[Plant Signaling & Behavior 3:3, 189-193; March 2008]; ©2008 Landes Bioscience

Research Paper

A diffusible signal from germinating *Orobanche ramosa* elicits early defense responses in suspension-cultured *Arabidopsis thaliana*

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Key words: *Orobanche ramosa*, *Arabidopsis thaliana*, cell death, hydrogen peroxide, secondary metabolism

In plant/parasitic plant interaction, little is known about the host plant response before the establishment of the parasite within the host. In the present work, we focused on host responses to parasitic plant, *O. ramosa* in the early stage of infection. We used a co-culture system of *A. thaliana* suspension cells and *O. ramosa* germinated-seeds to avoid parasite attachment. We showed that *O. ramosa* induced H_2O_2 generation and camalexin synthesis by *A. thaliana* followed by a drastic increase in cell death. We further demonstrated that a heat sensitive diffusible signal is responsible for this cell death. These data indicate that recognition of *O. ramosa* occurs before the attachment of the parasite and initiates plant defence responses.

Introduction

Parasitic plants cause severe yield losses, especially the parasitic plants belonging to the *Scrophulariaceae* and *Orobanchaceae* families.[1] Some *Orobanche* species have wide host ranges and grow on annual hosts.[2] These species are able to parasite a wide range of agricultural crops[1] and cause major yield losses.[1,3,4] *O. ramosa* is one of the most noxious especially in Europe but damages were also reported in Australia and Chile. In France, this parasite causes heavy losses in hemp, oilseed rape, tobacco and tomato.[5] *Orobanche* sp. is a root holoparasite completely dependent on the host plant for nutrients and water. It attaches and invades the host roots using a specialized structure, called haustorium. Parasite germination and host attachment are supposed to be the most critical steps required for the accomplishment of the parasite life cycle. They are dependent on different host plant molecules, sesquiterpenes or hydroquinones and benzoquinones.[6-9] These molecules are responsible for parasite germination and haustoria formation, respectively. On the other hand, the host perception of parasitic plants in this early stage of the interaction could be important as in numerous plant/pathogen interactions. In plant/pathogenic microorganism interactions, several pathogen-derived key molecules have been isolated. They are called elicitors because they induce plant defense responses. The general plant responses against pathogens are programmed cell death, oxidative burst, phytoalexin production and accumulation of antimicrobial proteins such as pathogenesis related (PR) proteins. In the last few years, some studies on plant responses to parasitic plants revealed that parasite attachment to the host root also promote the plant defense responses which observed in plant/pathogen interaction. Indeed, *PR-1* gene promoter is activated in transformed tobacco roots after the attachment of *Orobanche aegyptica*.[10] *Hmg2*, a related defense gene in tomato is also activated after root penetration by *O. aegyptica*.[11] *NRSA-1* encoding a putative disease resistance protein is induced in marigold roots parasitized by *Striga* spp.[12] The enhanced-expression of *LeAqp2,* an aquaporin-like gene, was reported in *Lycopersicum esculentum* in response to *Cuscuta reflexa*.[13] More recently, a proteomic study on pea responses to *O. crenata* reported the upregulation of PR proteins including β-1,3-glucanase and peroxidase.[14] Vieira Dos Santos et al.,[15,16] also showed the activation of *A. thaliana* defense genes in response to *O. ramosa* such as those coding for defensin (*pdf1*.2), PR-3, thionin (*thi2*.1), pectin methyl esterase inhibitor (PMEi), reactive oxygen species (ROS) detoxifying proteins, gst1, gst11 and peptide methionine sulfoxide reductase. These authors detected *A. thaliana* gene activation before the parasite attachment by the use of an *in vitro* co-culture system with *A. thaliana* callus.[16] This is consistent with the hypothesis that *A. thaliana* perceive the presence of *O. ramosa* without parasitic attachment. Although these studies indicate that parasitism promote the expression of defense related genes in host, the early plant defense responses against *O. ramosa* are still obscure. To clarify the early perception-based responses, we chose *A. thaliana* as the host of the parasitic plant *O. ramosa*, the most damaging species of dicotyledonous species in France.[5,17] An in vitro co-culture system using *A. thaliana* suspension cells allowed us to study the first stage of interaction preceding the parasitic attachment. In the last decade it has been demonstrated that use of suspension cultured cells is convenient for identifying early physiological events induced by pathogens.[18-22] Therefore, here we attempted to examine (i) the detection of cell death induction in *A. thaliana* cells by *O. ramosa,* which is known to occur both in compatible or incompatible host-parasite interactions, (ii) the quantification of oxidative burst represented by production of ROS, especially H_2O_2, which is one of the most commonly reported plant responses to biotic stresses,[23] and (iii) the evaluation of phytoalexin production.

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Submitted: 12/13/07; Accepted: 01/08/08

Previously published online as a *Plant Signaling & Behavior* E-publication: http://www.landesbioscience.com/journals/psb/article/5545

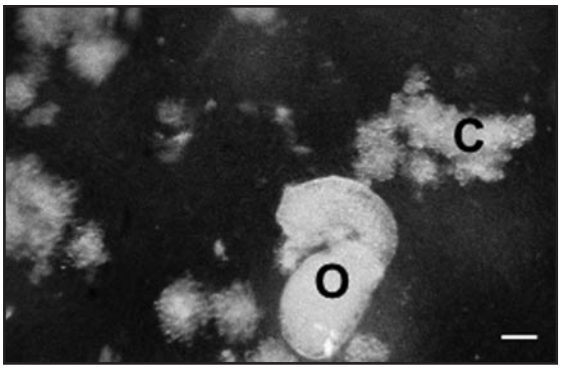

Figure 1. Five days co-culture of *A. thaliana* suspension cells (C) and *O. ramosa* germinated seeds (O). Bar = 280 µm.

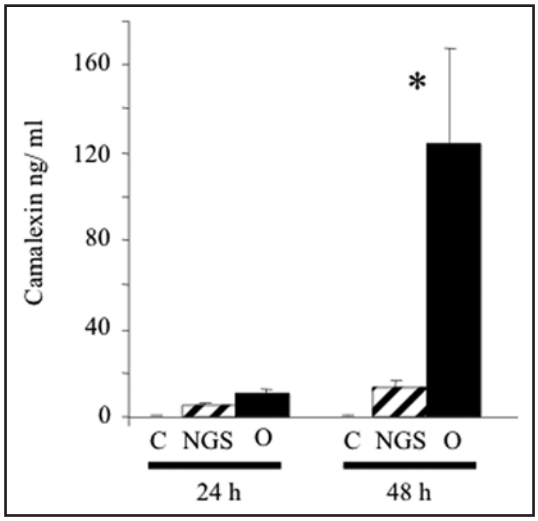

Figure 3. Effect of *O. ramosa* on camalexin production in *A. thaliana* suspension cells. Camalexin synthesis in response to 24 and 48 h of presence of *O. ramosa* germinated seeds (O), non germinated seeds (NGS) and without seeds (C). Data are mean of four independent samples. * significantly different from the control ($p < 0.05$).

Results

In this study, we used a co-culture system with *O. ramosa* seeds and *A. thaliana* cells to examine the induction of physiological defense related responses in *A. thaliana* (Fig. 1) independently of any physical attachment. Because the *O. ramosa* seeds remain inactive in absence of host root exudates,[6-9] they were treated, before co-culture, with a synthetic analog of strigol,[24] which allows their germination in absence of such exudates.

The production of ROS is one of the most common events related to cell death in plant-pathogen interactions. Thus, we tested the putative effect of germinating *O. ramosa* seeds on ROS production. A significant increase in luminol-mediated chemioluminescence caused by H_2O_2 release in the culture medium of *A. thaliana* cells infected by *O. ramosa* seeds was observed. This increase in H_2O_2 production occurred during the first day of interaction and then decreased consistent with an oxidative burst feature (Fig. 2). The control without seeds of *O. ramosa* did not show any significant increase in H_2O_2 production throughout the experiments. Non-germinated seeds also showed increase in H_2O_2 production

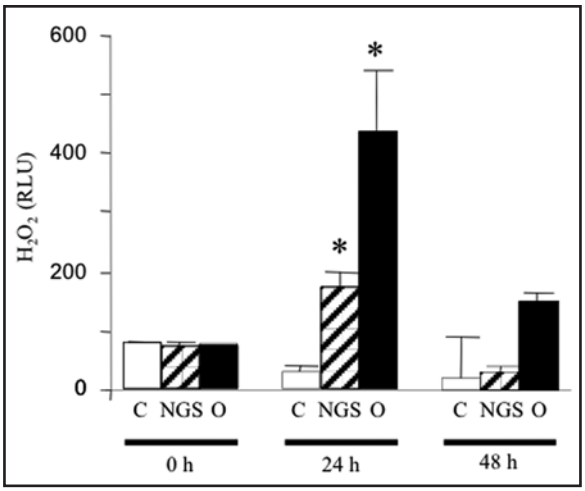

Figure 2. Effect of *O. ramosa* on production of H_2O_2 by *A. thaliana* cell suspensions. Accumulation of H_2O_2 in the medium of *A. thaliana* cell suspensions at 0, 24 and 48 hours of presence of *O. ramosa* germinated seeds (O), non-germinated seeds (NGS) and without seeds (C). Data were obtained from six independent experiments. *significantly different from the control ($p < 0.05$).

but only to a lesser extent.

O. ramosa germinated seeds also induced synthesis of camalexin, the main *A. thaliana* phytoalexin.[31] Even after 24 h of co-incubation, no significant change in camalexin level could be detected. After 48 h, germinating seeds induced a significant level of camalexin synthesis (Fig. 3).

Furthermore, we showed that *O. ramosa* germinated seeds induced death of *A. thaliana* suspension cells. Quantification of cell death was made with Evans blue staining. After 24 h, no significant variation of cell death could be observed between the controls and the suspension cells infected by germinated *O. ramosa* seeds. After 48 h, germinating seeds induced a large death of *A. thaliana* cells (Fig. 4A). Vital staining with neutral red further revealed that this cell death was accompanied with an important condensation and a vacuolization of the cell cytoplasm (Fig. 4B).

To assess the putative involvement of a diffusible signal secreted by the germinated seeds from *O. ramosa*, we tested the effect of a 12 or 24 h germinated *O. ramosa*/*A. thaliana* filtered co-cultured medium on *A. thaliana* cell death. Both filtered mediums were found to induce cell death after 24 h but at different extent (Fig. 5). These data indicate that a diffusible signal released by germinated *O. ramosa* seeds during co-culture could elicit cell death in absence of the seeds. The largest cell death was observed with the medium collected after 24 h of co-culture (Fig. 5) further suggesting that the quantity of a diffusible signal increased with the duration of co-culture. A heat treatment of this medium (5 min at 100°C) completely inhibited the *A. thaliana* cell death induction indicating that the diffusible signal is heat sensitive (Fig. 5).

Discussion

In this work, we used a sterile in vitro co-culture system of *A. thaliana* suspension cells and *O. ramosa* seeds to study the host responses prior host/parasite contact. We show for the first time that *O. ramosa* could induce host cell defense responses independently of any contact. *O. ramosa* stimulated secondary metabolism pathways

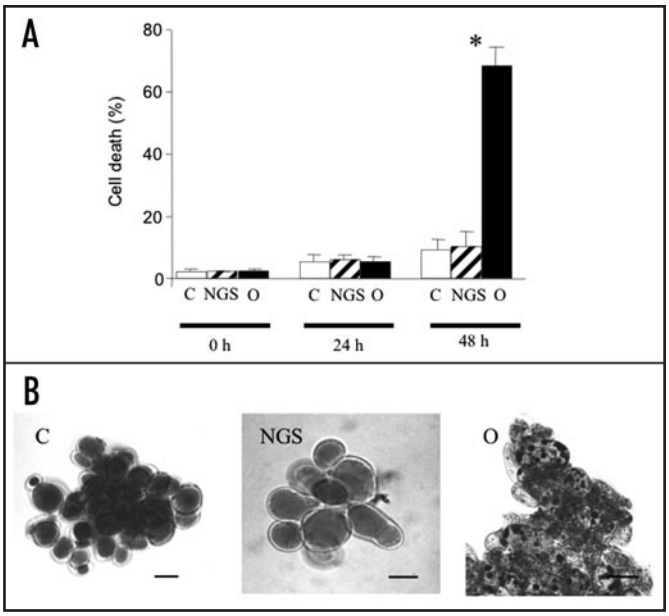

Figure 4. *O. ramosa* induced cell death in *A. thaliana* suspension cells. (A) Effect of *O. ramosa* germinated (O) and non-germinated seeds (NGS) on *A. thaliana* cell death evaluated with Blue Evans staining after 0, 24 and 48 h of co-culture. C corresponds to the control without *O. ramosa* seeds. Data were obtained from 10 independent experiments and error bars correspond to standard errors. *significantly different from the control ($p < 0.05$). (B) Neutral red staining of *A. thaliana* cells after 48 hours of the same treatments. Bar = 40 µm.

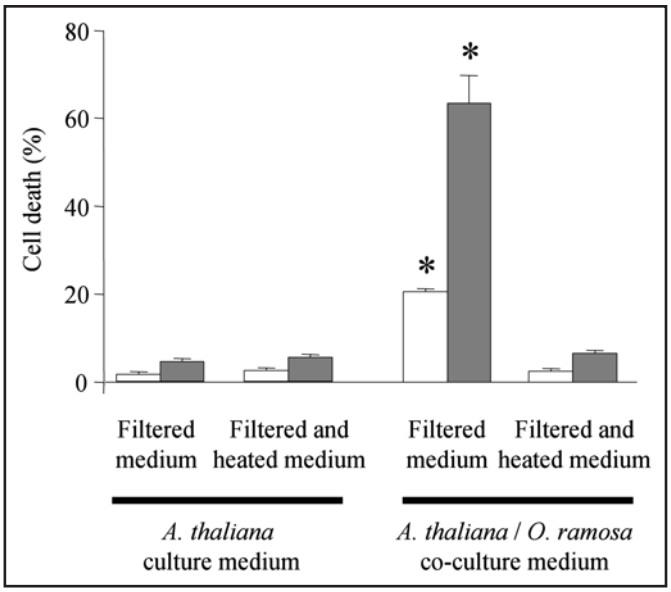

Figure 5. A diffusible signal from *O. ramose* seeds induced cell death in *A. thaliana* suspension cells. Co-culture mediums of O. ramosa germinated seeds and A. thaliana cells were filtered and heated, or not, after 12 h (white bars) or 24 h (grey bars) of co-culture. The effect of these mediums were assayed on cell death from fresh culture of A. thaliana cells with Blue Evans staining after 24 h of treatment. Culture medium of A. thaliana cells without O. ramosa was treated in the same way and used as a control. Data were obtained from 4 independent experiments and error bars correspond to standard errors. *significantly different from the control ($p < 0.05$).

leading to the synthesis of the phytolalexin, camalexin (Fig. 3). *O. ramosa* also induced an oxidative burst revealed by the generation of hydrogen peroxide (H_2O_2) in the culture medium (Fig. 2). The oxidative burst represented by production of reactive oxygen species (ROS) is one of the most common events related to pathogen-plant interaction. The generation of H_2O_2 in the culture medium precedes the induction of camalexin (Fig. 3) and could thus participate to the pathway leading to this induction. Several studies have effectively demonstrated that H_2O_2 modulates the gene expression and signaling pathways during defense responses.[32,33] The generation of H_2O_2 also precedes the induction of *A. thaliana* cell death (Fig. 3). In the same way, H_2O_2 could be a cell death-inducing signal in the plant/parasitic plant interaction. In fact, H_2O_2 increased following elicitor treatment or pathogen challenge[34,35] effectively acts as a signal triggering the cell death and systemic defense responses.[28,36] We show for the first time that *O. ramosa* could induce host cell death before the physical contact (Figs. 4 and 5). The dead cells present a condensation and a vacuolization of the cytoplasm (Fig. 4), which are morphological events that accompany programmed cell death during hypersensitive response in plants and apoptosis in animal.[37] This type of cell death appeared thus clearly different of the one observed during the crush of host cells in the parasite fixation site resulting from a mechanical pressure.[38-40] The existence of an induced cell death before parasitic plant contact could act in the beginning of establishment in order to facilitate its penetration into plant tissue. Such behavior was reported by Govrin and Levine[41] with the necrotrophic fungi *Botrytis cinerea* which induced an oxidative burst and a HR cell death in Arabidopsis. Although the aim of a parasitic plant, which could be considered biotrophic, is different of the one of a necrotrophic fungi these both kinds of organisms could exploit a host defense mechanism to facilitate their colonization of host tissue. The cell death we observed with the culture filtrate of germinated *O. ramosa* seed co-cultured with *A. thaliana* cells (Fig. 5) indicates that a diffusible signal is secreted by germinated *O. ramosa* seed. This hypothesis was proposed by Vieira Dos Santos et al.,[15] after observing the *A. thaliana* gene inductions in response to *O. ramosa* presented without attachment to host roots. Although there is no information about the existence of parasitic plant-derived elicitors analogous to those of microorganisms, the secretion of pectin methyl esterase (PME)[42,43] and the evidence of polygalacturonase and cellulase activities[44] by various parasitic plants could act as elicitors in addition to their role to open the way for intrusive parasite cells. Secretion of endopolygalacturonase by the germinated seeds could participate directly to cell death induction since this kind of enzyme was recently shown to induce signaling pathway leading to programmed cell death in soybean cells.[45] The heat sensitivity of the diffusible signal secreted by germinated *O. ramosa* seeds (Fig. 5) further suggests a proteinaceous nature for this parasitic plant derived signal.

In conclusion, we provide in this study the first evidences supporting the hypothesis that defense responses against *O. ramosa* germinated seeds are promoted in host plant before the physical contact. We further showed that the activation of *A. thaliana* physiological and molecular defence responses to *O. ramosa* is probably induced by a *O. ramosa*-derived signal molecule. The host plant response induced during this plant-plant interaction (summarized in Fig. 6) shared some cellular events comparable to those induced by invariant pathogen-associated molecular patterns (PAMPs) during plant microbe interactions.[46,47] Perception of a PAMPs-like parasitic

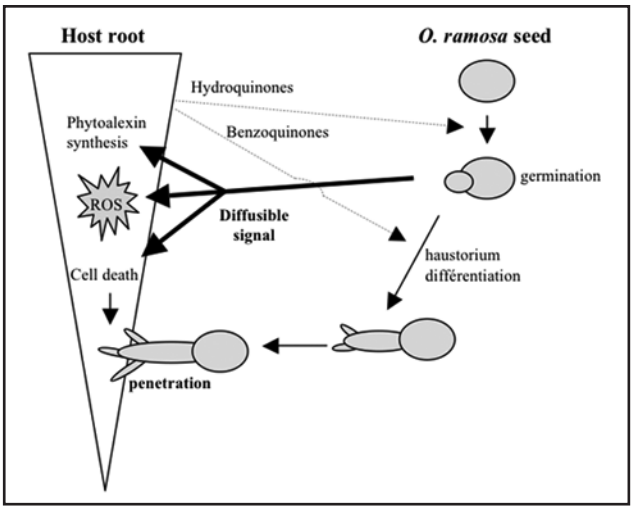

Figure 6. Putative model of early interactions during host root and *O. ramosa* seeds.

plant derived signal by host plant cells raises the question of the non-self discrimination between two plants. Further studies are thus need to purify this *O. ramosa*-derived signal molecule and analyze if host defense responses are governed by a specific recognition between the plant and this pathogen signal or induction of innate immunity.

Experimental Procedures

Plant material. *O. ramosa* L. seeds were collected from an infected winter rape field in Poitou-charente, France. Surface-sterilized seeds were preconditioned in Petri dishes containing glass fiber filter paper moistened with sterile distillated water. Preconditioning was performed in darkness at 20°C for 7 days. *O. ramosa* germinated seeds were obtained after a treatment with an aqueous solution of GR24 (5 ppm), a synthetic analog of strigol[24] which allows the germination in absence of host root exudates. Five days later, GR24 pre-treated seeds were collected, washed five times with sterile water to remove GR24 molecules and used for *A. thaliana* infestation.

A. thaliana L. (ecotype Columbia) suspension cells were grown at 24 ± 2°C, under continuous white light (40 $\mu E.m^{-2}.s^{-1}$) with rotation shaking, in a 1 liter round bottom flask containing 350 ml Gamborg culture medium.[25-27] The pH of the culture medium was 5.8. Cells were sub-cultured weekly by a 10-fold dilution. The experiments were conducted on 4-day-old cultures.

***A. thaliana* cells infestation.** Germinated *O. ramosa* seeds (30 mg) were placed in round bottom flask containing 25 ml of *A. thaliana* 4-day old cell cultures. *A. thaliana* cells and germinated *O. ramosa* seeds were incubated for various periods. Figure 1 show *A. thaliana* and *O. ramosa* after 5 days co-culture. We used non-infected *A. thaliana* cells (C) and *A. thaliana* cells incubated with the same amount of non-germinated *O. ramosa* seeds (non-treated with GR24) (NGS) to escape non specific response.

Experiments with culture filtrate. Co-culture of *A. thaliana* cells and germinated *O. ramosa* were conducted as described before. After 12 or 24 h of co-culture, cells and seeds were removed from the medium by filtration on 50 µm filter in sterile conditions. Four days old *A. thaliana* cells (1,25 mg) from a new culture were then placed in 25 ml of this medium previously heated (5 min at 100°C) or not.

Cell death detection. Cell viability was assayed using vital dye, Evans Blue.[28] *A. thaliana* cell cultures (50 µL) were incubated for 5 min in 1 ml phosphate buffer pH 7 supplemented with Evans Blue to a final concentration of 0.005% after 0, 24 and 48 h of treatment. Cells that accumulate Evans blue were considered dying. At least 1000 cells were counted for each independent treatment.

Death detection was confirmed with the vital staining dye neutral red as described by Wendehenne et al.[22] Cell cultures (1 mL) were washed with 1 ml of a solution containing 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4 and 2 mM Hepes, pH 7.0 and incubated for 5 min in the same solution supplemented with neutral red to a final concentration of 0.01%. Cells with no accumulation of neutral red were considered as dead cells.

H_2O_2 measurement. H_2O_2 released in the culture medium was quantified at different times by measuring the chemiluminescence of luminol reacting with H_2O_2.[29] The infected culture medium (200 µL) was added to 600 µL phosphate buffer (50 mM, pH 7.9) prior to addition of 100 µL luminol 1.1 mM (and 100 µL $K_3[Fe(CN)_6$ 14 mM]. Chemiluminescence was monitored at 5 min interval with a FB12-Berthold luminometer, with a signal integrating time of 0.2 s.

Camalexin determination. Camalexin determination was performed as described by Glazebrook and Ausubel.[30] For each sample, 20 mL of the infected suspension cells were filtered and heated in 350 µL of 80% methanol for 20 min. The cells were removed and the methanol was evaporated under vacuum. The aqueous residue was extracted with two 50 µL aliquots of chloroform, which were combined and evaporated to dryness. The residue was dissolved in 100 µL chloroform, applied to silica thin-layer chromatography plates and developed in ethyl acetate hexane 9:1 (vol/vol). Camalexin (Rf 0.81) was visualized by its blue fluorescence under a long-wave ultraviolet lamp (365 nm). The silica containing camalexin was scraped off the plate and camalexin was extracted into 1 mL ethanol. The emission at 385 nm after excitation at 315 nm was measured with a Hitachi F2000 fluorimeter and camalexin concentration was calculated by comparison with a standard curve obtained by using purified camalexin kindly provided by Jane Glazebrook (Torrey Mesa Research Institute, San Diego, California 92121).

Statistics. Mean separation was accomplished using Duncan's Multiple Range Test. Statistical significance was determined at p values <0.05.

Acknowledgements

The authors wish to thank Dr. Glazebrook for the kind donation of purified camalexin used for the standard curve. Thanks also go to the LEM team (EA 3514) for technical facilities. This work was supported by MENESR via the EA 3495.

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