Journal of Experimental Botany, Vol. 63, No. 8, pp. 3243–3255, 2012
doi:10.1093/jxb/ers048 Advance Access publication 29 February, 2012
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Evidence of a role for foliar salicylic acid in regulating the rate of post-ingestive protein breakdown in ruminants and contributing to landscape pollution

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Received 4 November 2010; Revised 30 January 2012; Accepted 31 January 2012

Abstract

Ruminant farming is important to global food security, but excessive proteolysis in the rumen causes inefficient use of nitrogenous plant constituents and environmental pollution. While both plant and microbial proteases contribute to ruminal proteolysis, little is known about post-ingestion regulation of plant proteases except that activity in the first few hours after ingestion of fresh forage can result in significant degradation of foliar protein. As the signal salicylic acid (SA) influences cell death during both biotic and abiotic stresses, *Arabidopsis* wild-type and mutants were used to test the effect of SA on proteolysis induced by rumen conditions (39 °C and anaerobic in a neutral pH). In leaves of Col-0, SA accumulation was induced by exposure to a rumen microbial inoculum. Use of *Arabidopsis* mutants with altered endogenous SA concentrations revealed a clear correlation with the rate of stress-induced proteolysis; rapid proteolysis occurred in leaves of SA-accumulating mutants *cpr5-1* and *dnd1-1* whereas there was little or no proteolysis in *sid2-1* which is unable to synthesize SA. Reduced proteolysis in *npr1-1* (*Non-expressor of Pathogenesis Related genes*) demonstrated a dependence on SA signalling. Slowed proteolysis in *sid2-1* and *npr1-1* was associated with the absence of a 34.6 kDa cysteine protease. These data suggest that proteolysis in leaves ingested by ruminants is modulated by SA. It is therefore suggested that influencing SA effects *in planta* could enable the development of forage crops with lower environmental impact and increased production potential.

Key words: *Arabidopsis thaliana*, cell signalling, environment, proteolysis, ruminants, salicylic acid.

Introduction

Ruminants have an important role to play in food supply, with ruminant products delivering not just high quality protein but also dietary nutrients which are unavailable or poorly utilized from other sources. Furthermore, domestic ruminants occupy a specific niche in the food chain, being able to produce food from land unsuitable for growing grain. This role is not likely to diminish in the near future; with an increasing global population, demand for milk and meat is predicted to double by 2050 compared with values from 2000 (FAOSTAT, 2009; Gill *et al.*, 2010). Ruminants are capable of digesting fibrous plant material because of the presence of a complex microbial community in the rumen (Hobson and Stewart, 1997). The rumen is an anaerobic, pH neutral chamber of ~100 litres in cattle which is maintained at 39 °C and supports the conversion of plant to microbial protein, which is digested later in the gastrointestinal tract to support animal growth. However, ruminants utilize feed protein extremely inefficiently, retaining as little as 20–30% of intake N for milk or meat production. The residual 70–80% of intake N is excreted onto the land in urine and faeces (MacRae and Ulyatt, 1974; Dewhurst *et al.*, 1996). This presents a significant global environmental problem having consequences directly in terms of nitrogenous pollution of land and water, and indirectly by contributing to the production of the greenhouse gas nitrous oxide through application of farmyard manures to soils (Dalal *et al.*, 2003; Subbarao *et al.*, 2006; Letica *et al.*, 2010). Production and handling of ruminant

manures is estimated to account for 35% of anthropogenic nitrous oxide (Dalal *et al.*, 2003; FAO, 2006). With ~300 times the warming potential of CO_2 and persisting for >100 years, it is clear that measures are needed to minimize nitrous oxide production. One way is to find novel approaches to minimize the application of N to farm land. It is estimated that grazing land currently occupies 38 million km^2, helping to support ~1.3 billion cattle and 1.7 billion sheep and goats (FAO, 2006). Therefore, because of the size of the global herd, mechanisms delivering even relatively modest improvements in protein retention by ruminants will have an immediate, direct impact on the production of climate change gases and in mitigating nitrogen pollution arising from ruminant systems.

Inefficiency in the ability of the rumen ecosystem to assimilate ingested N is believed to be caused by rapid post-ingestive generation of protein breakdown products during the initial colonization period immediately following ingestion in which fermentable carbohydrates are relatively inaccessible to the microbial population (MacRae and Ulyatt, 1974). Under these conditions, microbial deamination of amino acids delivers energy for microbial growth but liberates ammonia in excess of that which can be re-assimilated by the rumen microorganisms. Ammonia then enters the animal's bloodstream, is removed in the liver, and excreted onto the land in urine and faeces. Once present in the soil environment, *Nitrosomonas* and *Nitrobacter* species convert ammonia to nitrate, which causes hypoxic injury to fish when it enters watercourses, and results in decreased biodiversity in pastures as high nitrate soils favour proliferation of grass species (McGinn and Janzen, 1998).

It is widely considered that protein degradation in the rumen is entirely microbially mediated. However, work using fresh rather than preserved forage (e.g. silage or freeze-dried and ground feed) has identified that it is the plant rather than microbial proteases which are primarily responsible for the rapid generation of protein breakdown products from ingested forage (Zhu *et al.*, 1999; Kingston-Smith and Theodorou, 2000; Kingston-Smith *et al.*, 2005*b*, 2008). It is estimated that at least 50% of the cells ingested during grazing are intact on entry to the bovine rumen (A.H. Kingston-Smith, A. Gay, and E.J. Kim, unpublished) and these intact cells are not passive but can actively respond to the abiotic and biotic stresses presented by the rumen environment (Kingston-Smith *et al.*, 2008). In response to elevated temperature and lack of oxygen in the rumen, ingested plant cells undergo an induced, non-necrotic cell death process (autolysis) in which protein and DNA are degraded (Beha *et al.*, 2002; Kingston-Smith *et al.*, 2003*a*). Also, exposure of forage crop leaves to a combination of rumen temperature and anoxia results in enhanced rates of proteolysis compared with single stress treatments or ambient controls (Beha *et al.*, 2002; Kingston-Smith *et al.*, 2003*a*, 2008). Despite the huge environmental benefits which would be realized by being able to decrease intra-ruminal proteolysis of forage proteins, little is currently known about the regulation of post-ingestion autolysis by the plant cells. The rate at which forage protein is degraded on exposure to simulated rumen conditions has been found to depend on both season and genotype (Kingston-Smith *et al.*, 2002, 2003*b*; Shaw, 2005), but to date there is no evidence in support of a simple relationship between specific or total cellular protease activity and the rate of autolytic protein breakdown (Kingston-Smith *et al.*, 2003*b*; Pichard *et al.*, 2007). By gaining a better understanding of regulation of autolysis in ingested forage, it will be possible to identify suitable breeding targets by which to develop forage crops with decreased environmental impact (Theodorou *et al.*, 2008; Kingston-Smith *et al.*, 2010).

Ingested forage is exposed to rumen bacteria which colonize the apoplastic space after 2 h, albeit at relatively low numbers, but after 8 h incubation despite bacterial proliferation the intracellular spaces of the leaf (including vascular tissues) were still resistant to invasion, even though extensive changes to cell morphology have taken place by this time (Cheng *et al.*, 1980). This colonization pattern is similar to that adopted by some aerobic bacterial pathogens, for example *Pseudomonas syringae* (Preston, 2000), so that microbial plant colonization in the rumen could elicit host responses which have similarities to the pathogen-associated hypersensitive response (HR) or the broader response of systemic acquired resistance (SAR; Heath, 2000; Mur *et al.*, 2008). In particular, bacterial microbe-associated molecular patterns (MAMPs), which include lipopolysaccharides and flagellin, could initiate plant defences, at least partially through salicylic acid (SA) signalling (Tsuda *et al.*, 2008).

SA signalling is a key component in defence against microbial pathogens via activation of SAR (Ryals *et al.*, 1996) and the HR (Mur *et al.*, 1997, 2000). During HR, the foliar SA content of *Arabidopsis* [~100 ng g^{-1} fresh weight (FW)] typically increases 5- to 20-fold due to *de novo* synthesis (Raskin *et al.*, 1990; Silverman *et al.*, 1995; Chen *et al.*, 1997). This rise in foliar SA concentration stimulates expression of the pathogenesis-related (PR) proteins (Metraux *et al.*, 1990; Rassmussen *et al.*, 1991; Malamy *et al.*, 1992) and could be associated with induction of defence-related proteolysis (van der Hoorn and Jones, 2004; van Loon *et al.*, 2006). In addition to a role for SA in defending the plant against microbial pathogens, SA has been shown to be involved in defence against oxidative stress (Yang *et al.*, 2004) and protection against the damaging effects of heat shock, including determination of basal thermotolerance, the protection existing without exposure to pre-conditioning temperatures (Clarke *et al.*, 2004).

Given the pivotal role of SA in the stress responses of plant cells, *Arabidopsis* mutants with differential endogenous SA content were used to test the hypothesis that SA-mediated signal transduction is involved in induction of rumen stress-induced autolysis in plant cells. Using mutants *cpr5* and *dnd1*, where SA levels are constitutively elevated (Clarke *et al.*, 2000; Jurkowski *et al.*, 2004), *sid2* in which a lesion in isochorismate synthase means that SA cannot be produced (Wildermuth *et al.*, 2001), and *npr1* which is incapable of responding to the SA signal via the NPR1

(Non-expressor of Pathogenesis-Related genes1) transcriptional regulator (Cao *et al.*, 1994, 1998), SA was clearly established to influence proteolysis in rumen conditions. The level of SA accumulation could therefore be an important trait when breeding to improve forage crops for use in pasture-based ruminant systems.

Materials and methods

Plant material

Arabidopsis thaliana cv Columbia and mutant lines *npr1-1*, *sid2-1*, *dnd1-1*, and *cpr5-1* were grown from seed in compost in a controlled-environment cabinet maintained at 20±2 °C under an 8 h light period at an irradiance of ~165 μmol m^{-2} s^{-1}. Col-0 lines *npr1-1* and *crp5-1* were obtained from Xinnian Dong (Duke University, NC, USA), *dnd1* from Andrew Bent (University of Wisconsin-Madison, WI, USA), and *sid2-1* from the Nottingham Arabidopsis Stock Centre (UK; http://arabidopsis.info/). The derivation of PR1–β-glucuronidase (GUS) transformants is described in Clarke *et al.* (2004). Leaves were harvested for experimentation when plants were 6 weeks old.

In vitro simulation of the rumen environment

Whole leaves excised at the petiole, or leaf discs were placed in 20 ml of Hungate tubes containing anaerobic phosphate/bicarbonate buffer pH 6.8 (Van Soest, 1967) maintained at 39 °C in the dark in the presence or absence of 10% rumen microbial inoculum for between 0 h and 6 h. Tubes were backfilled with anaerobic gas (10% CO_2/10% H_2/80% N) and sealed with butyl rubber stoppers. Tissue was recovered by filtration and washed with ~50 ml of water per sample. Samples for cytological staining were analysed immediately. Samples for protein extraction were placed in 1.5 ml microfuge tubes and frozen in liquid nitrogen before storage at –80 °C until use. The rumen microbial inoculum was collected from dairy cows fitted with a rumen canula (as approved under the terms and conditions of the Home Office) and was filtered through two layers of muslin to remove solids, and thereafter maintained under a CO_2 stream to preserve the activity of obligate anaerobes.

Direct exposure to microorganisms

Arabidopsis leaves were directly inoculated as described previously (Mur *et al.*, 2000) except that 0.2–0.3 ml of a microbial solution or Van Soest buffer alone was used, so that the liquid visibly flooded the apoplastic space. Excised leaves were inoculated with 10% (v/v) rumen fluid (as described above) or axenic microbial cultures, and then placed into 20 ml Hungate tubes and subjected to anaerobic incubation at 39 °C (as described above). All axenic microbial cultures were grown anaerobically using the Hungate technique (Hungate, 1950). Bacterial cultures (*Fibrobacter succinogenes* strain S85, *Ruminococcus flavefaciens* strain Fd1, *Bacteroides vulgatus* strain 23, and *Clostridium proteoclasticum* strain B316) were grown to early stationary phase in Bellco tubes containing Hobson's M2 liquid medium (Hobson, 1969), and were used directly as an inoculum. The anaerobic fungal/methanogen co-culture was grown for 2.5 d on a barley straw growth medium as described previously (Cheng *et al.*, 2009) and the tube agitated (to release anaerobic fungal zoospores) prior to use of the liquid phase of the culture as an inoculum.

Effect of elevated SA

Mature leaves were excised from each of three individual Col-0 plants and placed in wells of an 8-well serum plate containing 2 ml of 0, 50, or 100 μM SA, or 50 μM or 100 μM of the inactive isomer *p*-hydroxybenzoic acid (pHA) in 0.1 M HEPES, pH 7.0. Uptake through the transpiration stream was allowed for 30 min before leaf discs were cut and placed in Hungate tubes for *in vitro* incubation as described above except that incubations were supplemented with SA at 0, 50, or 100 μM as appropriate. Samples were removed at 0, 1, 2, 4, and 6 h of anaerobic incubation at 39 °C, immediately frozen in liquid nitrogen, and stored at –80 °C until protein extraction.

Treatment with inhibitors of protein synthesis

Protein synthesis during *in vitro* incubation was inhibited by pre- and co-incubation with protein synthesis inhibitors 2-(4-methyl-2, 6-dinitroanilino)-*N*-methyl-propionamide (MDMP) and chloramphenicol. The isomer D-MDMP is an inhibitor of 80S protein synthesis whereas the isomer L-MDMP is inactive (Thomas, 1976). Chloramphenicol is an inhibitor of 70S protein synthesis. Leaflets were cut at the base of the petiole from Col-0 plants, and three leaves per aliquot of 2 ml were placed in wells of a 25-well serum plate containing uptake solution (10 μM L-MDMP, 10 μM D-MDMP, 50 μg ml^{-1} chloramphenicol, or water control) in the light for 1 h prior to use. After the uptake period, leaf discs were cut from the leaves and replicate sets of five discs were placed in Hungate tubes containing 5 ml of anaerobic buffer as described above, except that the buffer was supplemented with 10 μM L- or D-MDMP, or 50 μg ml^{-1} chloramphenicol, or were left untreated. At hourly intervals triplicate samples from each treatment were removed, blotted dry, and frozen at –80 °C for subsequent protein and protease analysis.

Biochemical measurements

Protein was extracted from recovered leaf discs by grinding in liquid nitrogen and homogenization into extraction buffer [0.1 M HEPES buffer, pH 7.5 containing 2 mM EDTA, 1 mM dithiothreitol, 0.1% (v/v) Triton X-100, 0.5% (v/v) protease inhibitor cocktail (Sigma UK, Ltd)] at the ratio of 10 ml g^{-1} FW. Where protease activity was to be determined, the protease inhibitor cocktail was omitted from the extraction buffer. Extracts were centrifuged at 10 000 *g* for 10 min at 4 °C, after which the supernatant was removed into clean tubes. The protein content of extracts was determined according to Bradford (1976). Protease activity was determined by cleavage of azocasein substrate as described previously (Kingston-Smith *et al.*, 2005*a*). Different protease activities were separated and visualized by substrate-containing semi-denaturing electrophoresis as described previously (Beha *et al.*, 2002). Briefly, 12.5% polyacrylamide gels were prepared containing 0.02% (w/v) bovine serum albumin. Aqueous protein extracts were prepared in the absence of SDS or 2-mercaptoethanol and peptides separated by electrophoresis (BioRad Mini Protean III; Bio-Rad, UK Ltd). For protease inhibitor treatments, samples were pre-incubated with specific inhibitors [final concentrations of 10 μM E64, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0 mM and 10 mM EDTA as indicated in the figures] for 15 min before electrophoresis. After electrophoresis, gels were incubated in 2.5% Triton X-100 for 30 min which was then replaced by development solution (25 mM phosphate buffer pH 5.0, 0.1% Triton X-100, 5 mM L-cysteine) containing 4 mM EDTA (control) or protease inhibitor treatments (final concentrations as above). After 16 h, gels were stained with Coomassie Brilliant Blue to reveal bands of clearing against a dark background. Immunological detection of PR1 protein within the mixed protein extract was detected by western blotting, conducted according to the manufacturer's instructions (Bio-Rad, UK Ltd). Protein samples for electrophoretic separation were prepared by combining protein extracted from three independent replicates at each time point during incubation. Gel images were recorded on a GS800 calibrated densitometer and analysed with Quantity One Software (BioRad UK, Ltd). Antisera to PR1 were a gift from John Antoniw (Rothamsted Research, UK). The SA content of

leaves was determined by quantitative mass spectroscopy as described previously (Clarke *et al.*, 2004).

Localization of PR1 protein

Leaves from *Arabidopsis* which had been transformed with the *uidA* gene coding for GUS were histochemically assayed using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc), which dimerizes and oxidizes to produce an insoluble blue dichloro-dibromo-indigo precipitate when cleaved by GUS. Briefly, leaves were placed in 7 ml plastic vials and immersed in 5 ml of 50 mM $NaPO_4$ pH 7, 10 mM EDTA, 50 μg ml^{-1} X-Gluc, and vacuum infiltrated for 5 min before overnight incubation at 37 °C. Before photographic recording of images, the chlorophyll was removed by immersion in repeated changes of 100% ethanol. Images were captured digitally and were analysed with the Matlab® image processing toolbox (The MathWorks Inc., Natick, MA, USA). For each leaf, the pixel values of the red layer of the image were subtracted from those of the blue layer, and the result visualized as a grey scale image. Where this difference was >50 pixels, the resultant area was coloured red to allow visual comparison with the original images, and the number of pixels in the red area counted and is shown in Fig. 7. The Matlab routines used are available on request from the authors.

Results

Protein degradation in Arabidopsis *Col-0*

Arabidopsis leaf discs were sensitive to exposure to a combination of heat and anoxia, which caused a rapid and extensive decrease in foliar protein content as compared with the initial content. *In vitro* incubation resulted in a net loss of 40% of the leaf protein over a 6 h period (Fig. 1). Endogenous leaf proteases were active during the 6 h incubation period and could be detected on substrate-containing gels. When developed at acidic pH, multiple bands of protease activity were detected in Col-0, the major activities resolving at 88.5 kDa (±0.95), 59.6 kDa (±1.08), 41.8 kDa (±3.20), 37.4 kDa (±3.41), and 34.6 kDa (±0.54) (Fig. 2). Incubation with protease inhibitors was used to identify the protease activities represented by the major bands. EDTA is a reversible inhibitor of metallo-proteases and also inhibits metal ion-dependent cysteine proteases. Partial inhibition of all protease activities was observed in the presence of increased EDTA (10 mM compared with 4 mM in control treatments; Table 1), but, surprisingly, inhibition of activity was greater when proteases were incubated in the absence of EDTA. Treatment with PMSF indicated that the activity resolving at 59.6 kDa was a serine protease (Table 1). Partial inhibition of activities resolving at 41.8, 37.4, and 34.6 kDa with PMSF and complete inhibition achieved by incubation with E64 suggested that these activities corresponded to cysteine proteases. Inhibition of the activity resolving at 88.5 kDa was achieved by co-incubation with both PMSF and E64 and in the absence of EDTA.

The requirement for de novo *protein synthesis during autolysis*

The requirement for autolysis for *de novo* synthesis of proteases was tested by incubation in the presence of inhibitors of protein synthesis. The L-isomer of MDMP has been previously shown to be inactive in plant tissue and so forms an additional control treatment (Thomas, 1976). Protein degradation was observed during 6 h incubation under rumen-like conditions regardless of treatment (Table 2). The rate of net protein decrease was determined and compared by fitting a linear rate expression, but analysis of variance revealed that rate values were not significantly different between treatments (overall mean –0.48±0.04 mg h^{-1} g^{-1} FW). Interestingly, after 3 h incubation, significantly less protein was recovered when leaves were treated with chloramphenicol than in the comparable water controls, but values after 6 h incubation were not significantly different (Table 2). This effect was not observed when comparing the values obtained for leaves treated with L- or D-MDMP (Table 2).

The effect of pre-treatment of Col-0 leaves with protein synthesis inhibitors on protease activity was determined. Analysis of protease activity was performed at pH 5 and 8 to estimate acidic and neutral proteolytic capacity of the vacuole and cytoplasm, respectively (Tables 3 and 4,

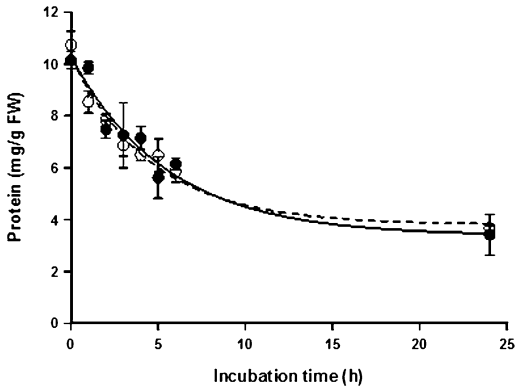

Fig. 1. Effect of the presence of rumen microorganisms on protein degradation during anaerobic *in vitro* incubation at 39 °C (open circles, buffer control; filled circles, 10% rumen fluid inoculum). Means ±SE of n=3 replicates are shown.

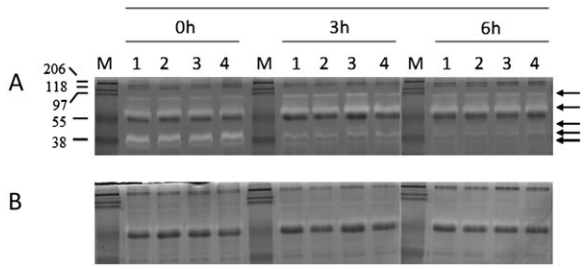

Fig. 2. The effect of protease synthesis inhibitors on resolution of protease activities. Protease activities (indicated by arrows in A) from leaves treated with 1, H_2O; 2, chloramphenicol; 3, L-MDMP; or 4, D-MDMP, recovered at 0, 3, or 6 h incubation in anaerobic Van Soest buffer at 39 °C, and resolved by semi-denaturing, substrate-containing PAGE. Gels were loaded with equivalent protease activity (8.7×10^{-3} ΔOD h^{-1}) per track and developed at (A) pH 5.0 or (B) pH 8.0. M, molecular weigh standards.

Table 1. Effect of protease inhibitors on protease isoform activities recovered from Arabidopsis thaliana as assessed by comparative calibrated densitometry of gels loaded with 1.7 mg of extractable protein where control treatment (25 mM phosphate buffer pH 5.0, 0.1% Triton X-100, 5 mM l-cysteine, 4 mM EDTA) resulted in bands of clearing which were equated to 100% activity, and activity of protease inhibitors resulted in increased band OD

Treatment	Activity of protease isoform relative to control (%)				
	88.5 kDa	59.6 kDa	41 kDa	37 kDa	35 kDa
0 mM EDTA	ND (–)	43.7 (13.2)	23.9 (1.04)	37.2 (6.38)	17.5 (2.25)
10 mM EDTA	59.2 (1.96)	66.8 (1.85)	64.8 (15.1)	81.9 (3.89)	83.3 (1.03)
E64	ND (–)	44.9 (2.04)	ND (–)	ND (–)	ND (–)
PMSF	ND (–)	ND (–)	29.2 (9.87)	59.7 (6.89)	48.5 (1.87)
Band density in control (mean OD)	0.0623	0.1272	0.0611	0.1179	0.0767

Means ±SE are presented; ND, not detectable.

Table 2. Effect of exogenous protein synthesis inhibitors on protein recovery from Col-0 leaf discs incubated anaerobically at 39 °C Differences between treatment means were compared by Student *t*-test.

Treatment	Protein content (mg g^{-1} FW)					
	Incubation in buffer (h)			Incubation in rumen inoculum (h)		
	0	3	6	0	3	6
H_2O	7.4 (0.19)	6.98 (0.21)	5.93 (0.22)	7.63 (0.42)	7.56 (0.39)	4.80 (0.37)
Chloramphenicol	6.29 (0.24)	5.11 (0.01)	5.32 (0.14)	7.09 (0.67)	5.50 (0.19)	4.69 (0.20)
Significance	NS	**	NS	NS	*	NS
L-MDMP	7.50 (0.36)	5.94 (0.46)	5.72 (0.20)	8.06 (0.10)	5.22 (0.05)	5.07 (0.21)
D-MDMP	7.1 (0.27)	5.50 (0.02)	5.14 (0.14)	8.15 (0.78)	5.59 (0.02)	4.79 (0.07)
Significance	NS	NS	*	NS	*	NS

NS, not significant; *$P < 0.05$; **$P < 0.01$. Means (±SE) of three independent replicates are shown.

Table 3. Effect of exogenous protein synthesis inhibitors on acidic protease activity in Col-0 leaf discs incubated anaerobically at 39 °C Differences between treatment means were compared by Student *t*-test.

Treatment	Protease activity (ΔOD h^{-1} g^{-1} FW)					
	Incubation in buffer (h)			Incubation in rumen inoculum (h)		
	0	3	6	0	3	6
H_2O	8.69 (0.60)	6.73 (0.02)	5.95 (0.28)	9.56 (0.17)	8.01 (0.07)	8.01 (0.43)
Chloramphenicol	8.93 (0.31)	6.34 (0.28)	5.18 (0.19)	9.00 (0.07)	8.01 (0.02)	7.96 (0.24)
Significance	NS	NS	NS	NS	NS	NS
L-MDMP	8.79 (1.61)	6.48 (0.14)	5.95 (0.11)	10.07 (0.30)	7.87 (0.03)	8.10 (0.24)
D-MDMP	7.87 (0.27)	6.31 (0.40)	5.04 (0.15)	9.33 (0.29)	7.80 (0.24)	7.46 (0.03)
Significance	NS	NS	**	NS	NS	NS

NS, not significant; **$P < 0.01$. Means (±SE) of three independent replicates are shown.

respectively). Comparable samples resulted in approximately half the initial protease activity at pH 8 than was observed at pH 5 (Tables 3, 4). Also, acidic protease activity tended to decrease with incubation time whereas neutral protease activities were more stable (Tables 3, 4). Significant differences in acidic proteolytic activity arising as a result of inhibition of protein synthesis were only detected after 6 h treatment with D-MDMP, which resulted in lower protease activity than in the equivalent L-MDMP control treatment. Significant differences in neutral protease activity were not detected as a result of inhibition of protein synthesis (Table 4). Further assessment of protease activities was obtained from protease activity gels. Although gels were loaded at equivalent protease activities as determined from azocasein degradation, protease band intensity was greater in gels developed at pH 5 than at pH 8 (Fig. 2). Multiple bands were detected when gels were developed at pH 5, but only one band at 59.6 kDa was detected when gels were

Table 4. Effect of exogenous protein synthesis inhibitors on neutral protease activity in Col-0 leaf discs incubated anaerobically at 39 °C
No significant differences between treatment means of each inhibitor and its respective control treatment were detected by Student *t*-test.

Treatment	Protease activity (ΔOD h^{-1} g^{-1} FW)					
	Incubation in buffer (h)			Incubation in rumen inoculum (h)		
	0	3	6	0	3	6
H_2O	4.25 (0.02)	4.15 (0.09)	4.24 (0.08)	5.72 (0.06)	5.77 (0.14)	5.54 (0.09)
Chloramphenicol	4.52 (0.11)	4.40 (0.09)	4.13 (0.11)	5.88 (0.10)	5.67 (0.07)	5.69 (0.10)
L-MDMP	4.47 (0.07)	4.46 (0.01)	4.13 (0.06)	5.85 (0.06)	5.72 (0)	5.64 (0.07)
D-MDMP	4.42 (0.01)	4.29 (0.07)	4.07 (0.05)	5.68 (0.13)	5.83 (0.11)	5.67 (0.09)

Means (±SE) of three independent replicates are shown.

developed at pH 8, regardless of treatment (Fig. 2). Treatment with either chloramphenicol or MDMP did not result in a change in the protease composition (Fig. 2).

The effect of SA on autolysis

In order to relate proteolytic changes to SA, the accumulation of SA in *Arabidopsis* was determined during anaerobic conditions and in response to rumen fluid (Fig. 3). These data suggested that accumulation of both free and glycosylated (total minus free) SA occurred within 6 h of treatment in rumen fluid-treated samples, but increased SA was not observed in incubations in which the microbial inoculum was omitted. Free SA is thought to be the active salicylate form and conjugated SA the storage form (Malamy *et al.*, 1992). To relate the physiological relevance of SA rises to autolytic proteolysis, mutants were identified with elevated or decreased endogenous SA concentrations. The mutants *cpr5* and *dnd1* contained significantly higher constitutive SA concentrations than those detected in Col-0 (Table 5). In contrast, free and total SA concentrations detected in *npr1* and *sid2* were not significantly different from those of Col-0 (Table 5). This indicates that *Sid2* is unable to synthesize SA, while Col-0 and *npr-1* do not constitutively synthesize SA, only in response to stress. Hence it is not unusual that levels in unstressed plants were similar. Also, because of the aqueous incubation system used to replicate the rumen environment, the true SA content of Col-0 as incubation progressed may have been underestimated due to leakage to the incubation medium. The endogenous starting SA concentration had a clear effect on net protein content, both initially and as a result of anaerobic incubation of leaf discs at 39 °C. The *Arabidopsis* mutants *cpr5* and *dnd1* showed rapid protein degradation on exposure to the *in vitro* incubation conditions, as seen for Col-0 (Figs 1, 4A, B). In contrast, decreased (relative to Col-0, Fig. 1) or negligible protein breakdown was observed when leaf discs from *npr1* and *sid2* plants were incubated anaerobically at 39 °C (Fig. 4C, D). These mutation-related effects on foliar protein content were independent of the presence or absence of rumen fluid in the incubation media.

The effect of the mutations on foliar protease composition was examined by activity zymograms. As seen with

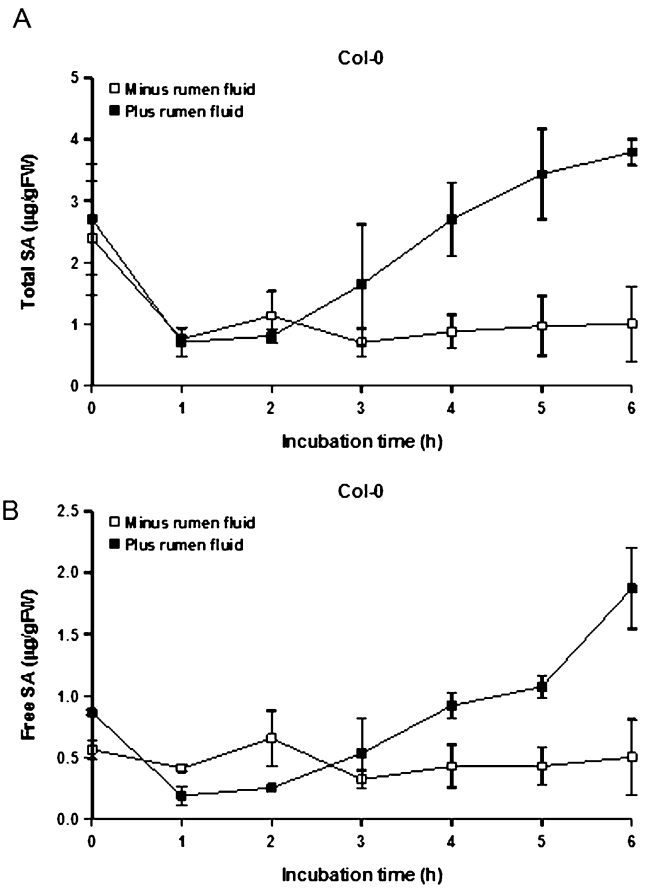

Fig. 3. Total (A) and free (B) SA accumulation in *Arabidopsis* Col-0 leaf discs incubated anaerobically at 39 °C in the presence (filled symbols) and absence (open symbols) of a 10% rumen microbial inoculum. Means ±SE for n=3 independent sample replicates are shown.

Col-0 (Fig. 2), multiple bands of protease activities were also detected in the mutant lines (Fig. 5). Gels were loaded on an equal fresh weight basis to reflect the relative abundance of protease activities between the mutant and control lines. Proteases were most active in Col-0 and *cpr5*, and least active in *sid2*. The activities in Col-0 resolving at 88.5 kDa and 59.6 kDa were persistent and relatively constant throughout the incubation, whereas the activity

Table 5. Total and free salicylic acid (SA) content of *Arabidopsis thaliana* wild-type (Col-0) and mutants before and after *in vitro* incubation in the presence or absence of a rumen inoculum
Significance of differences between means at comparable treatment/times (i.e, within columns) were determined by ANOVA and are presented together with least significant differences (LSD)

Genotype	Minus rumen fluid		Plus rumen fluid	
	0 h	6 h	0 h	6 h
Col-0				
Total SA	2.4	1.0	2.7	3.8
Free SA	0.6	0.5	0.9	1.9
cpr5				
Total SA	40.1	2.5	51.3	44.4
Free SA	9.8	1.3	17.5	19.4
dnd1				
Total SA	75.5	14.4	71.7	12.4
Free SA	25.4	8.7	37.3	6.7
npr1				
Total SA	8.4	3.4	8.0	5.1
Free SA	2.4	1.8	2.6	3.2
sid2				
Total SA	3.2	0	2.8	0
Free SA	1.5	0	1.6	0
Significance				
Total SA	<0.001	<0.001	0.010	<0.001
Free SA	<0.001	<0.001	0.005	<0.001
LSD				
Total SA	19.03	5.44	41.20	13.91
Free SA	6.29	3.72	18.47	5.01

Means (±SE) of three independent replicates are shown.

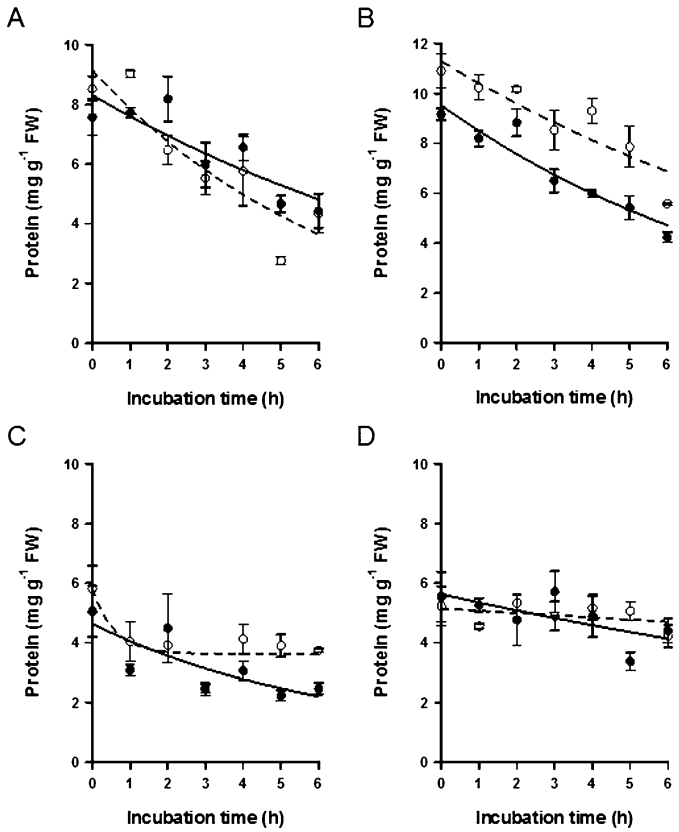

Fig. 4. Protein content recovered from leaf discs of *Arabidopsis* mutants (A) *cpr5*, (B) *dnd1*, (C) *npr1*, and (D) *sid2* as a result of anaerobic incubation at 39 °C in the absence (open symbols) or presence (filled symbols) of rumen fluid. Means ±SE of n=3 replicates are shown.

resolving at 41.8 kDa increased with increasing incubation time, and the activity resolving at 37.4 kDa decreased under rumen-like conditions (Fig. 5; Supplementary Fig. S1 available at *JXB* online). Interestingly, differences between the mutants in the abundance of the protease activity at 34.6 kDa were observed. This protease activity could be seen in the untreated leaves of Col-0, *cpr5*, *dnd1*, and *npr1*, but not in those of *sid2*. The activity of the 34.6 kDa band persisted but decreased throughout the 6 h incubation time for Col-0 and *cpr5*, but this activity band was absent or relatively poorly detectable in extracts from *sid2* leaves at any point during the incubations (Fig. 5; Supplementary Fig. S1).

PR1 expression in Col-0 and SA mutants

PR1 is expressed in response to SA (Metraux *et al.*, 1990; Rassmussen *et al.*, 1991; Malamy *et al.*, 1992) and hence is as an internal reporter of the functioning of the SA signalling pathway. The PR1 protein was detected by western blot in leaf discs from Col-0, *cpr5*, and *dnd1* plants throughout the duration of exposure to anaerobic conditions and elevated temperature in both the presence and absence of a rumen fluid inoculum (Fig. 6). In contrast, PR1 was not detectable in leaf discs taken from *npr1* or *sid2* plants during incubation, regardless of the presence or absence of a rumen inoculum (Fig. 6).

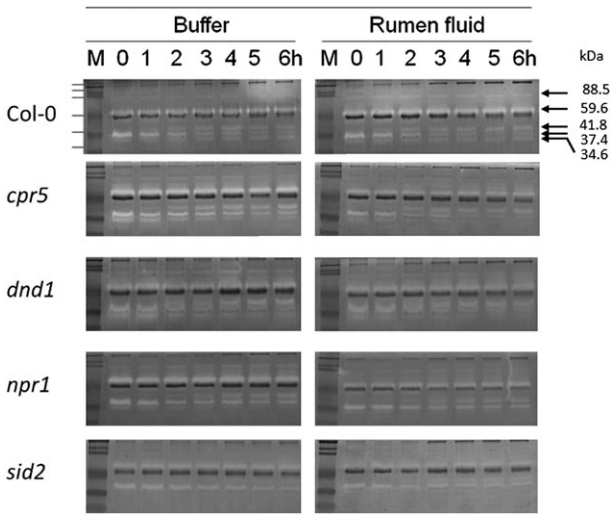

Fig. 5. Protease activities (visualized as bands of clearing on a dark background, indicated by arrows in the upper panel) present in the wild type (Col-0) and mutants of *Arabidopsis thaliana* with altered SA accumulation as a result of anaerobic incubation at 39 °C in the presence and absence of rumen inoculum. Molecular weight markers (M) at 206, 118, 97, 55, 38, and 29 kDa are indicated on the left of the upper panel. Each lane contains the protein present in 2 mg FW of leaf.

PR1 expression was investigated with PR1–GUS transformants. Microbial stimulation of PR1–GUS expression was induced by directly introducing rumen inoculum into the apoplast prior to incubation, in a manner routinely used for evaluation of the HR. In mock inoculations (controls) in the absence of microorganisms, GUS expression was localized predominantly at the leaf margins (Fig. 7A). In the presence of a 10% rumen microbial inoculum, PR1–GUS activity was widely distributed within the leaf, appearing as patches which were of greater abundance than in the controls (Fig. 7A). Expression of PR1–GUS could also be induced by individual microbial species which are present as part of the rumen microbial population. PR1 promoter activity was induced following inoculation by anaerobic fungi/methanogens and both Gram-positive (*C. proteoclasticum* and *R. flavefaciens*) and Gram-negative (*F. succinogenes* and *B. vulgatus*) bacterial species (Fig. 7B).

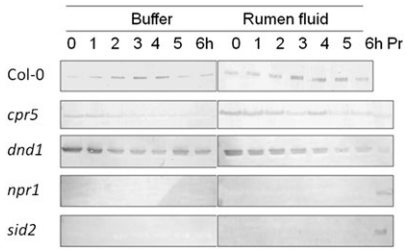

Fig. 6. Immunological detection (western blotting) of PR1 expression in the wild type (Col-0) and SA mutants of *Arabidopsis thaliana* as a result of anaerobic incubation at 39 °C in the presence and absence of rumen inoculum. Pr, PR1 control protein extracted from Col-0 undergoing a hypersensitive response. Each lane contains the protein present in 2 mg FW of leaf.

Discussion

Previous work has demonstrated that the plant processes induced by ingestion and exposure to the rumen environment can not only be significant in terms of impact on fermentation, but can also map onto innate cellular responses (Kingston-Smith *et al.*, 2008, 2010). Practically this means that nitrogenous pollution resulting from livestock could be minimized by production of a functional forage feed with a decreased proteolytic response to the rumen environment. Hence, understanding the stress responses of ingested plant cells will enable new strategies and generations of improved crops to be developed to

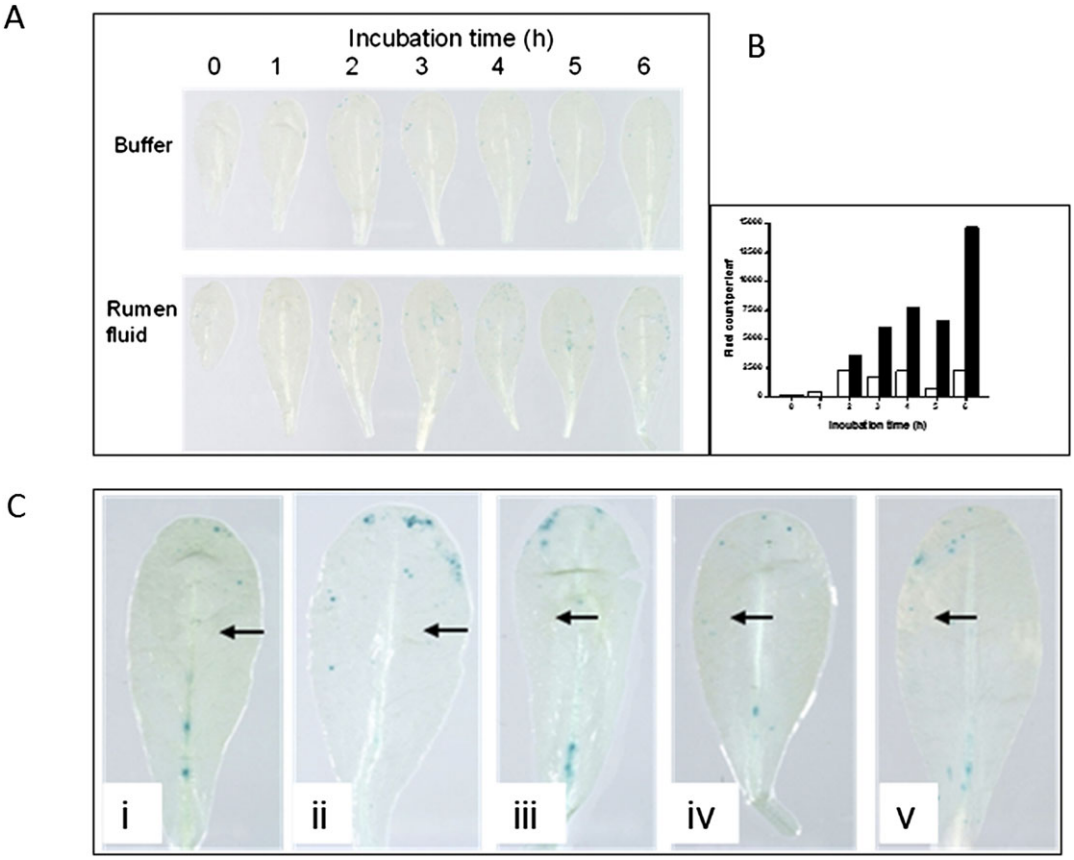

Fig. 7. PR1–GUS activity (A) as a result of anaerobic incubation at 39 °C for up to 6 h following inoculation of leaves with buffer or a 10% rumen fluid inoculum, (B) comparison of areas of blue staining in leaves shown in A by image analysis, and (C) PR1–GUS activity as a result of anaerobic incubation at 39 °C for 1–3 h following inoculation of leaves with cultures of (I) *Clostridium proteoclasticum*, (II) fungal –methanogen co-culture, (III) *Fibrobacter succinogenes*, (IV) *Bacteroides vulgatus*, or (V) *Ruminococcus flavefaciens*. Arrows indicate the site of inoculation.

mitigate adverse effects of livestock farming whilst maintaining food supply.

Previous investigations have been used to identify the role of SA in mediating the response to individual stress events. SA-deficient and SA-overproducing plants have been demonstrated to have opposing effects on the tolerance of plants to challenge by microbial pathogens (Gaffney *et al.*, 1993; Delaney *et al.*, 1995; Mauch-Mani *et al.*, 2000; Verberne *et al.*, 2000). Similarly, SA has been implicated in heat tolerance (Dat *et al.*, 1998; Hong *et al.*, 2003; Clarke *et al.*, 2004) and associated oxidative stress (Larkindale and Knight, 2002).

It has been demonstrated here that the ability to synthesize and respond to SA had a significant effect on protein degradation on exposure of leaves to rumen-like conditions, in response to challenges representing both the abiotic conditions alone and the combined abiotic and biotic conditions of the rumen environment.

SA in plants exists in both free and glycosylated forms and is an integral component in the signalling network utilized by plant cells which culminates in induction of cell death (Alvarez, 2000). In HR there is a temporal increase in cellular SA at ~6 h post-infection (Mur *et al.*, 2000), and endogenous SA increases with age/flowering (Martinez *et al.*, 2004). Increased SA was observed in Col-0 leaves only in the presence of a rumen microbial inoculum (Fig. 3; Table 5), consistent with pathogen-induced SA accumulation. In the rumen, initial colonization occurs rapidly (Koike *et al.*, 2003; Edwards *et al.*, 2007), with rumen-derived bacteria being identified on forage after just 15 min incubation. However, population growth of colonizing bacteria stabilizes after 30 min (Edwards *et al.*, 2007), before the rapid phase of dry matter degradation has begun (Sharon Huws, personal communication), so it would appear that to have an effect on endogenous SA content rather than inducibility is important.

There was a clear response of the rate of stress-induced proteolysis to endogenous SA concentration in the *Arabidopsis* mutants. Rapid proteolysis was observed in the constitutively SA-accumulating *cpr5* and *dnd1* mutants, whereas there was little or no proteolysis in *sid2* or *npr1* plants which are unable to synthesize (*sid2*) or respond (*npr1*) to SA. Induced SA biosynthesis was observed at 6 h (Table 5) in Col-0, most probably due to heat shock and/or the rumen fluid. This apparent anomaly between clear response of the mutants and that of Col-0 could be reflective of the need for prolonged exposure to a threshold level of SA to induce full proteolytic capacity and nonetheless parallels the response of PR1 to SA. With PR1, SA concentrations within solutions injected into leaves are required to be above a certain threshold before gene expression is activated (Bi *et al.*, 1995). This may also be the case with the influence of SA on the proteolytic response. It is proposed that SA acts according to a threshold accumulation, clearly demonstrated when proteolysis was examined in constitutive over- or non-SA-accumulating/responding mutants. If correct, physiological responders (i.e. control Col-0) may or may not be exposed to sufficient SA to trigger large-scale proteolysis. Indeed, it was observed that rates of proteolysis in Col-0 were variable between experiments, which was in contrast to the mutants where differences in SA were more marked (data not shown). SA can cause both up- and down-regulation of proteolytic activity in plants, with cysteine, aspartic, and serine proteases having been identified as targets (Van der Hoorn and Jones, 2004; Van Loon *et al.*, 2006; Prasad *et al.*, 2009; Raju *et al.*, 2009). *Arabidopsis* lines with elevated levels of SA (*cpr5* and *dnd1*) showed rapid protein breakdown, whereas protein degradation was significantly decreased in plants which were impaired in either SA synthesis (*sid2*) or NPR1-mediated gene expression (*npr1*) (Dong, 2004). Indeed the PR protein identified initially as PR7 is an SA-responsive subtilisin-like endoproteinase (Jordá *et al.*, 2000; Schaller, 2004; van Loon *et al.*, 2006). In *Arabidopsis*, exposure to SA has been shown to cause increased gene expression of the senescence-associated protein SAG12, which is a cysteine protease (Morris *et al.*, 2000).

Surprisingly, the effect of SA on the increased rate of proteolysis observed with the *cpr5* mutants could not be replicated by exogenous application of free SA to Col-0 leaves (data not shown). One explanation is that in these experiments the 30 min pre-incubation time of exposure to SA was insufficient for protease activation; another is that it is the glycosylated rather than the free form which is required for signal transduction under these conditions. Free SA is widely considered to be the active form, although glycosylated SA (which is localized to the vacuole; Enyedi and Raskin, 1993) has previously been indicated to be involved in heat signalling in mustard and *Arabidopsis* (Dat *et al.*, 1998; Clarke *et al.*, 2004). Also, SA synthesized *de novo* as a result of pathogenesis is in glycosylated rather than free acid form (Raskin *et al.*, 1990; Silverman *et al.*, 1995; Chen *et al.*, 1997) which is in agreement with the data for Col-0 in response to rumen fluid (Fig. 3) in which glycosylated SA accumulation equalled that of free SA. While it is possible that exogenous application was ineffective because of lack of uptake to the correct metabolic compartment, this is considered unlikely as SA has been shown to cross membranes readily (Clarke *et al.*, 2005).

Slowed proteolysis in the SA mutants appeared to be associated with the absence of a 34.6 kDa cysteine protease. The *sid2* mutants had noticeably less protease activity, and activity of this cysteine protease was not observed at any incubation time and was low in *npr1*, indicating that this protease is SA responsive. The slightly different protease activity undoubtedly reflects the difference in physiology between the two mutants. The mutant *sid2* is unable to synthesize SA but can respond to exogenous application (Wildermuth *et al.*, 2001), whereas *npr1* is unable to induce normal metabolic responses to SA (Pieterse and Van Voon, 2004; Peleg-Grossman *et al.*, 2009). The protein NPR1 is a transcriptional regulator of *PR* genes, and, if mutated, the plants can exhibit a compromised resistance. It acts in the SA signal transduction chain, and in unstressed tissue is located in the cytosol. In response to SA, or oxidative or

nitrosylative stress, it is translocated to the nucleus where it interacts with and activates TGA2, 5, or 6 class transcription factors to activate *PR* genes and a range of other defence genes. The low proteolytic activity in *npr1-1* mutants would suggest that expression of the 34.6 kDa protease was NPR1 dependent. NPR1 overexpression has been shown to increase resistance to disease in rice (Chern *et al.*, 2001), and it may be that naturally occurring NPR1 expression variants in grasses could exhibit enhanced protein degradation in the rumen. However, in the experiments reported here, treatment of leaves with inhibitors of protein synthesis did not result in an increase in the recovery of protein or decreased proteolytic capacity, indicating that proteolytic activities involved in autolysis are pre-existing and are either processed to confer activity or are normally located away from their protein substrates. The observation that less protein was recovered after 3 h but not 6 h incubation when leaves were treated with chloramphenicol than in water controls (Table 2) was not associated with enhanced protease activity (Tables 3, 4) or isoform abundance (Fig 2). Together, this can be interpreted as reflecting a time window in which ordinarily protective proteins such as heat shock proteins and anaerobic response proteins would be synthesized (Sachs *et al.*, 1996; Gurley, 2000; Wang *et al.*, 2004; Kotak *et al.*, 2007; Banti *et al.*, 2008). Regardless of their function, the fact that this effect was observed when leaves were treated with chloramphenicol but not when they were treated with D-MDMP indicates that these proteins would normally be synthesized within the organelles.

PR1 was used here as a reporter for the functioning of the SA-mediated signal transduction pathway in response to the combined abiotic and biotic stresses encountered by ingested forage. During HR, the plant defence responses are induced in response to inoculated bacterial pathogens over the first 6 h (de Torres *et al.*, 2003). Here, exposure to rumen microorganisms for the same time period enabled detection of PR1 in the vasculature and mesophyll cells, presumably in response to colonizing bacteria. This response probably reflects elicitation by conserved pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) which include lipopolysaccharides and flagella. Defences triggered by PAMPs/MAMPs are thought to represent the most rapid plant response when coming into contact with microbes (Zhang and Zhou, 2010).

In the experiments reported here, the presence of PR1 protein indicated that the SA signalling pathway was operational in Col-0, *cpr5*, and *dnd1*, with product being detected after biotic and abiotic incubations.. Although, PR1 was initially identified as being a key component of the defence response to pathogens (Hooft van Huijsduijnen *et al.*, 1985), it has subsequently been shown also to be involved in the heat stress response (Clarke *et al.*, 2004). It remains to be seen how far protein breakdown may be similarly not dependent on the presence of the rumen microbial population and can be induced by exposure of plant cells to temperatures of 39 °C in an anaerobic environment (Beha *et al.*, 2002; Kingston-Smith *et al.*, 2002, 2003*a*, 2005*a*).

Together, these data suggest that post-ingestive autolytic protein degradation in the rumen environment can be controlled post-translationally in response to endogenous SA concentration. Hence, in ingested plant cells, increased foliar SA resulting from the developmental stage, prior stress, or exposure to rumen conditions could act via the NPR1 signal transduction pathway to induce expression or activate SA-sensitive protease(s) and so contribute to excessive post-ingestive protein breakdown in the rumen. Regulation of SA content and responses of forage crops therefore appear to be an appropriate target for forage improvement if livestock production with decreased environmental impacts is to be achieved. Given the considerable variation in SA accumulation amongst genotypes and species, this should be a realistic objective.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Densitometric analysis of protease activities detected during *in vitro* incubation in anaerobic buffer or in anaerobic buffer containing a 10% rumen fluid inoculum.

Acknowledgements

The authors thank Gemma Griffin and Gurpreet Singh for technical assistance, the Glasshouse staff for growth of *Arabidopsis* plants, the staff at Trawscoed 'D-unit' for provision of rumen fluid, Jim Heald for the SA measurements, and Tony Pugh for photography. Dr R. John Wallace (Rowett Institute of Nutrition and Health, University of Aberdeen) is kindly acknowledged for the provision of the pure cultures of bacteria used in this study. This work was funded by BBSRC, with Gurpreet Singh funded by the Nuffield Bursary scheme.

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