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Research article

Molecular characterization of cell death induced by a compatible interaction between *Fusarium oxysporum* f. sp. *linii* and flax (*Linum usitatissimum*) cells

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Abstract

The cellular and molecular events associated with cell death during compatible interaction between *Fusarium oxysporum* sp. *linii* and a susceptible flax (*Linum usitatissimum*) cell suspension are reported here. In order to determine the physiological and molecular sequence of cell death of inoculated cells, reactive oxygen species (ROS) production, mitochondrial potential, lipoxygenase, DNase, protease and caspase-3-like activities, lipid peroxidation and secondary metabolite production were monitored. We also used microscopy, *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) and DNA fragmentation assay. Cell death was associated with specific morphological and biochemical changes that are generally noticed in hypersensitive (incompatible) reaction. An oxidative burst as well as a loss of mitochondrial potential of inoculated cells, an activation of lipoxygenase and lipid peroxidation were noted. Enzyme-mediated nuclear DNA degradation was detectable but oligonucleosomal fragmentation was not observed. Caspase-3-like activity was dramatically increased in inoculated cells. Phenylpropanoid metabolism was also affected as demonstrated by activation of *PAL* and *PCBER* gene expressions and reduced soluble lignan and neolignan contents. These results obtained in flax suggest that compatible interaction triggers a cell death sequence sharing a number of common features with the hypersensitive response observed in incompatible interaction and in animal apoptosis.

Keywords: Caspase; Flax (*Linum usitatissimum*); *Fusarium oxysporum* sp. *linii*; Programmed cell death; Nuclease; Reactive oxygen species; Secondary metabolites

1. Introduction

Plant defense mechanisms have been extensively analyzed in various plant–pathogen interactions. Indeed, understanding molecular events during plant–pathogen interactions is of great interest since these events are closely associated with plant disease resistance and can guide agricultural strategies. *Fusarium oxysporum* is ubiquitous in soil worldwide as both pathogenic and nonpathogenic strains. *F. oxysporum* f. sp. *linii*, causative agent of wilt disease in flax (*Linum usitatissimum* L.), is one of the most important diseases in this crop. It is characterized by its ability to enter the plant through the roots and to spread throughout the entire vascular system, inducing yellowing and wilting of the leaves, and eventually causing the death of the plant [37]. Host cell death that occurs during many interactions between plants and their pathogens

Abbreviations: DCG, dehydrodiconiferyl alcohol glucoside; HR, hypersensitive response; LOX, lipoxygenase; PAL, phenylalanine ammonia lyase; PCBER, phenylcoumaran benzylic ether reductase; PCD, programmed cell death; PLR, pinoresinol–lariciresinol reductase; ROS, reactive oxygen species; SECO, secoisolariciresinol; TUNEL, terminal deoxynucleotidyl transferase-mediated dUDP nick end labelling.

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can be associated with disease resistance (incompatible interaction) or with susceptibility (compatible interaction) [16].

The most studied plant–pathogen interaction model, the hypersensitive response (HR), an incompatible interaction, is associated with a phenomenon termed resistance. Resistance response in plant involves three steps: (a) recognition of the pathogen, (b) signal transduction, and (c) execution of the defense programs such as HR cell death, oxidative burst, transcriptional activation of defense genes, and subsequent induction of systemic acquired resistance (SAR). Recognition events often follow the gene-for-gene model discovered by Flor [11] in flax. The resistance response is triggered when the host has a dominant *R* gene corresponding to a dominant *avr* gene in the pathogen leading to a localized cell death and the rapid activation of defense responses [19,41]. This hypersensitive cell death is regarded as a defensive suicide of the plant cells against an avirulent pathogen that prevents pathogen spreading. In plants, cell death during HR has been shown to be genetically controlled (programmed cell death, PCD) and to share some features with animal cell apoptosis [14]. It includes the generation of reactive oxygen species (ROS) leading to the so-called oxidative burst that plays a central role for the generation of transduction signals for cell death [15,30]. It is followed by lipid peroxidation, as a result of free radical peroxidation or lipoxygenase enzyme activity, which reflects membrane degradation or dysfunction [36]. Activation of nucleases that degrade the chromosomal DNA into oligonucleosomal DNA cleavage has also been reported in some plant tissues undergoing PCD [28,31]. Recently, there has been growing evidence of caspase-like activity in plants [24,26,32], for example, during HR in fungus-infected cowpea plant [7,10].

Another component of the plant's defense system is the production of secondary metabolites such as constitutive antifeedants and phytoanticipins, or inducible phytoalexins. Lignin deposition is generally induced in response to microbial attack and acts as a physical barrier against pathogen invasion [8,34]. Lignans and neolignans, that share monolignols as common precursors with lignin, can be formed *de novo* in response to fungal attack and act as phytoalexins in both woody and non-woody species [13]. Activation of genes of the early steps of the phenylpropanoid pathway by biotic or abiotic stresses occurs in different plant systems. Phenylalanine ammonia lyase (PAL) has a crucial role in plant defense mechanisms as the starting point for the synthesis of defense/stress-related compounds such as lignin, lignans and neolignans from phenylalanine [8]. Pinoresinol–lariciresinol reductase (PLR) and phenylcoumaran benzylic ether reductase (PCBER), involved in lignans and neolignans' metabolism, are both correlated with functions associated with plant defense [13]. In flax cell culture, elicitation with *F. oxysporum* extracts triggered a strong incorporation of monolignols in the non-condensed labile ether-linked lignin fraction concomitantly with a decrease in lignans and neolignans' accumulation [17].

Plant cell cultures are a useful model system to investigate biosynthesis of plant secondary metabolites; for example *Linum* cell cultures produce a wide range of putative defense-related lignans of pharmaceutical interest [2,12]. The use of direct pathogen infections of plant cell cultures enables the identification of signaling components and the determination of the relationship between cell death and other defense-related events [16], because this system, contrary to a whole plant, allows uniformity, accessibility and reduced complexity. Recent studies suggest that cell death during interactions between susceptible plant and pathogen (compatible interaction) exhibits some features characteristic of apoptosis [16]. For example, the compatible interaction between *Puccinia coronata* f. sp. *avenae* or *Magnaporthe grisea* and oat host cells induced chromatin condensation and DNA cleavage [46]. The authors hypothesized that both compatible and incompatible interactions may have overlapping pathways [16]. However, little is known about the morphological and molecular events associated with cell death that occurs in susceptible plant cells. Indeed most of the published work focused on incompatible interaction or/and a small set of the above mentioned cell death markers. Therefore in the present work, we investigate the kinetics of the biochemical changes and molecular mechanisms of cell death occurring in a cell suspension of a susceptible flax cultivar inoculated with spores of a virulent strain of *F. oxysporum* f. sp. *linii*. We put effort in monitoring a wide range of molecular and biochemical markers.

2. Results

2.1. Morphological and physiological changes

To evaluate the effects of *F. oxysporum* on both plant cell morphology and viability, we examined flax suspension-cultured cells inoculated with conidiospores at the end of their exponential growth phase. Conidiospores began to germinate 24 h after inoculation and hyphae surrounding cell clusters were visible at 48 h (Fig. 1A). Since 24 h after inoculation, a browning of treated-cell suspension was observed and accompanied by shrinkage and finally membrane disruption, but apoptotic bodies were not observed (Fig. 1A). Changes in cell morphology were associated with a growth arrest 24 h after inoculation (Fig. 1B) and an increased cell death (Fig. 1C). Cultures exhibited *per se* a certain ratio of dead cells, before inoculation, however, this level remained constant in control cell culture while it increased since 24 h post-inoculation in treated suspensions (Fig. 1C). A significant alkalinization of the extracellular medium was noted in inoculated cells when compared to control (Fig. 1D). Conductivity measurements reflect the ion content in culture medium which results from the equilibrium between uptake and leakage. Contrary to control cells, ion leakage in inoculated cells at least compensates ion uptake (Fig. 1E). Conductivity measurements suggest that inoculated cells no longer metabolized their culture medium.

2.2. Oxidative burst and membrane dysfunction

An oxidative burst, corresponding to a sudden rise in intracellular superoxide anions' level, occurred after inoculation. Indeed, when compared to control cells, intracellular