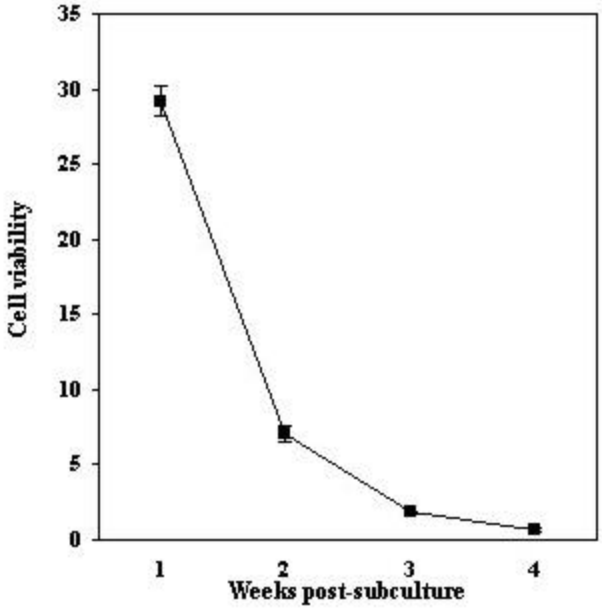

Figure 2. Cell viability measured by TTC assay (n=5, error bars represent standard error).

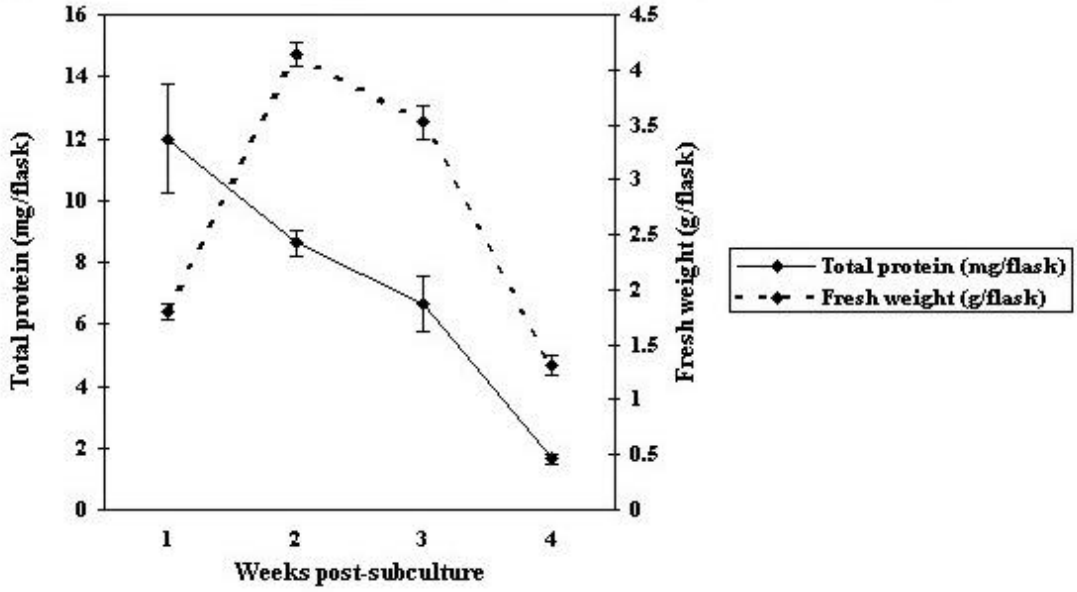

Figure 3.Total protein content of Taipei 309 cells (n=4, error bars represent standard error).

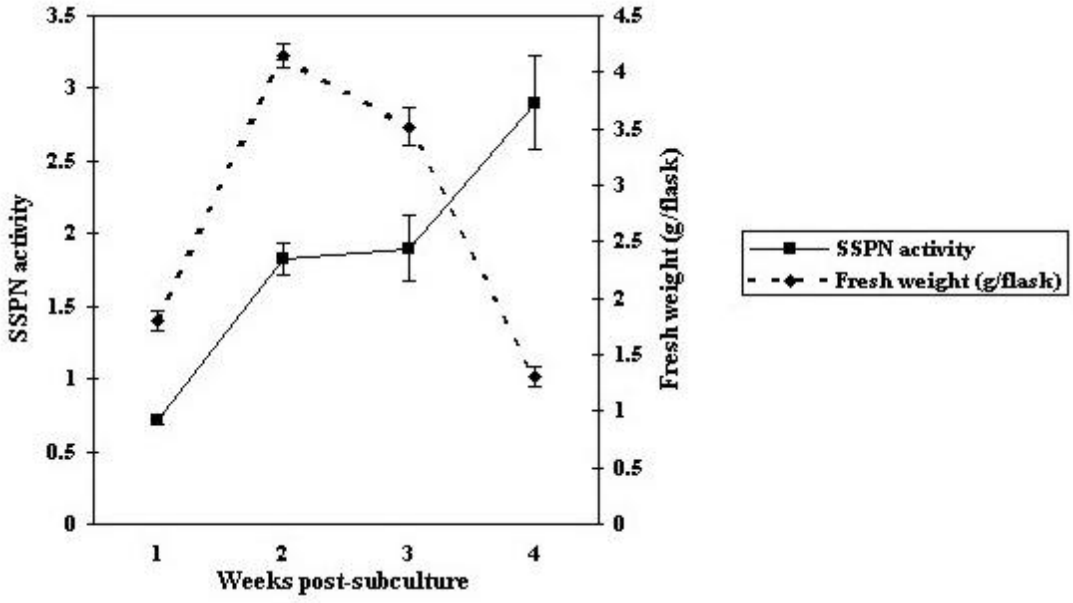

Figure 4. SSPN activity of Taipei 309 cells (n=3, error bars represent standard error).

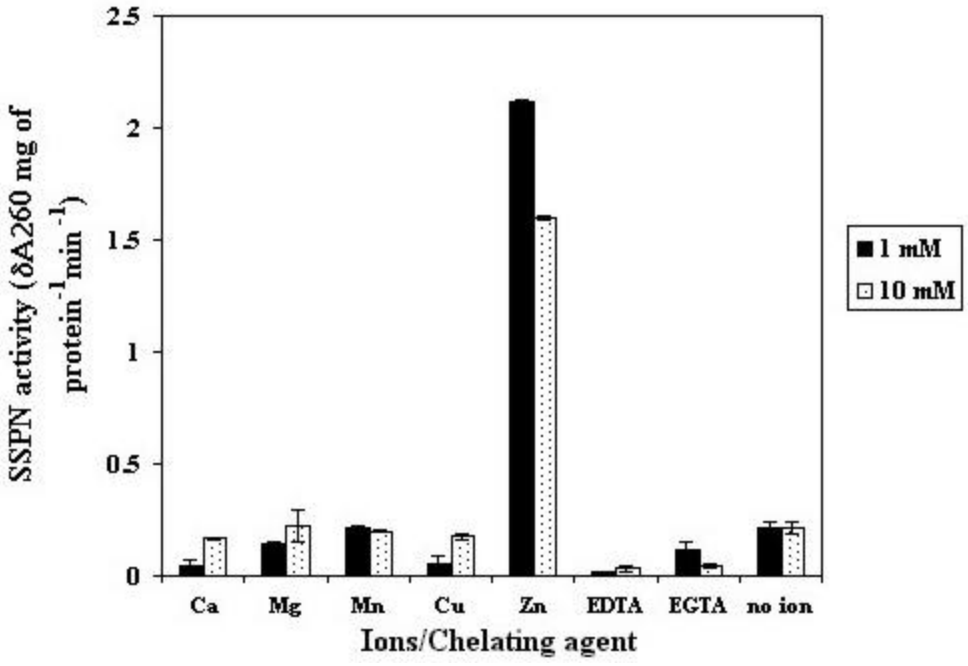

Figure 5. The effect of ions, EDTA and EGTA on SSPN activity of Taipei 309 cell extract (3 replicates).

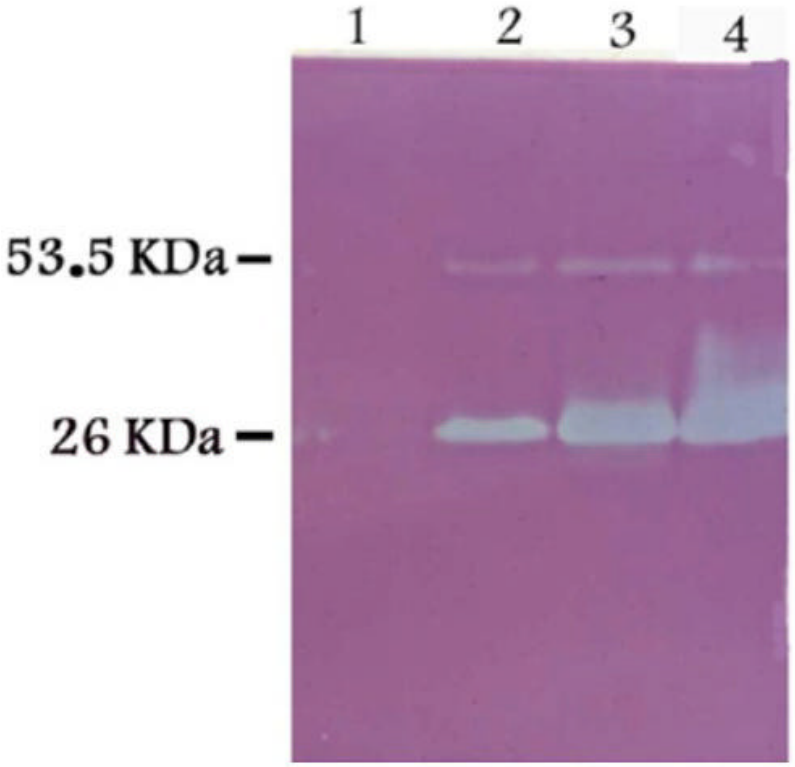

Figure 6. DNA-SDS-PAGE analysis of rice SSPNs. Protein samples (5 mg) were loaded on a 12.5% (v/v) polyacrylamide gel, containing 0.02% (w/v) ssDNA. after electrophoresis and renaturacion of proteins, the gel was incubated in 10 mM citrate buffer, pH 5.5 containig 10mm zinc, overnight. Lanes 1-4, samples from 1-,2-,3- and 4-week-old cells.

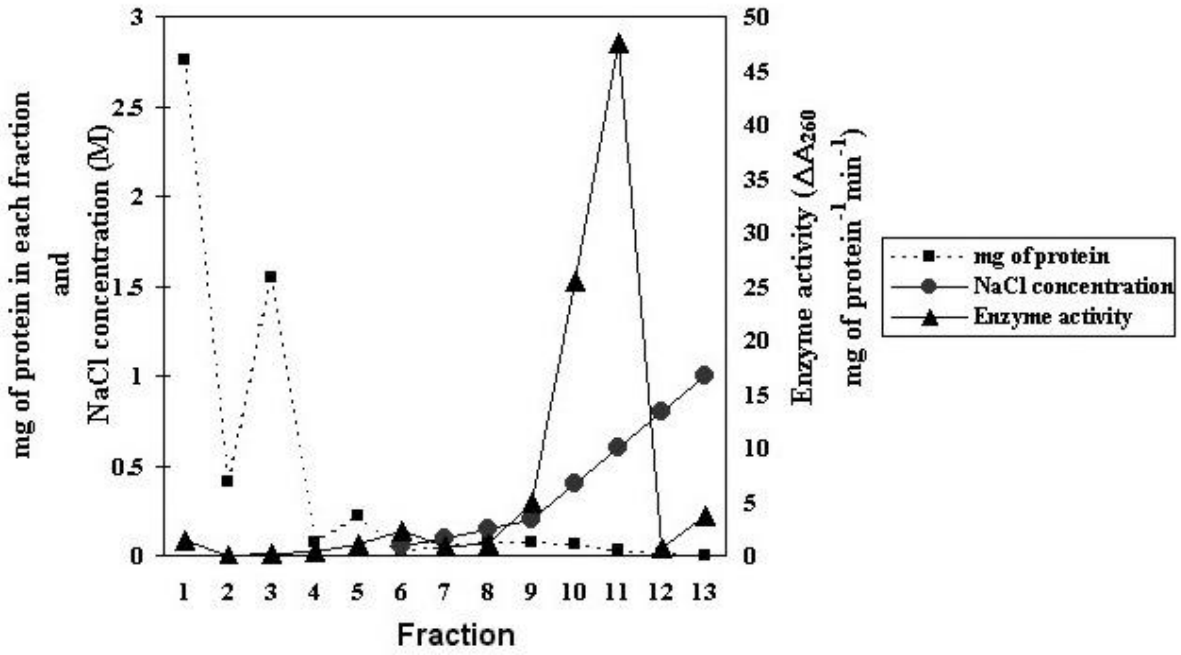

Figure 7. Purification of the 26 KDa nuclease from the 3-week-old Taipei 309 cell filtrates by heparin-Sepharose column chromatography.

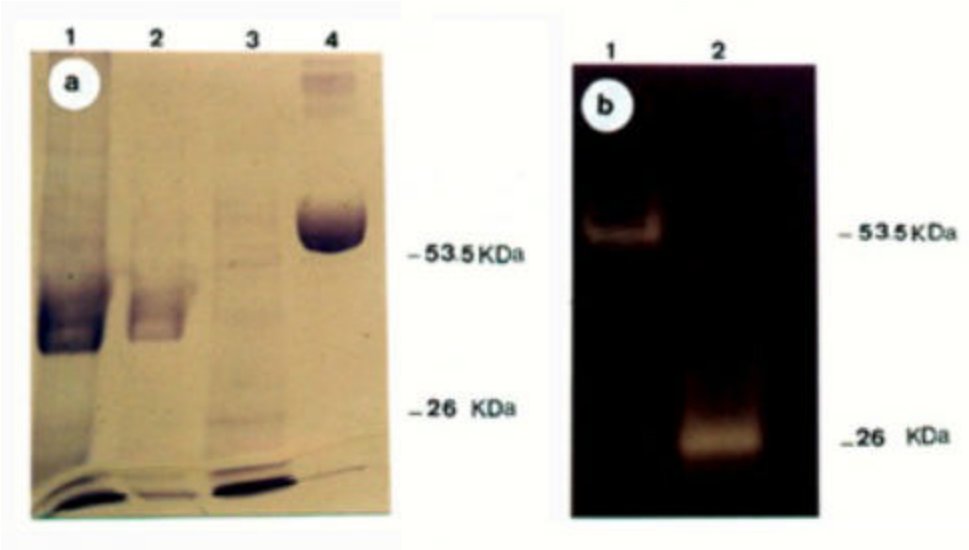

Figure 8. SDS-PAGE and DNA-SDS-PAGE analysis of the 3-week-old Taipei 309 cell filtrate before and after purification.

a. Sample before purification, lane 1, sample before concentration by aceton, lane 2, sample after concentration by aceton, lane 3, sample after purification by heparin-Sepharose column and lane 4, 10 mg BSA

b. DNA-SDS-PAGE analysis of the purified sample. Lane 1, concentrated sample before purification, lane 2, sample after purification.