The potential role of apoptosis in the host-parasite interaction of *Leptosphaeria maculans* with *Brassica napus*

Wallace A. Cowling[*1,3], Caixia Li[1], Susan J. Barker[1], and David G. Gilchrist[2]

[1]School of Plant Biology, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia. Telephone +61-8-9380 7979. Facsimile +61-8-9380 1108. Email wcowling@cyllene.uwa.edu.au

[2]Department of Plant Pathology, University of California, Davis, CA 95616, USA. Telephone +1-530-752 6614. Facsimile +1-530-752 6523. Email dggilchrist@ucdavis.edu

[3]Canola Breeders Western Australia Pty. Ltd., 15/219 Canning Highway, South Perth, WA 6151, Australia.

ABSTRACT

The fungus *Leptosphaeria maculans* (Phoma or blackleg disease) causes lesions on leaf, stem and crown tissue of oilseed rape (*Brassica napus*). It is similar to many other necrotrophic fungi that rely on plant cell death for growth and reproduction. Recent published evidence supports the theory that some, if not all, necrotrophic bacteria and fungi stimulate apoptosis as a prerequisite for infection of host tissue. Apoptosis in animals is characterized by the activation of an enzyme pathway (the caspase cascade) that leads to ordered proteolytic inactivation of specific cellular repair and structure proteins. This in turn leads to nuclear disintegration and cellular collapse. Caspases belong to a conserved family of cysteine proteases with aspartate specificity – one of the most specific protease groups known in animals. Sequence searches in plants have not identified close homologues, although functional assays suggest caspase-like proteases occur in plants.

We examined the effect of an animal caspase inhibitor on development of leaf lesions caused by *L. maculans* in susceptible *B. napus* 'Westar'. Leaves were co-infiltrated with a conidial suspension of *L. maculans* and the tetrapetide caspase inhibitor acetyl-asp-glu-val-asp-aldehyde (Ac-DEVD-CHO) in 10 µL containing an average of 100 or 1000 conidia. Lesions increased in severity from 10-14 days after inoculation, and from 100 to 1000 conidia per inoculation site. In every case, the inhibitor reduced lesion severity and area in paired inoculations across a range of inhibitor concentrations from 50 to 500 µM.

The inhibitory effect of Ac-DEVD-CHO, which is highly specific to caspase enzymes, occurred at low concentrations that are known to affect caspases in animal systems. The inhibitor had no affect on conidial germination or growth *in vitro*. By understanding the role of apoptosis in susceptibility, plant breeders will be armed with molecular genetic information to select novel genes for resistance to these diseases.

Key words: Programmed cell death – canola

INTRODUCTION

The interaction of pathogens and plants leads to a disruption in cellular homeostasis, often leading to cell death in both compatible and incompatible interactions. Programmed cell death (PCD) is now recognized to play important roles in plant disease (Gilchrist, 1998; Greenberg, 1997; Richael et al., 2001) and shares many of the morphological characteristics of apoptosis as defined in animal systems (Gilchrist, 1998; Wang et al., 1996). Current data suggest that activation or suppression of apoptosis may underlie disease in plants as it does in animals. Cross-kingdom interactions cause activation or suppression of PCD in plants (Dickman et al., 2001; Gilchrist, 1998; Heath, 2000; Lincoln et al., 2002; Richael et al., 2001).

Apoptosis was first characterized in animals as the gene-directed orderly disintegration of a cell under control of a set of preformed gene products functioning in transmembrane signalling, specific proteolytic cleavage of key cellular substrates, nuclear disintegration and cellular collapse. Critical components of the apoptotic machinery are cysteine proteases with aspartate specificity known as caspases (Grütter, 2000). Caspases are well characterized in animal systems but have not been studied in plants. Initial sequence search algorithms have identified potential relatives known as metacaspases but thus far have failed to find direct

sequence homologues of animal caspases. However, as is true in many DNA sequence-protein function studies, the failure to detect close sequence homologues does not preclude the conservation of caspase-like functions in plants. In fact, specific tetrapeptide inhibitors of caspase enzymes from animal systems, such as acetyl-asp-glu-val-asp-aldehyde (Ac-DEVD-CHO), were found to reduce symptoms of a necrotrophic bacterial disease in tobacco and tomato (Richael et al., 2001) and a specific caspase inhibitor, the baculovirus p35 gene, blocked PCD in transformed cells and resulted in resistance to a range of fungal and bacteria pathogens when transformed into tomato (Lincoln et al., 2002).

We examined the effect of an animal caspase inhibitor on development of leaf lesions by *Leptosphaeria maculans* in susceptible *Brassica napus* 'Westar'.

MATERIALS AND METHODS

Plants of *B. napus* 'Westar', susceptible to blackleg, were grown to the three-leaf stage (three weeks old) in a growth chamber at 18/13 C (16 h light/8 h dark) before inoculation with a highly aggressive isolate of *L. maculans*. The fungus was grown on V8 juice agar for two weeks under 12 h fluorescent and blacklights per day to induce asexual sporulation. Culture plates were flooded with sterile distilled water and the conidial suspension was filtered through sterile cheesecloth before collecting conidia on a 0.22 µm filter and resuspending to a final concentration of 10^6 conidia mL^{-1}. This suspension was stored at –20 C before inoculation.

In the "plus inhibitor" ("+I") treatments, Ac-DEVD-CHO was added to a final concentration of 50, 100, 250 and 500 µM, and the stock suspension of conidia was added to achieve a final concentration of 10^5 and 10^4 conidia mL^{-1} in each treatment. Approximately 10 µL of these solutions were infiltrated into the intercellular spaces of the mesophyll of leaves 1-3 using a flat-ended syringe with minimal damage to the leaves. The four "+I" treatments were randomized on one side of the leaf midvein, and equivalent sets of "-I" inoculations were located on the opposite side of the leaf midvein in a paired arrangement. The site of infiltration was visible as a watersoaked mark with an area of approximately 0.75 cm^2 that dissipated within 1 h. Water with or without inhibitor was infiltrated into leaves of one plant as a control.

This arrangement of paired inoculations was repeated on 6 plants for each concentration of inoculum. Plants were subjected to high humidity in the growth chamber by covering with clear plastic for 4 days, and severity of lesions were recorded at days 10, 12 and 14 after inoculation according to the following scale:

0 = no visible symptoms.
1 = slight chlorosis at the site of inoculation.
2 = moderate chlorosis and slight necrosis at the site of inoculation.
3 = necrotic spots scattered across the inoculation area, not amalgamated.
4 = extensive necrosis (gray-white lesion in the inoculation area).
5 = 100% necrosis, lesions spreading, pycnidia present.

Lesion diameter (cm) was recorded at 14 days. Data were analysed by pair-wise t-tests to directly compare "+I" and "-I" treatments. The effect of Ac-DEVD-CHO on *L. maculans in vitro* was assessed by placing 100 µl of conidial suspension "+I" and "-I" on water agar medium and assessing conidial germination and growth after 24 hours.

RESULTS

Co-infiltration with Ac-DEVD-CHO delayed symptom development and reduced severity of leaf lesions caused by *L. maculans* on *B. napus* 'Westar'. Nine days after inoculation, the first necrosis appeared in the "-I" treatments, whereas only chlorosis was evident in the "+I" treatments. No symptoms were present on the water-only controls with or without inhibitor.

Lesion severity increased over time, but the inhibitor reduced lesion severity in each pair of "+I" and "-I" treatments 10, 12 and 14 days after inoculation (Fig. 1A). The inhibitor was equally effective from 50 to 500 µM (Fig. 1B). Lesion severity increased as the inoculum dose increased from 100 to 1000 conidia per infiltration site, but the inhibitor reduced severity at both doses (Fig. 1C). At 100 conidia per infiltration, average lesion area at day 14 was reduced from 1.34 cm^2 in "-I" to 0.86 cm^2 in the "+I" treatment.

Conidial germination *in vitro* was 80% in both "+I" and "-I" treatments.

DISCUSSION

The specific animal caspase tetrapeptide inhibitor Ac-DEVD-CHO reduced average lesion severity and lesion area in the compatible interaction of the fungal necrotroph *L. maculans* with *B. napus*. The inhibitor did not affect fungal germination and growth *in vitro*, nor did it cause any toxicity to leaves. This confirms similar results in tomato, tobacco and pea with a wide range of diseases (Lincoln et al., 2002; Richael et al., 2001) and demonstrates that Ac-DEVD-CHO has disease-inhibiting properties across a wide range of plant systems. The properties of Ac-DEVD-CHO in inhibiting caspase enzymes and apoptosis were discovered in animal systems, and our results suggest that the compatible interaction of *B. napus* with *L. maculans* occurs as a result of apoptosis in plant cells induced by the fungus. Further work is required to confirm this hypothesis. If correct, the hypothesis leads to many interesting research areas for improving resistance of *B. napus* to blackleg disease.

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Fig. 1A. Effect of time after inoculation on lesion severity +/- inhibitor (I)

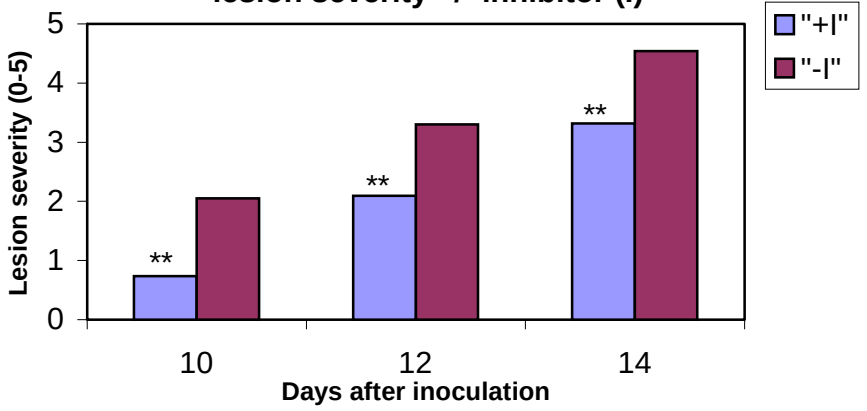

Fig. 1B. Effect of inhibitor concentration on lesion severity +/- inhibitor (I)

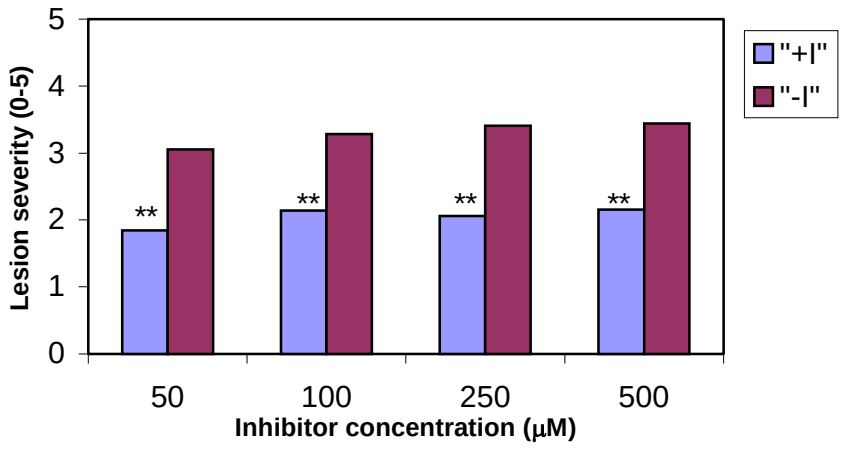

Fig. 1C. Effect of inoculum concentration on lesion severity +/- inhibitor (I)