A novel assay for the detection of anthelmintic activity mediated by cuticular damage to nematodes: validation on *Caenorhabditis elegans* exposed to cysteine proteinases

A. M. PHIRI[1,2], D. I. DE POMERAI[1], D. J. BUTTLE[3] *and* J. M. BEHNKE[1]*

[1] *School of Life Sciences, University of Nottingham, Nottingham NG7 2RD, UK*
[2] *Department of Clinical Studies, School of Veterinary Medicine, University of Zambia, P.O. Box 32379, Lusaka, Zambia*
[3] *Department of Infection, Immunity & Cardiovascular Disease, University of Sheffield Medical School, Sheffield S10 2RX, UK*

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SUMMARY

Plant cysteine proteinases (CPs) from *Carica papaya* kill parasitic and free-living nematodes *in vitro* by hydrolysis of the worm cuticle, a mechanism that is different to all commercially available synthetic anthelmintics. We have developed a cheap and effective, rapid-throughput *Caenorhabditis elegans*-based assay for screening plant CP extracts for anthelmintic activity targeting cuticular integrity. The assay exploits colorimetric methodology for assessment of cuticular damage, and is based on the ability of viable cells to incorporate and bind Neutral red dye within lysosomes and to release the dye when damaged. Living worms are pre-stained with the dye, exposed to CPs and then leakage of the dye through the damaged cuticle is quantified by spectrophotometry. In contrast to motility assays and semi-subjective interpretation of microscopical images, this colorimetric assay is independent of observer bias. Our assay was applied to a series of *C. elegans bus* mutant strains with leaky cuticles and to cystatin knockout mutants. At ambient temperature and over 0.5–24 h, both *bus* mutants and the cystatin knockouts were highly susceptible to CPs, whereas wild-type Bristol N2 worms were essentially unstained by Neutral red and unaffected by CPs, providing validation for the utility of this assay.

Key words: *Caenorhabditis elegans*, Neutral red, cuticular damage, cysteine proteinases.

INTRODUCTION

The free-living nematode *Caenorhabditis elegans* has been used previously to screen plant cysteine proteinases (CPs) from *Carica papaya* for nematicidal activity (Phiri *et al*. 2014). Plant extracts containing active CPs kill parasitic and free-living nematodes *in vitro* by hydrolysis of the worms' cuticles. This mode of action is different from that of all commercially available synthetic anthelmintics. Although *C. elegans* has already been used to screen for compounds or drugs with anthelmintic properties (Simpkin and Coles, 1981; Boyd *et al*. 2010), measurement of efficacy is largely based on assays of motility, effects on lethality and on the number of damaged worms (Katiki *et al*. 2011; Phiri *et al*. 2014), most of which are dependent on the observer's subjective interpretation of microscopical images. However, to enable rapid-throughput screening of multiple samples, observer-independent, and preferably automated methods are required. One such recently developed method relies on automated measurement of worm motility (Buckingham and Sattelle, 2009; Buckingham *et al*. 2014).

To develop an alternative observer-independent assay for assessment of cuticular damage to *C. elegans*, we investigated the uptake, retention and release of Neutral red by the worm. Our project was motivated by the precedent of cellular Neutral red retention in coelomocytes derived from earthworms (*Lumbricus rubellus*) (Weeks and Svendsen, 1996) and pioneering work in *Daphnia* (Koehring, 1930). Assays based on uptake of Neutral red are widely used with cell lines as measures of cytotoxicity and have been employed with many other applications in the biomedical and environmental sciences (Borenfreund and Puerner, 1984; Babich and Borenfreund, 1990; Cavanaugh *et al*. 1990; Repetto and Sanz, 1993; Liebsch and Spielmann, 1995; Repetto *et al*. 2008). The success of Neutral red based assays is dependent on the ability of viable cells to incorporate dye by passive diffusion leading to its subsequent binding within the lysosomes. Up to 90% of Neutral red is taken up by lysosomes (Koehring, 1930; Winckler, 1974; Nemes *et al*. 1979), and subsequent release of this dye in quantifiable amounts when the cells harbouring the dye are damaged by toxic agents, provides quantitative assessment of damage, hence Neutral red leakage assay (NRLA; Repetto and Sanz, 1993; Weeks and Svendsen, 1996; Repetto *et al*. 2008).

The availability of bacterially unswollen (*bus*) *C. elegans* strains (Gravato-Nobre *et al*. 2005; Partridge *et al*. 2008), with their characteristically fragile cuticles, was exploited in this study to

* Corresponding author. School of Life Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK. E-mail: jerzy.behnke@nottingham.ac.uk

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explore the cuticular response to CPs of Neutral red-stained worms. The *bus* phenotype can arise from mutations in several genes affecting the cuticle, bacterial adhesion or colonization, the host swelling response and glycoconjugate biosynthesis (Gravato-Nobre and Hodgkin, 2005; Gravato-Nobre *et al*. 2005; Partridge *et al*. 2008; Palaima *et al*. 2010). The reorganization of the epidermis and cuticular layers of these worms results in a more fragile surface as revealed by increased bleach sensitivity, cuticle permeability and multi-drug sensitivity (Partridge *et al*. 2008). These qualities afford an opportunity for *bus* strains of *C*. *elegans* to be used in drug screening (Partridge *et al*. 2008; Palaima *et al*. 2010).

It must be noted that all parasitic nematodes tested *in vitro*, thus far have proved sensitive to plant-derived CPs (Stepek *et al*. 2004, 2005, 2007; Behnke *et al*. 2008; Buttle *et al*. 2011; Luoga *et al*. 2015). However, *C*. *elegans* is normally relatively resistant through cystatin inhibition of CP action (Phiri *et al*. 2014). Therefore, using *bus* or *cpi* null mutants offers the practical advantage of rendering *C*. *elegans* sensitive to CP attack, so that it can act as a surrogate (and much cheaper) model for parasitic species when screening CP-containing plant products for anthelminthic activity.

In order to validate the assay that we report here, intact *bus* mutant *C*. *elegans* worms were stained with Neutral red, and then following exposure to CPs, the stain that leaked into the incubation medium was measured spectrophotometrically to estimate the extent of CP damage to the cuticles and underlying tissues, and concurrent physical damage to the cuticle was assessed by scanning electron microscopy (SEM). Further evidence in support of the usefulness of the NRLA was provided by assessment of dye release by cystatin null mutants (*cpi*-$1^{-/-}$ and *cpi*-$2^{-/-}$) which, lacking cystatin gene products encoded by the *cpi*-1 or *cpi*-2 genes, cannot as readily resist externally applied CPs (Phiri *et al*. 2014). Since wild-type *C*. *elegans* are also highly susceptible to damage by CPs at 0 °C, a temperature at which it has been hypothesized cystatins fail to protect the worms, we also conducted some experiments at both 0 °C and at ambient temperature (a temperature at which wild-type worms are resistant to CPs) (Phiri *et al*. 2014).

MATERIALS AND METHODS

Source and maintenance of worm strains and papaya latex supernatant (PLS)

All strains were maintained on Nematode Growth Medium (NGM) agar plates containing a lawn of 0P50 *Escherichia coli* at 15 °C as described previously (Guven *et al*. 1994). Worms were harvested from agar plates using K medium (53 mM NaCl, 32 mM KCl; Williams and Dusenbery, 1990); Table 1 shows the strain name and genotype of worms employed in this study. All *bus* strains (Table 1) were provided by Professor Jonathan Hodgkin (University of Oxford, Oxford, UK), and both cystatin null mutants [*cpi*-$1^{-/-}$ (ok1213) and *cpi*-$2^{-/-}$ (ok1256)] as well as the wild-type Bristol N2 strain, were supplied by the *Caenorhabditis* Genetics Centre (University of Minnesota, Minneapolis, USA). All preparations of PLS, containing four CPs (Buttle *et al*. 1990), were prepared as described previously (Buttle *et al*. 2011), and 1 mM L-cysteine was included in all incubations with PLS. Once the preparations were made, aliquoted and frozen, there was no detectable deterioration of the enzyme activity (Phiri *et al*. 2014). Preparation and active-site titration of micromolar concentrations of CPs was carried out as previously described (Buttle *et al*. 2011; Luoga *et al*. 2015), using the CP-specific inhibitor L-trans-epoxysuccinyl-leucylamido- (4-guanidino) butane (E-64) (Sigma-Aldrich Ltd., Poole, UK).

Neutral red dye

Neutral red (Sigma®, St Louis, USA) stock solution (4 mg mL^{-1}) was prepared by dissolving 40 mg Neutral red dye in 10 mL K medium. The stock solution was kept at ambient temperature protected from light by aluminium foil for up to 2 months until use. To remove any precipitated dye particles from the 4 mg mL^{-1} stock solution, the stain was filtered through a 0·2 μm Millipore® filter.

Assessment of Neutral red uptake and staining of C. elegans

Worms at different stages of growth were washed off NGM plates with ice-cold K medium and handled as described previously (Phiri *et al*. 2014). The worm pellets were then transferred into a 50 mL centrifuge tube containing K medium plus a dense bacterial suspension (optical density = 1·0–1·5 at 550 nm) mixed with a 40 μg mL^{-1} final concentration of Neutral red. The worms and dye were incubated at 20 °C for at least 2 h to allow for consistent staining of the worms, though only a minority of wild-type N2 worms showed any signs of taking up the dye. The suspension was agitated gently using a slow speed on a tube roller during staining. Thereafter, worms were washed three or four times in ice-cold water and centrifuged for 3 min at 1619 × ***g*** between washes to remove excess dye.

Assessment of Neutral red retention

Successful uptake and retention of Neutral red was evaluated microscopically. Worm samples of all strains were inspected under an inverted microscope to check for successful and consistent staining.

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Table 1. Strain names and genotypes of *Caenorhabditis elegans* used in this study

Strain name	Gene of interest	Genotype
Bristol		Wild type
DC 19	*bus*-5	*bus*-5 (br19)
AQ 351	*bus*-8	*bus*-8 (lj22)
CB 6208	*bus*-8	*bus*-8 (e2887)X
CB 5696	*bus*-15	*bus*-15(e2709)
CB 5680	*bus*-16	*bus*-16 (e2802) I
CB 6081	*bus*-17	*bus*-17 (e2800)X
CB 6125	*bus*-18	*bus*-18 (e2795) V
CB 6596	*bus*-19	*bus*-19 (e2964) V
CB 6757	*bus*-21	*bus*-21 (e2992)
CB 7014	*bus*-21	*bus*-21 (e3016)
CB 7031	*agmo*-1	*agmo*-1 (e3016)
cpi-1-/-	*cpi*-1	*Ce-cpi*-1 (ok1213)
cpi-2-/-	*cpi*-2	*Ce-cpi*-2a(ok1256)

Photographs were taken using a Hiro High Resolution Microscope camera (American Lab and Science, Minnesota, USA) with TSView Digital imaging software.

Treatment of worms and measurement of Neutral red absorbance

Treatments involved 200 μL of stained living worm suspension added to 1000 μL of PLS to give the stated final active CP concentration in 1·5 mL micro-tubes, with or without the addition of E-64 at double the molar concentration of PLS; the concentration of PLS (and E-64), test temperature and duration of exposure were varied, depending upon the aim of the experiment. After treatment, the micro-tubes were shaken and placed on ice for 10–15 min or centrifuged at 11 586 × ***g*** for 1 min, to allow worms to settle at the bottom of the tubes. One millilitre of the supernatant was carefully removed from the micro-tube and transferred into a clean plastic cuvette with a 1 cm optical path length. The absorbance of the supernatant was then measured at 540 nm in a spectrophotometer (model-Libra 6, Biochrom, Scientific Laboratory Supplies, Nottingham, UK) that had been zeroed on a K medium blank.

Assessing cuticular damage of Neutral red stained worms using Trypan blue dye

As an independent measure of cell death, samples of the worms treated with CPs as described above and their controls were counterstained with 40 mg L^{-1} Trypan blue (Sigma-Aldrich, Steinheim, Germany) for microscopy. Controls included unstained worms and worms stained with Neutral red but not treated with CPs. Immediately after Trypan blue staining, worms were placed on a glass slide and cuticular damage was viewed and photographed using a Hiro High Resolution Microscope camera as above.

Effect of PLS on bus and cpi null mutant strains assessed by SEM

The *bus* strains used were CB 6081, CB 6125, CB 6757, CB 7014, DC 19, CB 5696, CB 7031, AQ 351 and CB 5680 (Table 1), together with N2 controls and both *cpi* null mutants. Mixed life-stage worms of each strain were prepared from NGM plates and handled separately as described previously. They were left unstained and exposed in duplicate to 45 μM PLS with or without 100 μM E-64 for 30 and 60 min. For each strain, an appropriate K medium control was employed. Assays were placed in 24-well plates and incubated at ambient temperature or at 0 °C, because the earlier work had shown that at temperatures above 15 °C wild-type *C. elegans* resist damage from CPs (it is hypothesized by secretion of cystatins), whereas as 0 °C they are highly susceptible (Phiri *et al*. 2014). A rapid microscopical assessment of the worms was made at each time point before samples were processed for SEM as described by Phiri *et al*. (2014), the latter essentially stopping any further enzyme reaction. Briefly, worms were fixed in 2·5% (v/v) glutaraldehyde for 1 h, and then placed in 0·15 M sodium phosphate buffer (pH 7·2) for a further hour before the phosphate buffer was changed and worms left overnight at 4 °C. After removal of the phosphate buffer, worms were further fixed in 1% (w/v) osmium tetroxide (Agar Scientific Ltd., Stansted, Essex, UK) before being washed three times with distilled water. This was followed by dehydrating the worms for 1 h in ascending concentrations of acetone (30–100%) before drying with a Polaron E3000 critical point drier. All samples were then coated with gold to a thickness of 50 nm using a Polaron sputter-coating unit (Polaron E5100). Worms were examined using a scanning electron microscope (Jeol JSM 840) and images were collected using a digital camera.

Statistical analysis

For the Neutral red assays, optical density measurements (± SEM) were analysed in Graphpad Prism 6 by one-way or two-way ANOVA, with Dunnett's or Tukey's *post hoc* multiple comparisons tests against corresponding K medium controls (no CP). Statistical significance was ascribed at $P < 0{\cdot}05$.

RESULTS

Successful Neutral red uptake and staining in C. elegans

Figure 1 shows a sample of wild-type N2 worms exhibiting successful uptake of Neutral red and consistent staining of the worms at 2 h when feeding bacteria were incorporated, and contrasts this with worms cultured without bacteria, which had

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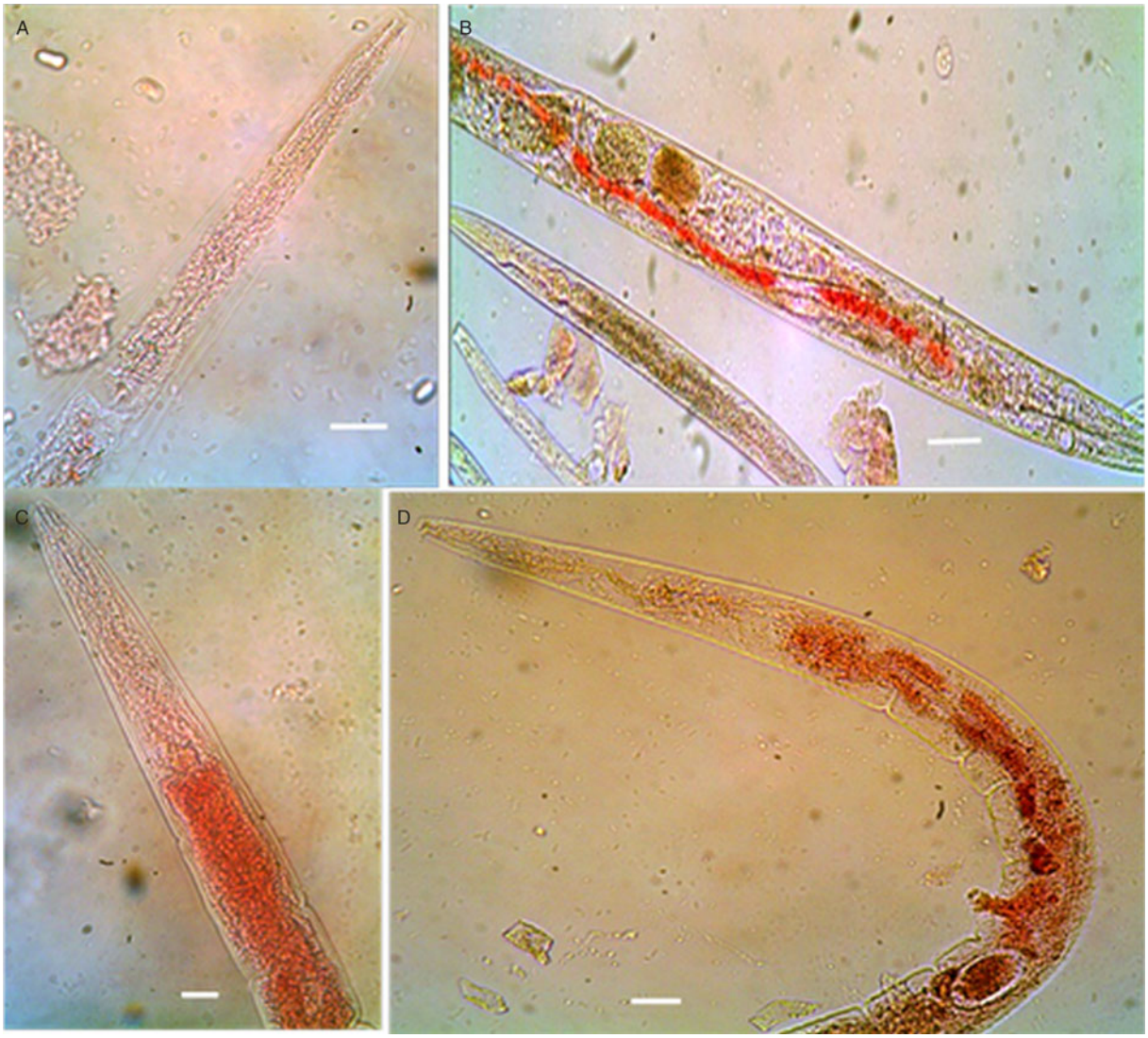

Fig. 1. Incorporation of OP50 *E. coli* feeding bacteria in the Neutral red dye enhanced dye uptake. Retention of dye was imaged in N2 wild-type worms after 2 h of incubation at 20 °C in K medium in the presence of 40 μg mL^{-1} of Neutral red, and was less apparent in the absence (A, B) as compared to the presence (C, D) of feeding bacteria. Scale bars = 63·1 μm.

minimal staining of the gut, the dye mostly failing to penetrate the cuticle of wild-type N2 worms. All the stained worms were alive prior to CP treatment. In general, only a minority of wild-type N2 worms showed detectable staining with Neutral red, even when abundant food bacteria were present. This may reflect their tough and impermeable cuticle; staining may only be possible when this is weakened, shed and replaced during moulting – a stage through which only some of the larvae present will pass during any given incubation period in the dye.

Caenorhabditis elegans bus strains were highly susceptible to the nematicidal effects of PLS

Caenorhabditis elegans bus strains were exposed to PLS for either 30 or 60 min. In contrast to the wild-type N2 controls, all the *bus* strains were affected by the CPs within 30 min of exposure at ambient temperature. Cuticular damage was evident in all strains except for N2 (Fig. 2), with previously retained Neutral red escaping through the damaged cuticles of the PLS-treated *bus* strain worms but not wild-type N2 worms. CP-induced damage was not confined to the cuticle, but started with it. Since the worm interior is under high hydrostatic pressure, once the cuticle was breached, the worms gave way and split outwards from that break. In that event, the worm can be completely destroyed without showing the initial site of cuticular damage. Even though damage was most obvious in the large adult worms, similar damage to much smaller larval worms was also present and much easier to see by SEM. Overall, in quantitative terms, it was easy to count 50 worms in untreated controls, but the number was significantly reduced

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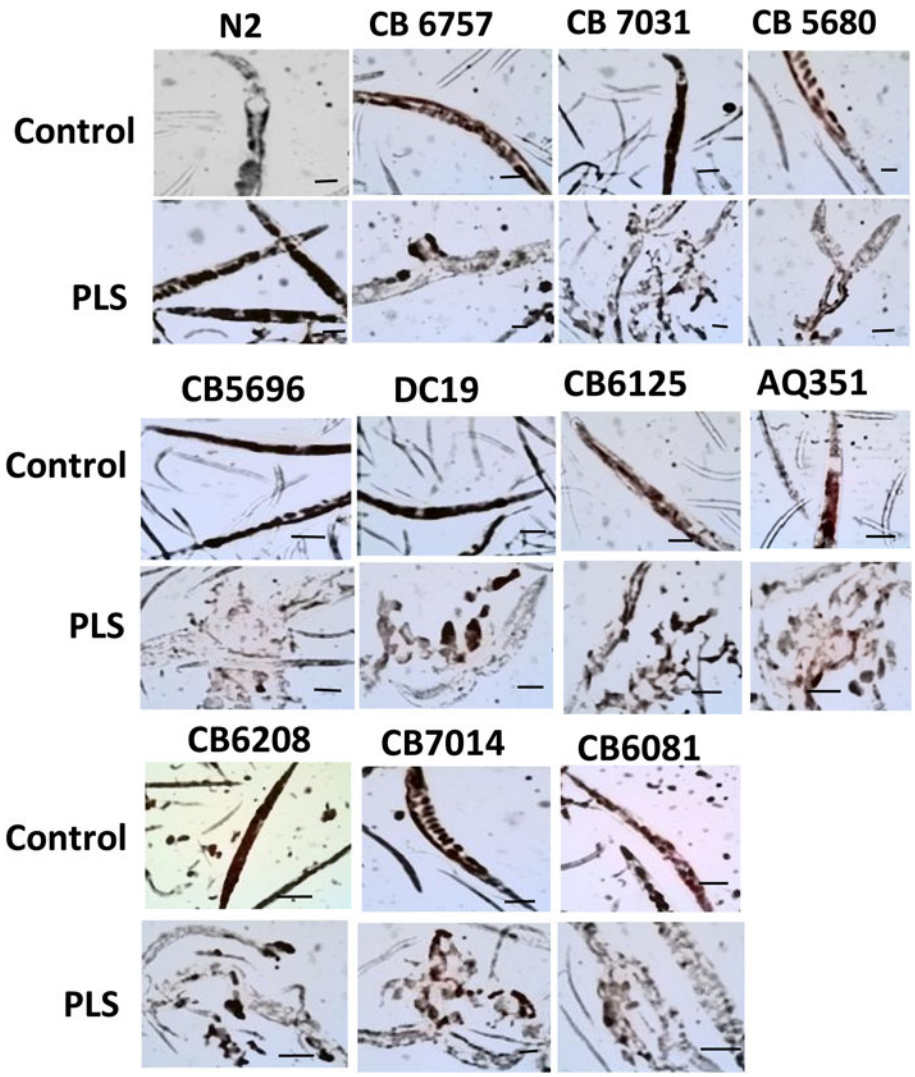

Fig. 2. Damage by PLS in mixed-stage *C. elegans bus* mutant strains: worms were stained with Neutral red and exposed to 45 μM PLS for 30 min at 20 °C. The panels show the control worms alongside the PLS-treated worms. Scale bars: 63·1 μm.

to single digits in the treated samples as a result of damaged cuticles and disintegration of whole worms.

Neutral red leakage is reduced when PLS is blocked with E-64

Neutral red leakage after 1 h at 20 °C was significantly greater for PLS-treated groups than for K medium controls in all of the tested *bus* strains ($P < 0{\cdot}01$ in all cases), but was non-significant in N2 controls ($P > 0{\cdot}05$), as shown in Fig. 3. However, Neutral red leakage from *bus* strains was reduced significantly when PLS was present together with a molar excess of the specific CP inhibitor, E-64 ($P < 0{\cdot}01$ for all *bus* strains). However, in the case of N2 there was no effect ($P > 0{\cdot}05$). Although PLS-induced leakage of dye and its blockade by E-64 was clearly seen in all *bus* genotypes, this was not the case in the wild-type N2, which showed the least response to CP treatment and no reduction when exposed to CP in the presence of E-64.

Effect of PLS on bus strains by SEM

All *bus* strains showed extensive physical damage to their cuticles when exposed to PLS after 30 min at 20 °C (Fig. 4). However, worms incubated in K medium and those exposed to PLS plus E-64 did not show any obvious signs of damage. N2 did not show any damage from PLS alone and no great difference when E-64 was also included (not shown).

CP-induced cuticular damage highlighted by use of both Neutral red and Trypan blue dyes

Figure 5 confirms extensive CP-induced cuticular damage in cystatin null mutants, and to some extent in wild-type N2 worms, with Neutral red escaping through the damaged cuticle of pre-stained *C. elegans* (red background) at 20 °C. Although Trypan blue was used to counterstain worms in order to highlight the damage more clearly, the blue

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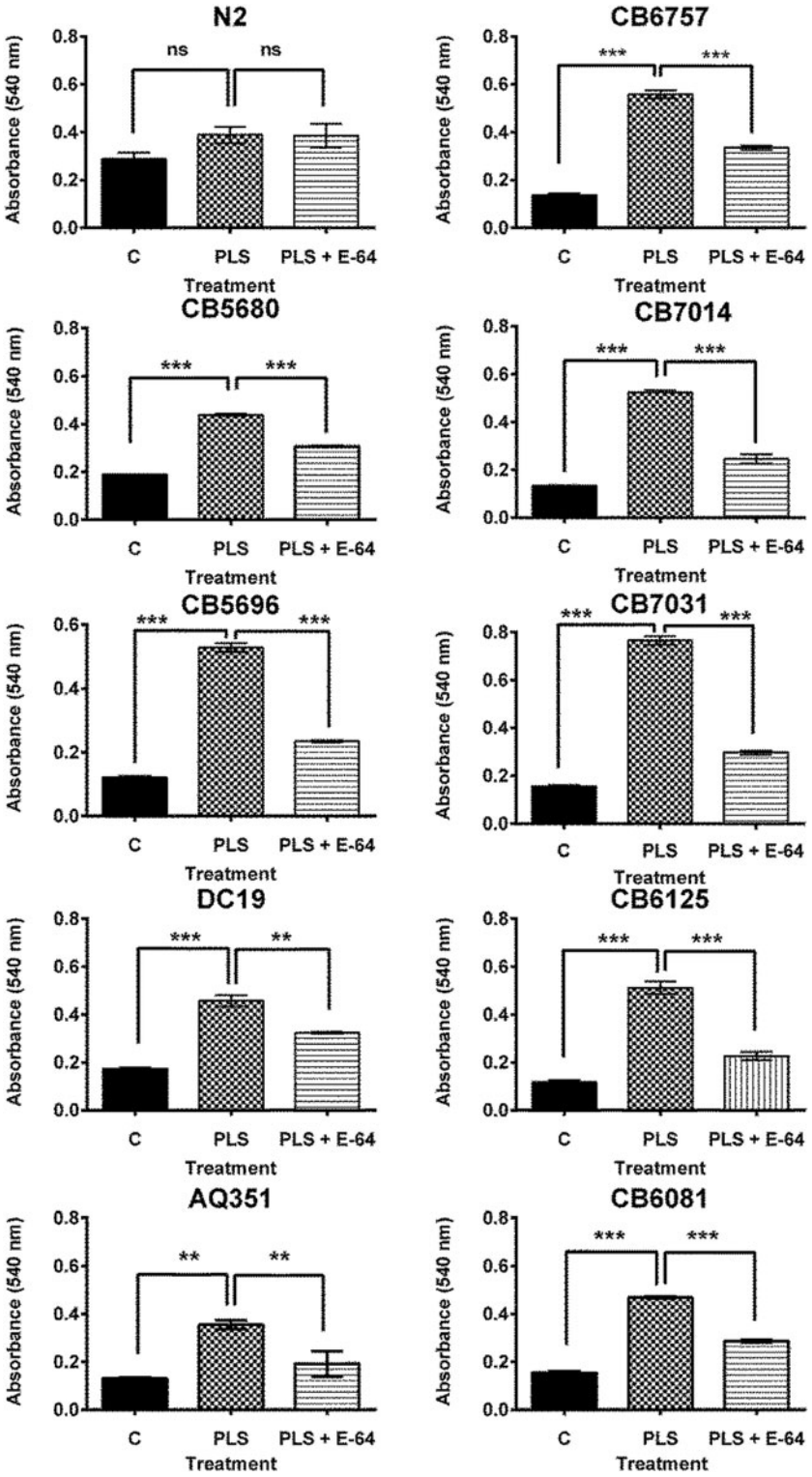

Fig. 3. Responses of *bus* strains and wild-type N2 *C. elegans* to cysteine proteinases: Worms stained with Neutral red were treated with 100 μM PLS and 100 μM PLS plus 200 μM E-64 and incubated at 20 °C for 1 h. Absorbance of Neutral red in the supernatant from exposed worms was measured in a spectrophotometer. The data are expressed as mean and SEM derived from three replicates (four for N2 controls). * = $P < 0{\cdot}05$, ** = $P < 0{\cdot}01$, *** = $P < 0{\cdot}001$, or ns = not significant ($P > 0{\cdot}05$) (Tukey's multiple comparisons test).

stain in the composite images in Fig. 5 is far from obvious, perhaps confirming that these tissues were still viable at the time the worms burst open, and implying that they had not been poisoned as such (through Neutral red or bacterial toxicity) but rather burst open at some point before cell death. Substantial Trypan blue staining of dead cells was not seen in any of the panels shown in Fig. 5.

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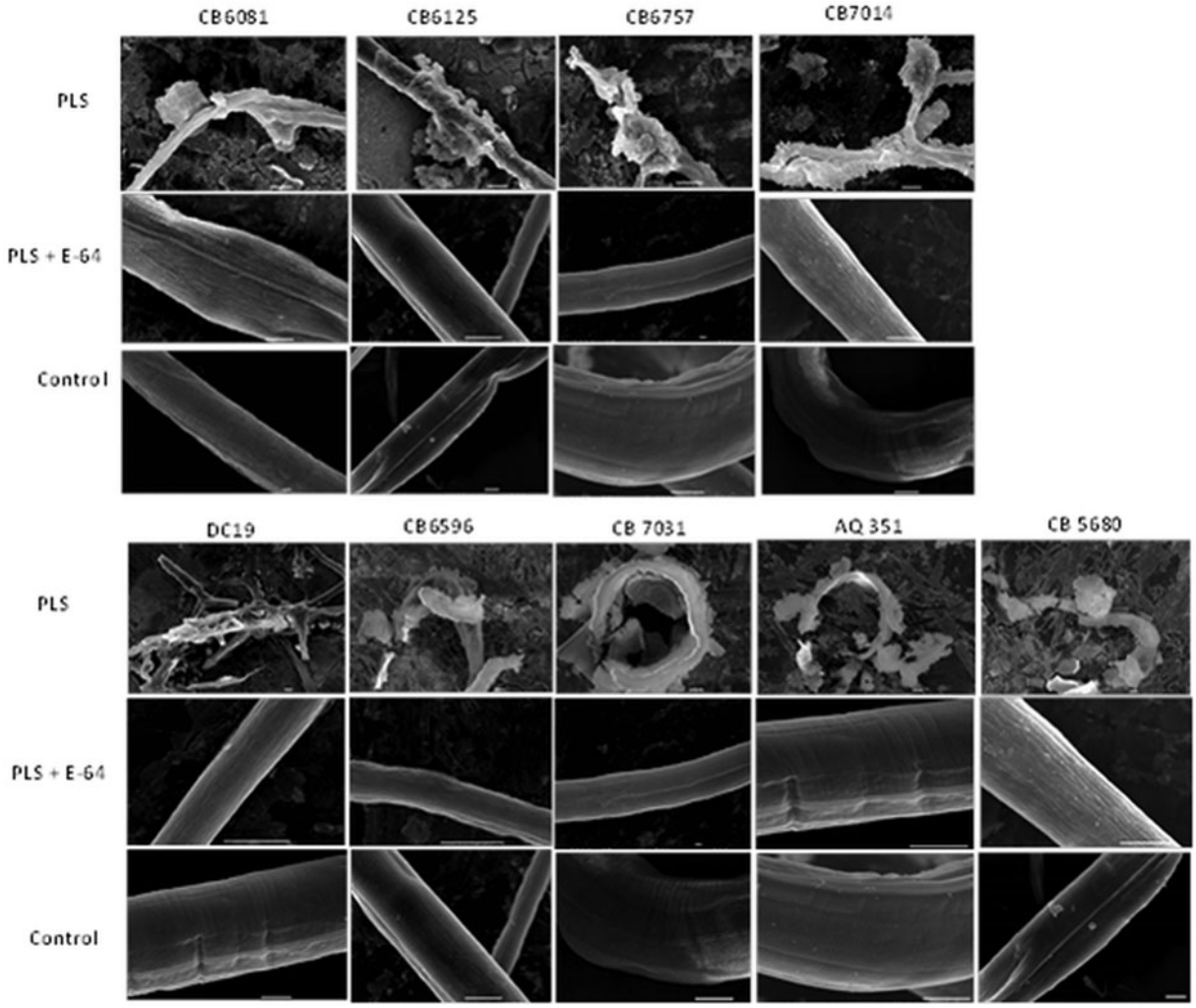

Fig. 4. Scanning electron microscopy of bacterially unswollen (*bus*) strains of *C. elegans* exposed for 30 min to papaya latex supernatant (PLS): Worms were exposed to 45 μM PLS and incubated at 20 °C for 30 min. The upper row shows electron micrographs of PLS-treated worms and depicts PLS damage to the cuticle; the middle row shows the blocking effect of incubating PLS with cysteine proteinase inhibitor (E-64), while the lower row shows control worms in K medium only. Scale bars: 10 μm.

Time course of NRLA with cystatin null mutant strains

There were no significant effects of PLS (45 μM PLS and 45 μM PLS in the presence of 90 μM E-64) on N2 worms at any time point tested at 20 °C ($P > 0{\cdot}05$), whereas both *cpi* mutant strains showed a significant increase in dye leakage following PLS treatment, even after 1 h ($P < 0{\cdot}05$), as shown in Fig. 6. However, there was seemingly no blocking effect of the CP inhibitor E-64 at 1 h and only a small, non-significant inhibition at 3 h ($P > 0{\cdot}05$). After 24 h, however, CP treatment of both *cpi* mutants released large amounts of dye ($P < 0{\cdot}001$), and this was largely blocked by E-64 ($P < 0{\cdot}001$), whereas no such effect was seen with N2 control worms.

Effects of PLS on cystatin null mutants at the SEM level

The cuticles of *cpi*-1$^{-/-}$, *cpi*-2$^{-/-}$ and wild-type N2 worms were observed at equivalent points along the body length after exposure to K medium (controls), 45 μm PLS, or 45 μm PLS + 100 μM E-64. Figure 7 shows scanning electron micrographs of cuticular damage in these null mutants, which is not seen in N2 controls, demonstrating the protective effect of the endogenous cystatins against exogenous CP attack. Null mutant worms treated with PLS exhibited extensive cuticular damage and disruption and digestion of cuticular structure after just 30 min of incubation at ambient temperature, but this effect was largely blocked by E-64.

DISCUSSION

All parasitic nematodes tested to-date have proved to be susceptible to digestion by CPs *in vitro* (Stepek *et al*. 2004, 2005, 2007; Behnke *et al*. 2008; Buttle *et al*. 2011; Luoga *et al*. 2015), but screening for novel compounds depends on the provision of live parasitic stages for suitable *in vitro* assays and

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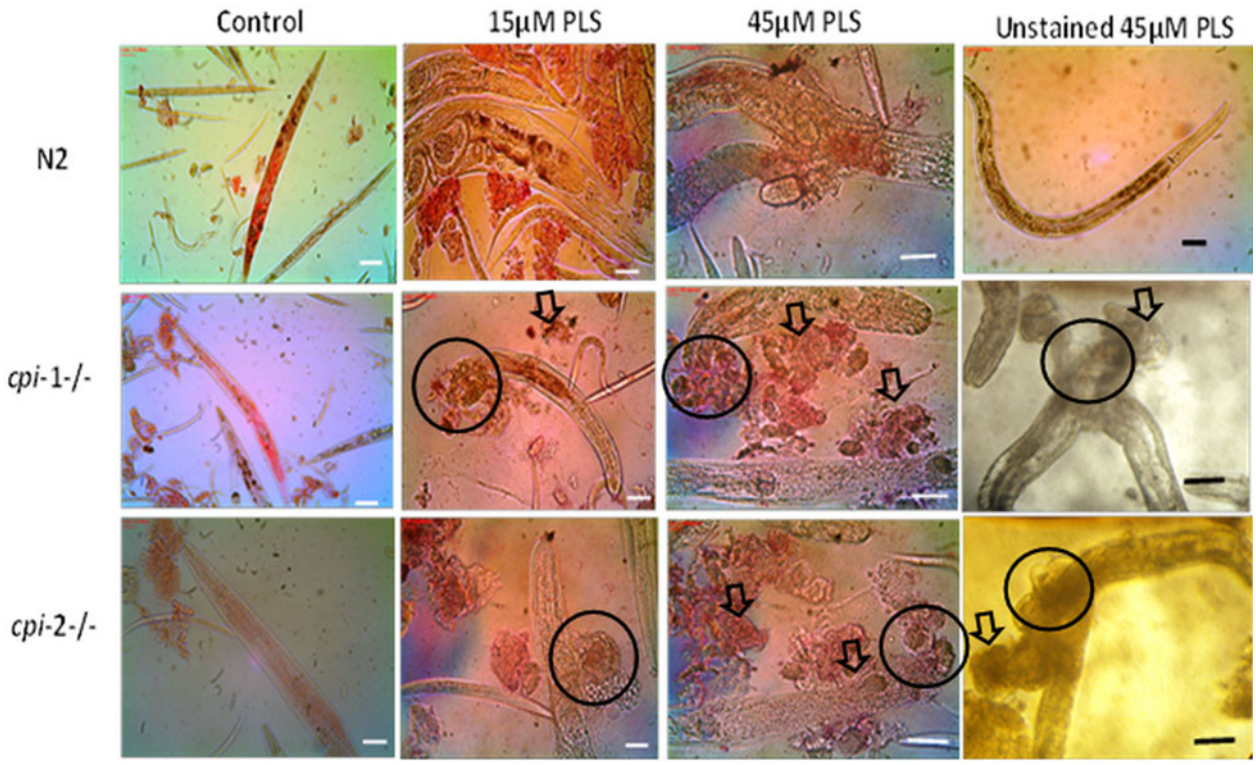

Fig. 5. Assessment of cuticular damage in cystatin mutant worms stained with Neutral red as a result of cysteine proteinase (CP) activity. After 30 min at 20°C, cuticular damage from the action of CPs was evaluated microscopically in worms pre-stained with Neutral red dye. The different rows show wild-type (N2; top), *cpi*-1$^{-/-}$ (middle) and *cpi*-2$^{-/-}$ (bottom) strains of *C. elegans*. In addition, determination of cell death was demonstrated in PLS treated worm samples counter-stained with Trypan blue. All strains were inspected under an inverted microscope. Unstained worm samples are included for comparison. The circles show the part of the worm that was initially damaged and the worms burst or disintegrated from there. The arrows show worm debris after cuticular damage. Scale bars: 63·1 μm.

therefore entails high costs (e.g. infection and culling of animal hosts) and is extremely labour intensive. Here, a cheap, technically simple and rapid NRLA based on *C. elegans* has been developed for screening plant CP extracts for anthelmintic activity targeting the cuticular surface of worms, and its suitability for screening purposes has been demonstrated in this study. Although an automated method for assessing worm viability based on motility has been described (Buckingham *et al.* 2014), our method does not require the use of software and can be carried out using a spectrophotometer as well as a plate reader. Neutral red has been widely applied to mammalian cells in culture (Parish and Müllbacher, 1983; Zhang *et al.* 1990; Repetto *et al.* 2008; Girishkumar *et al.* 2010; Thorne *et al.* 2014), and exploited in *in vitro* cytotoxicity assays based on leakage of the dye from damaged stained cells. Here this approach was successfully adapted for *C. elegans* and the study of CPs, although at this stage it is not known whether leakage of the dye from damaged worms reflects leakage from cells or through the damaged cuticle from within the worms. This is the first report showing the use of Neutral red as a readily quantifiable way of identifying nematicidal activity of plant products on *C. elegans* that is independent of investigator bias. It complements previously reported assays that involved counting the number of worms damaged by CPs (Phiri *et al.* 2014). Using *bus* strains, this assay provides a cheap, sensitive and simple procedure to run at ambient temperature (rather than at 0 °C as with wild-type worms – see Phiri *et al.* 2014). The Neutral red leakage protocol reported here required less equipment than conventional incubation and microscopy-based assessment and about 30 min to run to completion; however, as a result of the leaky structure of their cuticles, some absorbance was also noted in the untreated *bus* controls. As a quantitative measure of the extent of cuticular damage, the absorbance of leaked Neutral red can be measured on a conventional spectrophotometer at 540 nm; alternatively, this assay can be adapted for use in 96-well plates where the dye concentration in supernatants can be read automatically with a spectrophotometric microplate reader. Additionally, it has been reported that sensitivity could be enhanced by measuring the fluorescence of Neutral red in supernatants with excitation at 530 nm and emission read at 645 nm (Repetto *et al.* 2008).

In the development of a suitable protocol for measuring the uptake and leakage of Neutral red, it was important first to find a concentration of the dye that was not toxic to *C. elegans*, and that was

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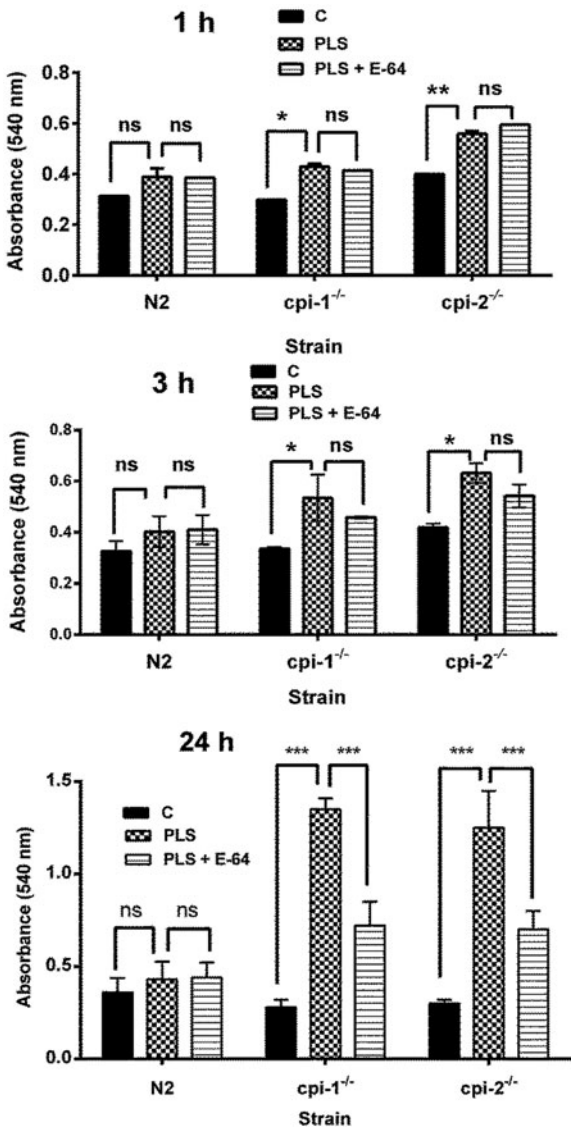

Fig. 6. Time course of Neutral red release from wild-type and cystatin knockout worms exposed to PLS at 20 °C. Cuticular damage as a result of PLS treatment (45 μM PLS) and the blocking effects of E-64 (45 μM PLS in the presence of 90 μM E-64), were evaluated after 1, 3 and 24 h of incubation by measuring the absorbance of Neutral red in supernatants from wild-type N2, *cpi*-1$^{-/-}$ and *cpi*-2$^{-/-}$*C. elegans*. Bars show the mean and SEM derived from 3 replicates (4 in the case of N2). * = $P < 0{\cdot}05$, ** = $P < 0{\cdot}001$, *** = $P < 0{\cdot}0001$, or ns = not significant ($P > 0{\cdot}05$) (Tukey's multiple comparisons test).

less prone to precipitation yet sufficient to achieve successful uptake and staining. Secondly, standardization of the time needed for adequate staining was required. Thirdly, K medium plus bacterial suspension was incorporated as a medium for dissolving the Neutral red, which in turn enhanced feeding and stain uptake by the worms. The use of this specific staining protocol incorporating feeding bacteria in K medium offered better and more consistent results than using K medium alone. Furthermore, a combination of the staining, exposure to CPs and measurement of Neutral red absorbance in the incubation supernatant was successful when employed with a series of *bus* strains.

Agents that attack the external layers of a worm are potential anthelmintics (Craig *et al*. 2007). CPs have been shown to act in this manner, with remarkable success in mammalian hosts (Buttle *et al*. 2011; Levecke *et al*. 2014; Luoga *et al*. 2015) and are capable of killing parasitic nematode worms *in vitro* as well as *in vivo* (Stepek *et al*. 2005, 2007; Levecke *et al*. 2014). However, in contrast to parasitic nematodes, free-living *C. elegans* worms, across their optimal temperature range (15–25 °C), are known to resist CP attack, at least in part through the action of the secreted cystatins CPI-1 and CPI-2 (Phiri *et al*. 2014). When the secretion of cystatins is impaired in *C. elegans* (as in *cpi*-null mutants), the worms become incapable of mounting effective resistance against CP attack (Phiri *et al*. 2014). Both of the available cystatin null mutants were far more sensitive to CP attack than wild-type N2 *C. elegans*. Using the NRLA described herein, the *cpi*-1$^{-/-}$ mutant in particular was shown to provide an effective rapid-throughput *C. elegans*-based assay for screening the nematicidal activities of plant-derived CPs. However, the time course for *cpi* mutants suggests that dye release is slower as compared with *bus* mutants. The characteristics and severity of cuticular damage are consistent with those reported in parasitic nematodes (Stepek *et al*. 2004, 2005; Behnke *et al*. 2008). While cystatin null mutants have proven to be good models for screening CPs (Phiri *et al*. 2014), the quantification of cuticular damage using microscopy had limitations. Adoption of the NRLA with this model ensured that nematicidal effects could be quantified independently of the investigator.

When *bus* strains that had been exposed to PLS for 30 min at ambient temperature were examined, their susceptibility to CPs was clearly evident through the changes in cuticular integrity that were visualized by SEM. The observed damage seemed even more severe and extensive than that observed in cystatin null mutants (Phiri *et al*. 2014). However, cuticular damage was prevented when worms were incubated with both PLS and the CP inhibitor, E-64. This finding provides strong support for the conclusion that CPs were indeed the cause of the cuticular damage and led to the leakage of the Neutral red and thus increased absorbance. We hypothesize that the mode of action of CPs in this case was facilitated by the fragile cuticle integrity (mechanical and structural) of *bus* strains, thus allowing easier access by CPs to the underlying structural proteins that they attack. In N2 worms, the cuticle on its own is a formidable barrier that, coupled with cystatins, inhibits CP attack. The presence of active cystatins (CPI-1 and CPI-2) at the surface of the cuticle on their own is unlikely to provide much protection if it is much easier for the CPs to permeate and attack the cuticle from within.

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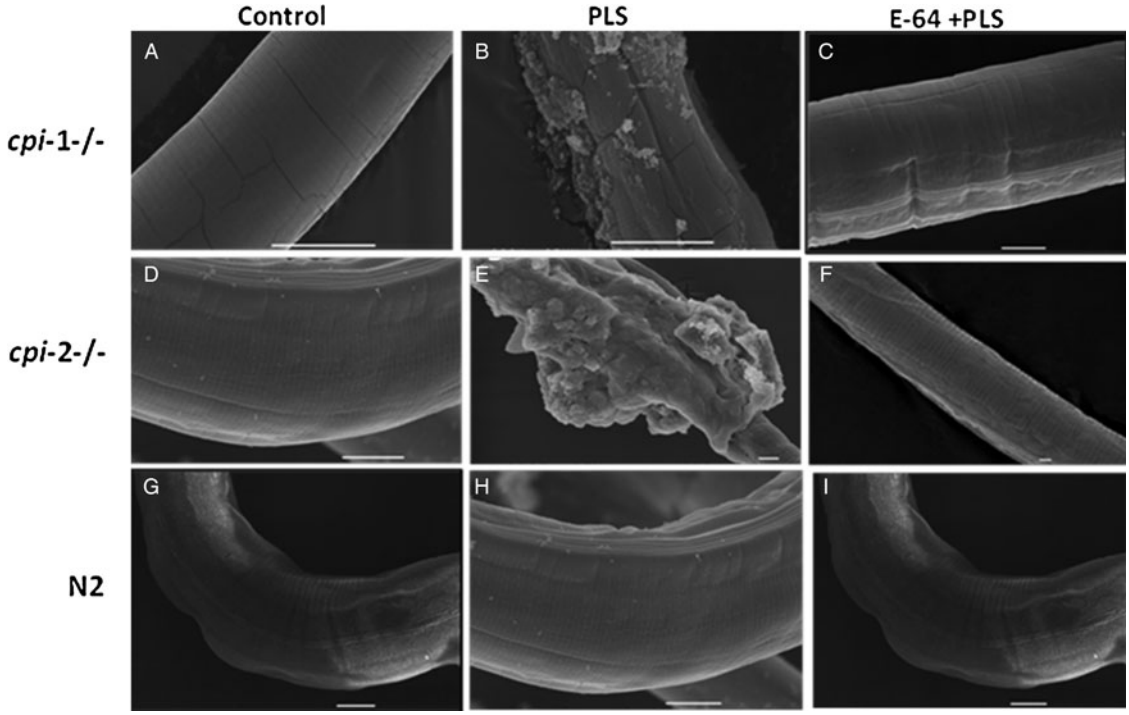

Fig. 7. Scanning electron micrographs of unstained cystatin null mutants (*cpi*-$1^{-/-}$ and *cpi*-$2^{-/-}$) worms incubated with (45 μm PLS) or without PLS, in the presence (45 μM PLS + 100 μM E-64) or absence of excess E-64 at ambient temperature for 30 min. Micrographs were taken as far as possible at equivalent points along the worm's surface near the mid-point. Panels (A–C) show *cpi*-$1^{-/-}$, (D–F) show *cpi*-$2^{-/-}$, and (G–I) show wild-type N2. The disruption and digestion of the worms' cuticle by PLS exposed the internal structures (B and E), but this was largely blocked by E-64 (compare C and F). The cuticles of K medium controls (A, D and G) remain intact. Scale bars, 10 μm.

In conclusion, a NRLA has been developed for assessment of damage to the cuticular surface of *C. elegans*. It involves the pre-staining of worms with Neutral red dye in the presence of bacteria for 2 h, followed by exposure of the stained worms to CPs. Subsequent assessment (through spectrophotometric measurement of absorbance in the supernatant) of the amount of dye released following leakage through the damaged cuticle is then used as a quantitative estimate of cuticular damage. This assay can be carried out at ambient temperature and is independent of any observer-dependent variation. When it incorporates strains with fragile cuticles (*bus* strains) especially CB 7014, CB 6757 and CB 7031, screening can be completed within 30–60 min after exposure to CPs at concentrations over 15 μM. The assay requires controls, including untreated worms and those with CP treatment blocked by E-64. Further adaptations to the assay, such as replacement of a conventional spectrophotometer with 96-well plates and a spectrophotometric microplate reader, would increase the speed and number of tests that can be carried out.

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