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Proteolysis and cell death in clover leaves is induced by grazing

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Summary. Programmed plant cell death is a widespread phenomenon resulting in the formation of xylem vessels, dissected leaf forms, and aerenchyma. We demonstrate here that some characteristics of programmed cell death can also be observed during the cellular response to biotic and abiotic stress when plant tissue is ingested by grazing ruminants. Furthermore, the onset and progression of plant cell death processes may influence the proteolytic rate in the rumen. This is important because rapid proteolysis of plant proteins in ruminants is a major cause of the inefficient conversion of plant to animal protein resulting in the release of environmental N pollutants. Although rumen proteolysis is widely believed to be mediated by proteases from rumen microorganisms, proteolysis and cell death occurred concurrently in clover leaves incubated in vitro under rumenlike conditions (maintained anaerobically at 39 °C) but in the absence of a rumen microbial population. Under rumenlike conditions, both red and white clover cells showed progressive loss of DNA, but this was only associated with fragmentation in white clover. Cell death was indicated by increased ionic leakage and the appearance of terminal deoxynucleotidyl transferase-mediated dUTP-nick-end-labelled nuclei. Foliar protein decreased to 50% of the initial values after 3 h incubation in white clover and after 4 h in red clover, while no decrease was observed in ambient (25 °C, aerobic) incubations. In white clover, decreased foliar protein coincided with an increased number of protease isoforms.

Keywords: *Trifolium pratense*; *Trifolium repens*; Proteolysis; Rumen; Cell death.

Introduction

Many articles have been published exploring the mechanisms of cell death in plants as they relate to cellular and whole-organ processes such as senescence, the hypersensitive response, and formation of morphological features (for example, Ryerson and Heath 1996, Beers 1997, Del Pozo and Lam 1998, Fukuda et al. 1998, Mittler et al. 1999). In addition, it has recently been proposed that plant cell death has an important role in determining the efficiency with which grazing ruminants use ingested feed (Theodorou et al. 1996, Kingston-Smith and Theodorou 2000). Production of forage crops, such as grasses and clovers, for animal feed is necessary to support the populations of cattle and sheep produced worldwide (McKersie 1997). Unfortunately the combination of excessive proteolysis of plant protein and microbial deamination of amino acids in the rumen often results in an accumulation of ammonia in excess of that which can be assimilated by the rumen microbial population (Theodorou and France 1983, Siddons et al. 1985, Beever and Siddons 1986, Wetherall et al. 1995, Dewhurst et al. 1996). Excess ammonia is passed from the rumen into the bloodstream, where it is not absorbed by the animal but converted to urea in the liver and expelled in urine (Beever and Siddons 1986, Wetherall et al. 1995) representing both a waste of nitrogen input and a serious environmental pollutant.

When animals are grazing at pasture, they take in large portions of leaf containing numerous intact cells (Hill et al. 1993, Wilson and Mertens 1995, Baumont 1996). These cells are subject to a number of simultaneous stresses including elevated temperature, oxygen deficiency, constant darkness, and the presence of an invasive microbial population. The rumen in cattle is a large liquid-filled organ with a capacity of about 100 litres which is maintained anaerobically at 39 °C and neutral pH and contains a diverse microbial popula-

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tion (Hobson 1988, Theodorou and France 1993, Flint 1997). Although it is widely considered that proteolysis in the rumen is mediated by rumen microbial proteases (Wallace and Cotta 1988, Attwood and Reilly 1996, Forsberg et al. 1999, Wallace et al. 1999), evidence is emerging to indicate that the initial stages of proteolysis are, in fact, mediated by plant proteases contained within the ingested forage (Zhu et al. 1999, Beha et al. 2002). Hence, previous attempts to modify the rate of proteolysis of plant protein in the rumen have targeted the proteolytic rumen microorganisms (Flint 1997, Rooney et al. 1997, Forsberg et al. 1999, Wallace et al. 1999, Forano and Flint 2000) but have left the potential contribution of plant enzymes largely unexplored. This overlooks the fact that living plant cells contain proteolytic capacity within lytic vacuoles, cytosol, and chloroplasts (Feller 1986, Matile 1997, Thomas and Stoddart 1980, Salter et al. 1992, Shipton and Barber 1994, Adam 1996, Andersson and Aro 1997, Noodén et al. 1997, Thomas 1997, Clarke 1999). Furthermore, proteolysis is an integral part of both the senescence and cell death processes in plants (Thomas and Stoddart 1982, Pennell and Lamb 1997, Buckner et al. 1998, Feller 1986, Solomon et al. 1999, Beers et al. 2000, Thomas and Donnison 2000) and apoptosis in animals is regulated in part by a proteolytic cascade mediated by the caspase family of cysteine proteases (Vaux 1993, Kidd 1998, Depraetere and Goldstein 1998, Stennicke and Salvessen 1998). In combination, or singly, the stresses plant cells encounter in the rumen can lead to eventual death of the ingested plant cells (Kingston-Smith and Theodorou 2000, Beha et al. 2002). Therefore, it is important to determine the interaction between cell death and protein breakdown under the very specific stress encountered in the rumen.

It has been suggested that plant cell death in the rumen might be akin to an enforced, premature senescence (Theodorou et al. 1996, Zhu et al. 1999). This is an attractive hypothesis given the extensive upregulation of cysteine proteases commonly observed during senescence (Thomas and Stoddart 1980, 1982; Smart 1994, Morris et al. 1996; Wagstaff et al. 2002). However, results obtained with grass suggest that proteolysis in ingested forages was mediated by the onset and progression of a cell death process which does not strictly adhere to the processes of natural or induced senescence (Beha et al. 2002). Here we have investigated further the relationship between proteolysis and cell death in clover leaves placed under rumenlike conditions in an in vitro system in the absence of a rumen microbial population. Through understanding the processes occurring in plant tissue soon after ingestion, new plant breeding targets will be established, aiming to simultaneously improve nitrogen supply to the animal and decrease land pollution.

Material and methods

Plant material

White clover (*Trifolium repens* cv. Aran) and red clover (*Trifolium pratense* cv. Sabatron) were grown from seed, in 6-inch pots of "John Innes No. 1" compost, in a controlled environment chamber with 8 h day length and 300 $\mu mol/m^2 \cdot s$ illumination. Plants were grown to maturity for six to eight weeks. Trifoliate leaves judged to be mature (fully expanded and not visibly yellow) were harvested at mid-light period and used in subsequent experimentation.

In vitro incubation under rumenlike conditions

The petioles were removed from clover trifoliates and leaflets separated. Leaves were weighed (approximately 0.5 g of fresh weight) into perforated tubes which were then placed into bottles containing 100 ml of anaerobic phosphate buffer (50 mM Na_2HPO_4-KH_2PO_4, 1 mM dithiothreitol, pH 6.8) equilibrated to 39 °C as described previously (Beha et al. 2002). Perforated tubes containing leaflets were removed at intervals and recovered leaf material was drained, blotted, and stored in microcentrifuge tubes at –80 °C until further use. Residual protein in leaflets was extracted by grinding tissue to a fine powder in liquid nitrogen, which was thawed into 2 ml of extraction buffer (50 mM Tris, 2 mM dithiothreitol, 1 mM EDTA, pH 7.5, 0.1% [v/v] Triton X-100) followed by further grinding. The extract was centrifuged for 5 min at 10,000 **g** and the protein content of the supernatant was determined according to Bradford (1976) in a total reaction volume of 1 ml.

Gel electrophoresis

Protein extracts were denatured and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli (1970). Protease activity zymograms were prepared and run as described in Beha et al. (2002). Gel scans were performed on a Bio-Rad GS710 scanning densitometer (Bio-Rad, Hemel Hempstead, U.K.) and polypeptide molecular weights were determined with the Quantity One software package (Bio-Rad).

Measurement of ion leakage

Leaf discs (9 mm diameter) were cut with a cork borer. Ten discs were placed in each of a series of Hungate tubes (Scientific Laboratory Supplies Ltd, Nottingham, U.K.) containing 10 ml of deionised water maintained at either 25 °C or 39 °C and prepared aerobically or anaerobically, in the latter case by extensive gassing with anaerobic gas mix (80% N_2, 10% CO_2, 10% H_2). This was designated the treatment period, and at intervals between 0 to 6 h, three replicate tubes from each treatment were removed, cooled to 25 °C where necessary, and the conductivity of the released electrolyte in the bathing liquid was measured with a potassium ion electrode (Jenway 4020 conductivity meter and probe). The leaf discs were then removed and placed in screw-cap vials (volume, 20 ml) containing 8 ml of deionised water at 25 °C for 4 h, after which time a

further conductivity measurement was taken. This was designated the measurement period. The total leaf disc electrolyte pool was then determined by sealing the tubes containing the leaf discs and placing them in a boiling water bath for 10 min. After cooling to 25 °C, the conductivity of the total electrolyte in the bathing medium was measured. These three measurements of conductivity were required for accurate determination of electrolyte loss because preliminary studies had shown that although the total electrolyte pool size did not change during the treatment period, a loss of electrolyte to the liquid did occur. Thus the data presented take into account a loss of electrolyte during treatment and measurement periods, calculated in proportion to the total electrolyte pool of the tissue.

Flow cytometry

Leaf discs (0.9 cm diameter) were cut from red and white clover leaflets. Under anaerobic conditions five leaf discs of each species were placed in each of eight Hungate tubes (Scientific Laboratory Supplies) containing 10 ml of buffer (50 mM Na_2HPO_4-KH_2PO_4, 1 mM dithiothreitol, pH 6.8) previously equilibrated to 39 °C which were then sealed. At intervals between 0 h and 12 h one tube for each species was opened and leaf discs removed. Each leaf disc was placed in a shallow dish together with 0.5 ml of buffer A (50 mM citric acid, 0.5% [v/v] Tween 20) and chopped exactly 100 times with a razor blade. To this suspension 0.5 ml of buffer B (0.35 M Na_2HPO_4 containing 2 µg of 4′,6-diamidino-2-phenylindole per ml) was added and after 1 min the solution was filtered through mesh (pore size, 0.2 mm) and the filtrate loaded into a flow cytometer (Partec PA; Partec GmbH, Münster, Federal Republic of Germany). The programme was run for exactly 2 min. Red and white clover showed a diploid peak at x-axis values of 50 and 140 respectively (see Fig. 5). Minor peaks indicative of small fragments of DNA were not resolved away from cell debris present at the beginning of the elution. The peak area was calculated by the Partec internal software (Partec PA).

Microscopy

Clover leaf discs were incubated under rumenlike conditions as described for flow cytometry. At the intervals specified on figures, discs were removed, fixed, and dehydrated through an ethanol series before they were embedded in acrylate according to Kronenberger et al. (1993). Thin sections (2 µm) were cut with a microtome (Reichert-Jung, Vienna, Austria) and placed on polylysine-coated slides. Prior to staining the embedding resin was removed by addition of 50 µl of acetone per section which was allowed to evaporate before the section was rinsed with 50 µl of deionised water. To visualise changes in cell structure, sections were stained with methylene blue and examined by light microscopy. To localise nuclei with nicked DNA, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) was performed with the TUNEL detection kit (Roche Diagnostics, Lewes, U.K.). Initially in our investigations control reactions (without the terminal transferase) showed extensive false positive reaction. For this reason the terminal deoxynucleotidyl transferase solution was diluted one in three with the appropriate buffer according to manufacturers instructions (to minimise nonspecific fluorescence) such that staining was not observed in control or time-zero samples. Fluorescence microscopy was performed after excitation with UV illumination and images recorded on Kodak Ektachrome 400 slide film.

DNA extraction and visualisation

Red and white clover leaflets were incubated under rumenlike conditions in a volume of 100 ml of buffer as described previously, except that each incubation bottle contained 3 g of fresh weight of leaflets. At intervals, tissue was removed and stored at –80 °C in microcentrifuge tubes (volume, 1.5 ml) until extraction. DNA was extracted with Qiagen DNeasy Plant Maxi kit according to manufacturers instructions with the exception that the separation of high- and low-molecular-weight DNA by microcolumn chromatography was not performed. Purified DNA was concentrated by ethanol precipitation, resuspended in 10 µl of TE (10 mM Tris, pH 8.0, 1 mM EDTA) and quantified by absorbance of an aliquot at 260 nm. 1 µg of genomic DNA for each time point was electrophoresed in a 0.7% agarose gel, 1× TBE (89 mM Tris-borate, pH 8.3, 2 mM EDTA), at 2 V/cm for 3 h. Gels were stained with ethidium bromide and photographed under UV illumination. Alternatively, in order to increase resolution, gels were used for Southern blotting. Gels were denatured in 0.5 M NaOH, 1.5 M NaCl for 1 h and neutralised in 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl for 30 min. The DNA was transferred to a positively charged nylon membrane (HybondN$^+$; Amersham, Little Chalfont, U.K.) by capillary blotting overnight in 20× SSC (1× SSC is 0.015 M sodium citrate, pH 7.0, plus 0.15 M NaCl) and fixed by baking at 80 °C for 2 h. The DNA samples were detected by hybridisation using labelled DNA from the 5 h time point as a probe. Labelling and detection were carried out using the enhanced-chemiluminescence direct nucleic acid labelling and detection system (Amersham) with a final concentration of 50 ng of labelled probe per ml of hybridisation solution. The signal was visualised by exposure to HyperfilmECL (Amersham) for 10 min.

Results

Proteolysis in red and white clovers under rumenlike conditions

When red or white clover leaves were placed in an in vitro incubation under conditions resembling those of the rumen (39 °C, anaerobic) but in the absence of rumen microorganisms, there was a marked decrease in foliar protein content compared with that measured for ambient controls (leaves incubated at 25 °C under aerobic conditions) at each time point (Fig. 1), suggesting stress-induced autolysis of leaf protein. Furthermore, after 4 h incubation under rumenlike conditions, the protein content had decreased to 29.8% and 57.2% of the initial values for white and red clover, respectively (Fig. 1).

While protein content decreased during incubation, this decrease was accompanied by changes in the composition of the residual protein pool. On an equal protein basis, protease zymograms from both red and white clover revealed an increase in the number of isoforms with increasing duration of incubation under rumenlike conditions, as compared with the profile when leaves were incubated under control conditions (Fig. 2). This indicates that proteases were retained preferentially during the period of decreasing total protein pool size. Under control conditions two major activities were detectable in white clover and three

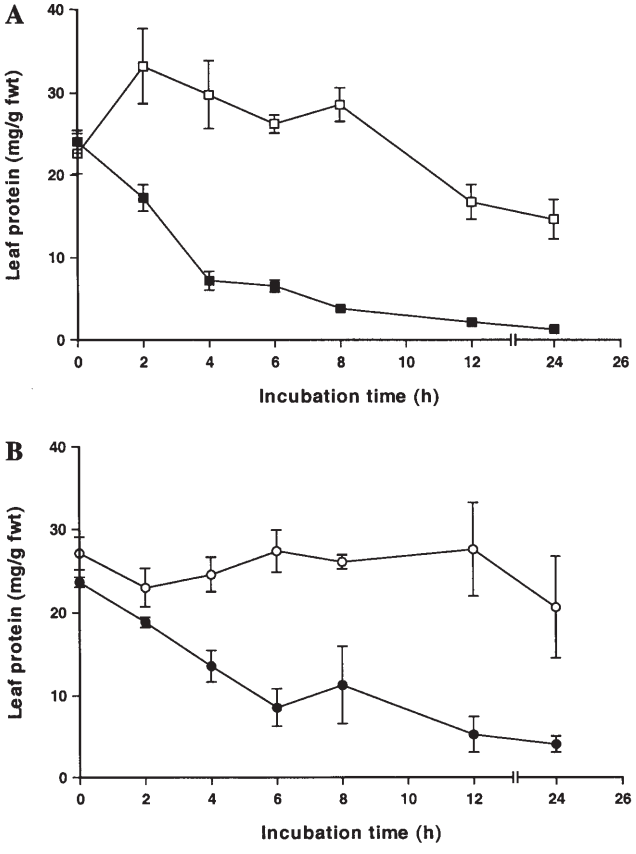

Fig. 1. Changes in foliar protein content of white clover (**A**) and red clover (**B**) as a result of in vitro incubation in buffer maintained at ambient (25 °C aerobic; open symbols) or rumenlike (39 °C anaerobic; closed symbols) conditions. Mean values with standard errors for n = 3 are presented

in red clover. Incubation of white clover under rumenlike conditions resulted in an increased relative activity of the upper band and appearance of four additional lower-molecular-weight bands (at 38, 34, 30, and 27 kDa) which were clearly visible within 6 h of incubation (Fig. 2 C). Alternatively, the protease isoforms in red clover incubated under rumenlike conditions showed little change in activity relative to the activity observed under ambient conditions until after 8 h (Fig. 2 D). Such changes in activity indicate a degree of specificity in protein degradation.

Cell death in red and white clovers under rumenlike conditions

Measurements of ion leakage were used to investigate the influence of temperature and anaerobiosis on white clover leaves. This revealed that ion leakage from leaf discs into deionised water was enhanced in response to increased incubation temperature (39 °C) and lack of oxygen, as compared with controls maintained aerobically at 25 °C (Fig. 3). These data suggest that the combination of heat and anaerobic stress affected membrane integrity.

In comparison with untreated (zero-time incubations) tissues, incubation of red and white clover leaves under rumenlike conditions was accompanied by cytological changes (Fig. 4). Stained sections from untreated red and white clover leaves showed the typical arrangement of cell layers and mesophyll cells containing plentiful chloroplasts. The chloroplasts were positioned close to the cell wall, indicating intact vacuoles. However, after 4 h of incubation at 39 °C

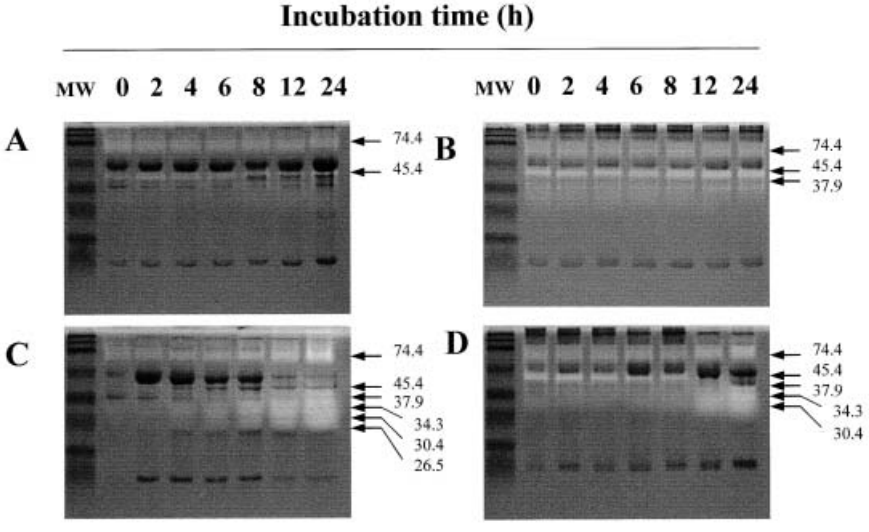

Fig. 2. Protease activities in white (**A** and **C**) and red clover (**B** and **D**) leaves incubated under ambient (**A** and **B**) or rumenlike (**C** and **D**) conditions revealed by activity-stained zymograms. 10 µg of protein was loaded per sample track. Molecular-weight standards were included as indicated

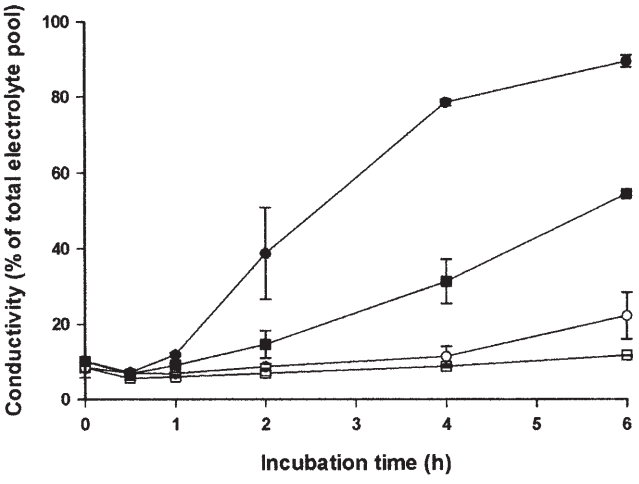

Fig. 3. Change in conductivity of bathing solution observed in response to incubation of white clover leaf discs under aerobic (open symbols) or anaerobic (closed symbols) conditions at either 25 (squares) or 39 °C (circles). Means with standard errors of three determinations are shown

under anaerobic conditions, the cell organisation was showing signs of disruption. Methylene blue staining of chloroplasts was less intense than in zero-time samples, suggesting a loss of protein. After 8 h of incubation the cytoplasm and contents of some cells appeared to have pulled away from the cell wall. After 12 h of incubation chloroplastic material coalesced into darkly staining bodies which are reminiscent of gerontoplasts (Matile 1992).

In order to determine if the observed changes in protein content and cell morphology were associated with events synonymous with programmed cell death, a number of investigations focused on changes in the nucleus. Flow cytometry was used to quantify the DNA content of red and white clover leaves during incubation under rumenlike conditions (Fig. 5). Peaks of diploid DNA were clearly resolved for both species (Fig. 5 A, B). Additional significant peaks were not detected on the chromatograms during the course of rumenlike incubations (data not shown). From integrations of the major peak, it was clear that the DNA loss from red clover leaf cells was less rapid than from white clover leaf cells (Fig. 5 C), reflecting the observed differential rates of protein degradation (Fig. 1).

In mammalian cells, apoptotic DNA cleavage results in a specific end sequence (3′-OH groups) which can be detected in situ by the TUNEL reaction (Gorczyca et al. 1994, Bortner et al. 1995). Incubation of both red and white clover leaves under rumenlike conditions was found to promote progressive increase in TUNEL positive labelling of nuclei, as distinct from autofluorescence of cell walls (Fig. 6). After 4 h at 39 °C under anaerobic conditions, about 25% of the nuclei present in white clover cells contained cleavage sites recognised by the labelling, some of which fluoresced so brightly that other cell contents were illuminated; this effect was even more obvious after 8 h of incubation. The lack of labelling in white clover sections after 12 h of incubation suggests that extensive DNA degradation had removed the free 3′-OH ends of DNA strand breaks recognised by the TUNEL reaction (Fig. 6). Similar staining patterns were observed with red clover leaf sections, but the events appeared to proceed more slowly than in white clover and TUNEL staining was never as intense as was observed for white clover sections (Fig. 6).

Qualitative changes to DNA from red and white clover leaves were examined during the initial few hours of in vitro incubation (Fig. 7). When white clover leaves were subjected to rumenlike incubations, DNA fragmentation occurred resulting in the appearance of DNA ladders resolved on agarose gels (Fig. 7 A). Such fragmentation was not observed in controls where white or red clover leaves were incubated under aerobic conditions at 25 °C (data not shown), or when red clover leaves were incubated anaerobically at 39 °C (Fig. 7 B, D). To increase the resolution of the gels, Southern blots were performed with the DNA extracted after 5 h of incubation (for each clover as appropriate) as a probe. The results show that under rumenlike conditions, DNA ladders were observed in white clover cells as early as 2 h into the incubation (Fig. 7 C). Laddering was not observed for red clover, although the smear of DNA extracted from tissue after 5 h of incubation suggests the activity of nonspecific endonucleases (Fig. 7 D).

Discussion

Homologies have been drawn previously between mammalian apoptosis and plant cell death (Gray et al. 1997, Orzaez and Granell 1997, Mitsuhara et al. 1999, Lacomme and Santa Cruz 1999) and it has been suggested both that programmed cell death in plants represents the final stage of senescence (Delorme et al. 2000, Yen and Yang 1998) and that senescence is distinct from apoptosis (Lee and Chen 2002). Despite considerable effort, the pathway of programmed cell death in plants is incompletely defined at present

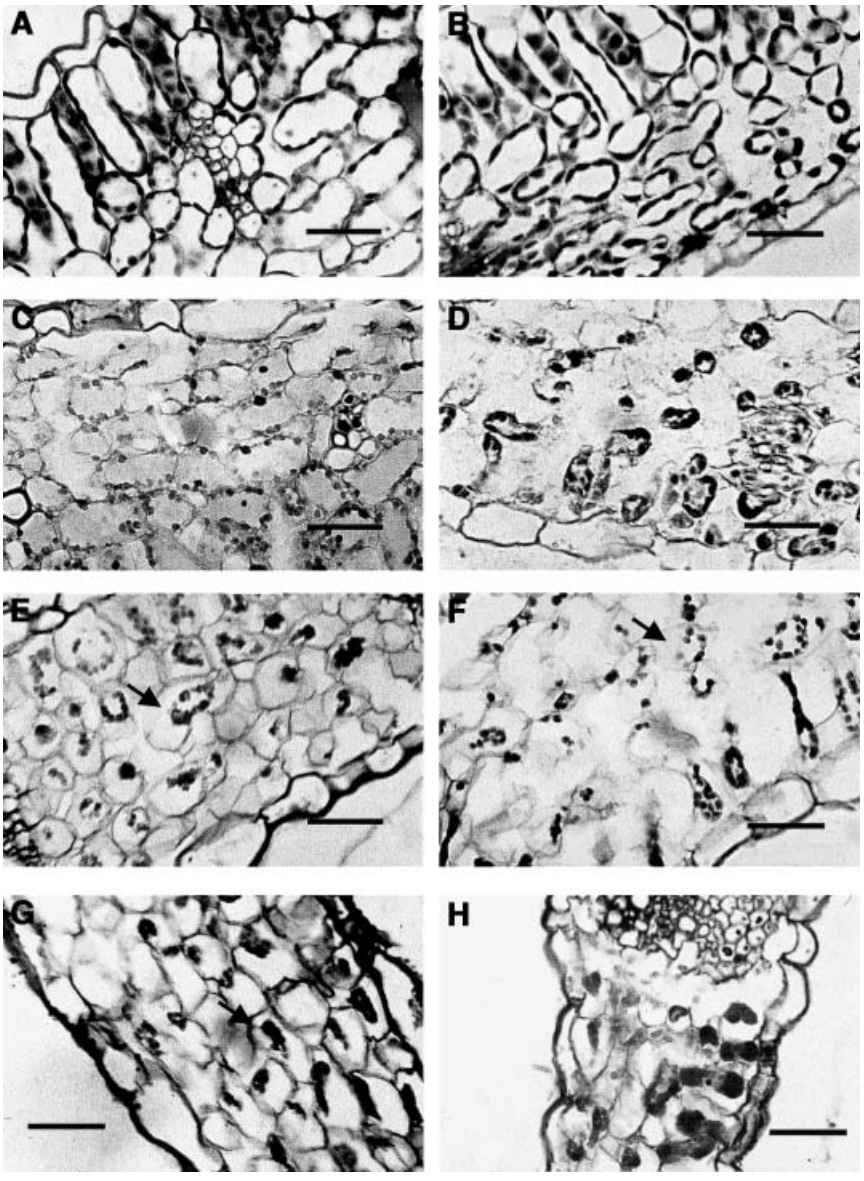

Fig. 4. Cytological changes occurring in white clover (**A**, **C**, **E**, and **G**) and red clover (**B**, **D**, **F**, and **H**) leaves during in vitro incubation in anaerobic buffer at 39 °C for 0 (**A** and **B**), 4 (**C** and **D**), 8 (**E** and **F**), or 12 h (**G** and **H**). Sections (2 μm) were cut with a microtome and stained with methylene blue. Bar: 25 μm. Arrow indicates cells in which contents appear to have pulled away from the wall

(Jones 2000, Lam et al. 2001) and is often described in terms of the presence of symptoms of apoptosis in animal cells. For example, the ability to cleave poly(ADPribose) polymerase (Tian et al. 2000), caspase-like activity (Chen et al. 2000), release of mitochondrial cytochrome *c* (Balk et al. 1999, Sun et al. 1999), and induction of DNA fragmentation patterns commonly associated with apoptosis have been observed as a consequence of senescence (Yen and Yang 1998), biotic stress (Tada et al. 2001), or abiotic stress (Yamada et al. 2001; Chen et al. 2000, 1999; McCabe and Leaver 200). Such fragmentation patterns have been associated with up-regulation of endonucleases as part of the cell death programme during xylogenesis, pathogenesis, and senescence (Kawai and Uchimiya 2000, Mittler and Lam 1995, Thelen and Northcote 1989). Nuclease activity results in DNA degradation, particularly in the interchromatin regions, leading to the appearance of "ladders" after agarose gel electrophoresis (Wyllie et al. 1984) and could account for the DNA laddering observed in white clover. It is also possible that the actions of preexisting nucleases by chance produce a banding pattern similar to that observed during apoptosis. In addition, not all cell death in plants resembles apoptosis and it is possible for cell death to result in decreased content of consistently high-molecular-weight DNA (Fath et al. 1999). The latter is similar to

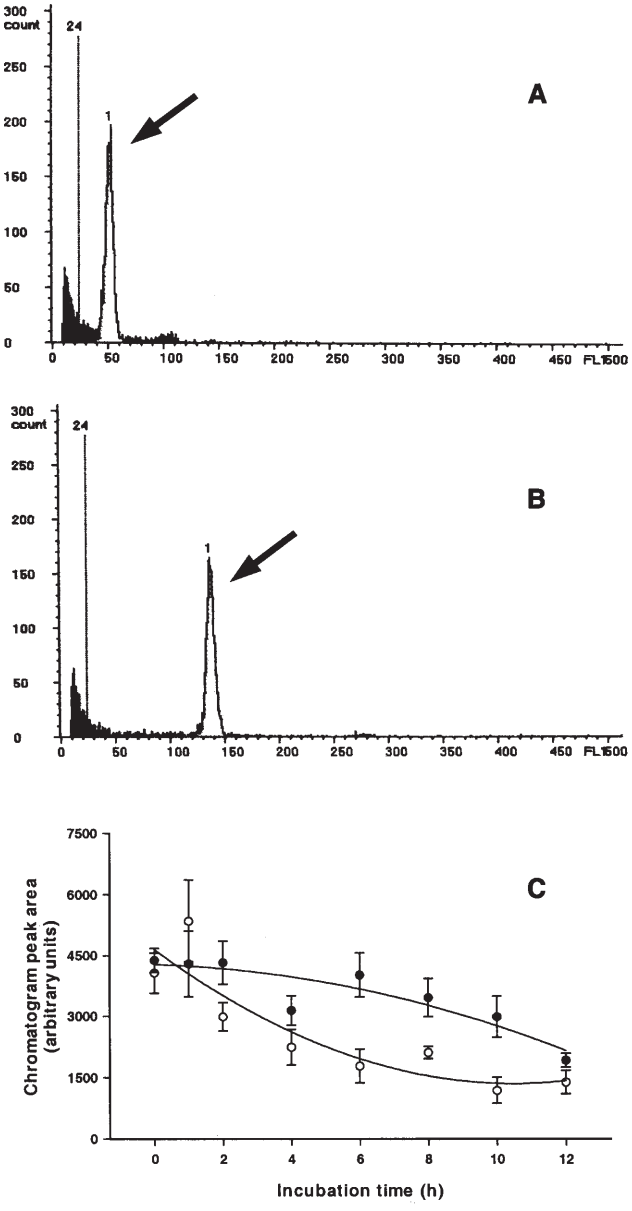

Fig. 5 A–C. Quantitation of DNA content by flow cytometry. Flow cytometer chromatogram peaks relating to nuclei from red (**A**) and white clover (**B**) were integrated and plotted against duration of incubation under rumenlike conditions (**C**; ○, white clover; ●, red clover). Means with standard errors of five replicate determinations are shown

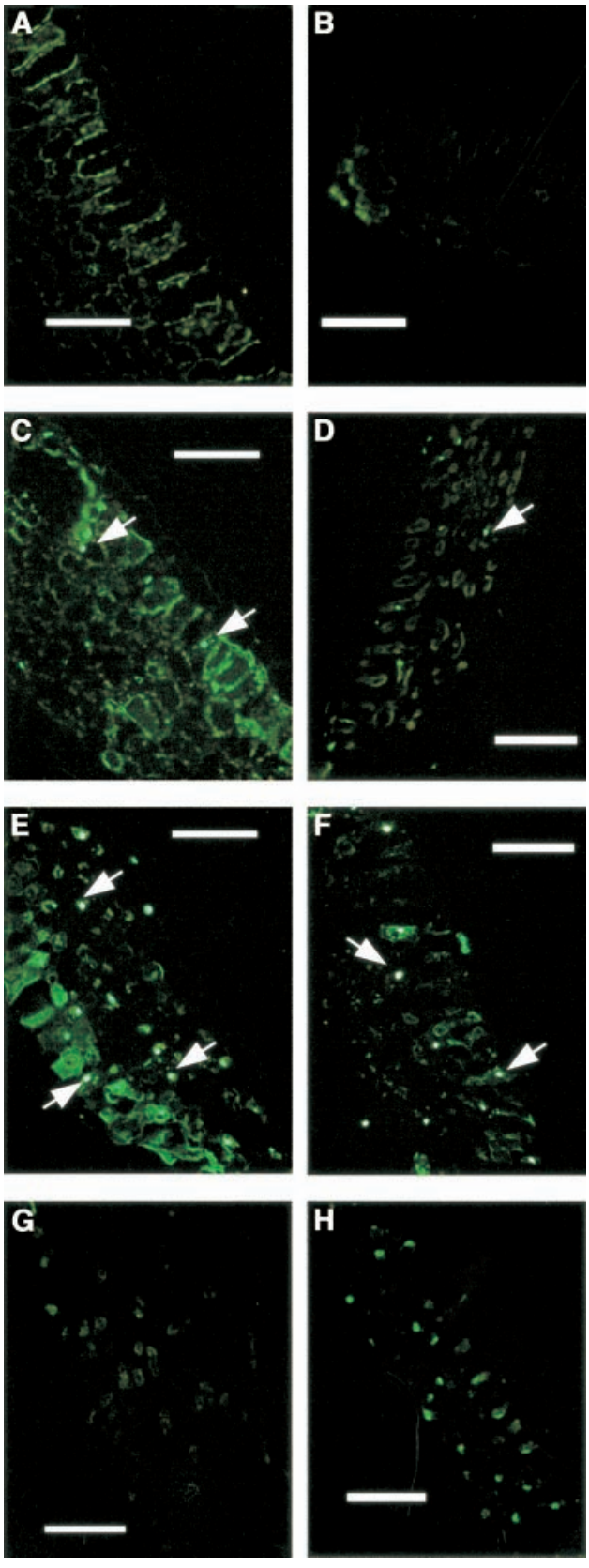

Fig. 6. DNA strand breaks (stained by TUNEL labelling) in the nucleus of white (**A**, **C**, **E**, and **G**) and red clover (**B**, **D**, **F**, and **H**) leaf cells during in vitro incubation in anaerobic buffer at 39 °C for 0 (**A** and **B**), 4 (**C** and **D**), 8 (**E** and **F**) or 12 h (**G** and **H**). Arrows indicate representative labelled nuclei. Bar: 50 μm

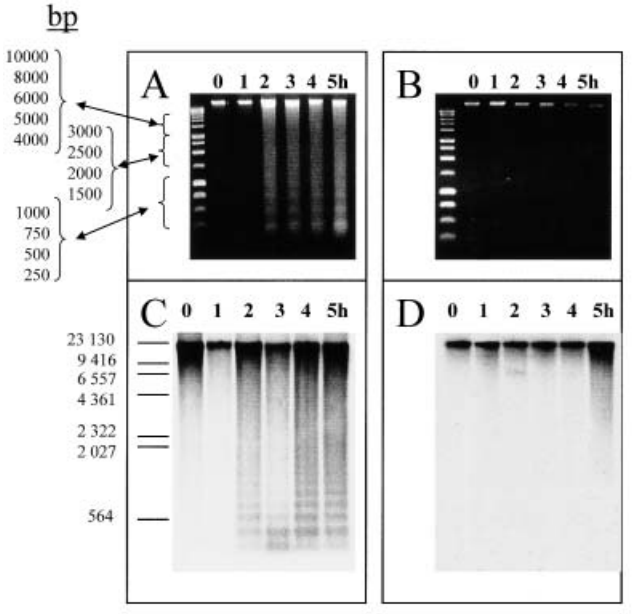

Fig. 7. In vitro degradation of DNA from white (**A** and **C**) and red clover (**B** and **D**). Gels were loaded with 1 μg of DNA extracted from leaves which were removed at the times indicated from anaerobic incubation at 39 °C. After electrophoresis, bands of DNA were visualised by staining with ethidium bromide (**A** and **B**) or visualised by Southern blotting (**C** and **D**). The left-hand lane of each gel shows molecular-weight markers; A and B, 1 kb ladder (Promega, Southampton, U.K.); C and D, λ DNA cut with *Hin*dIII (Roche Diagnostics, Lewes, U.K.)

our observations with red clover. It is not possible to say at present whether the lack of laddering in red clover is due to an interaction of DNA with one or more secondary metabolites. Red clover is rich in tannins and other polyphenols which could bind or prevent access of nucleases to substrate DNA.

It has been proposed that plant cell death is a result of intercellular oxidative stress (Moon et al. 2002, Maccarrone et al. 2001) especially during the hypersensitive response (Sandermann 2000, Mittler et al. 1999). However, such signals cannot be essential for plant cell death (programmed or otherwise) as here we have reported the presence of common indicators of cell death even though plant cells were maintained anaerobically. Under such conditions it is unlikely that oxidative signalling would be extensive, especially if a period of anaerobic stress also results in up-regulation of the antioxidant defence system (Amor et al. 2000, Mittler et al. 1999).

Leaves of both red and white clover (Figs. 2 and 3) and ryegrass (*Lolium perenne*) (Beha et al. 2002) contain proteases which have the potential to cause large-scale protein catabolism before microbial invasion of the intact cells (Wilson and Mertens 1995, Theodorou et al. 1996, Zhu et al. 1999, Kingston-Smith and Theodorou 2000, Boudon and Peyraud 2001). Indeed, the rate of protein loss observed here is consistent with observed rates of protein degradation in grazing ruminants (Beever 1986, Holden et al. 1994, Hristov 1998). In clover, the appearance of TUNEL positive nuclei with increasing duration of incubation under rumenlike conditions and the rapid breakdown of DNA coincided with the most rapid phase of proteolysis and occurred in tissues containing structures resembling chloroplasts. It has been proposed by Minamikawa et al. (2001) that during senescence an interaction of vacuoles and chloroplasts facilitates the degradation of Rubisco. Conductivity measurements suggested that membranes of the tonoplast and plasmalemma are significantly disrupted within 2 h of exposure to the rumen stress, and thus it is unclear if a tonoplast–chloroplast interaction could occur in the experiments described here, or whether intrachloroplastic proteolysis could occur during the initial few hours that plants are exposed to the rumen environment. TUNEL labelling was very intense at later stages of incubation of white clover. It is possible that this is a result of partially degraded DNA becoming more distributed throughout the cell as heat and anaerobic damage occur to remove limitations of compartmentation.

Up-regulation of cysteine proteases is integral to the process of senescence (Smart 1994, Morris et al. 1996, Buchanan-Wollaston 1997, Del Pozo and Lam 1998, Guerrero et al. 1998, Xu and Chye 1999, Wagstaff et al. 2002) and a cysteine protease cascade (caspases) occurs during apoptosis (Vaux 1993, Noodén et al. 1997). Caspase-like activities have been detected in plants following a number of stimuli (Lam and del Pozo 2000, Thomas and Donnison 2000, Del Pozo and Lam 1998, Solomon et al. 1999, Fukuda et al. 1998), but are not an essential part of plant cell death (Jones 2000). Previously, the cysteine proteases appeared to play a minor role during proteolysis and cell death of grass leaves incubated under rumenlike conditions (Beha et al. 2002). Protease isoform gels loaded on an equal protein basis allowed the investigation of the relationship between protein content and protease activity. These revealed that the relative abundance of protease isoforms increased in clover cells maintained at 39 °C under anaerobic conditions, even though the total protein content was decreasing. This could occur either as a result of de novo synthesis of protease

isoforms or could represent protection of normally minor-abundance proteases from proteolytic attack, possibly through sequence specificity or posttranslational modification such as glycosylation.

The observed differential pattern of DNA degradation in red and white clover could have implications for the use of genetically modified forage. It is possible that a gene introduced into white clover would be quickly broken down in intact cells in the rumen into short, subcoding-length, fragments such that the incorporation of the introduced gene into either microbial or animal DNA would be unlikely. The same gene introduced into red clover would persist for much longer in the rumen environment, hence increasing its probability for incorporation. Phylogenetic analysis has revealed the potential horizontal gene flow between bacteria of genes coding for antibiotic resistance and enzymes (Shoemaker et al. 1991, Forano and Flint 2000, Garcia-Vallvé et al. 2000). Although the potential for exchange of genetic material to occur in the rumen has been demonstrated in vitro (Morrison 1996, Scott et al. 1997), such events might be rare in vivo because of nucleases present in the rumen fluid (Morrison 1996, Mercer et al. 1999). However, horizontal gene transfer could contribute to the evolutionary success of colonising bacteria of ruminants and humans (Morrison 1996, Flint 1997, Garcia-Vallvé et al. 2000). As yet, the actual risks associated with possible plant-to-microorganisms or microorganism-to-host animal gene transfer remain undetermined.

In summary, quantitative and qualitative observations indicate that a relationship between proteolysis and cell death exists in plant material entering the rumen. During anaerobic incubation of clover leaves at 39 °C, disruption of cell organisation and DNA damage occurred over a time-span corresponding to the major loss of leaf protein content. The observation that proteolysis and cell death occurred in the absence of a rumen microbial population discredits the widely held belief that microorganisms are the causal factor in the initial stages of plant degradation in the rumen. It is clear from these experiments that plant enzymes can make an active contribution to rumen function and this substantially alters how regulation of proteolysis in the rumen should be interpreted and addressed.

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