Photosynthetic Declines Are Induced by *Phytophthora ramorum* Infection and Exposure to Elicitins[1]

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Abstract

Infection of compatible plants by *Phytophthora* spp. often leads to a decline in stomatal conductance and photosynthesis, although the mechanistic basis for such declines is not completely understood. In many cases, declines in leaf gas exchange rates have been linked to losses in water supply capacity associated with root and/or xylem. However, the reductions in gas exchange may not be proportional to changes in hydraulic capacity, and may be observed in non-invaded regions, suggesting the presence of a toxin, or host-derived signal, that is responsible for some of the physiological impairment.

In the current study, we first conducted a series of experiments to determine if toxins secreted by *P. ramorum* are likely contributors to physiological injury in the host by examining the temporal changes in photosynthesis, stomatal conductance, and hydraulic conductivity of *Rhododendron macrophyllum* G. Don (rhododendron) artificially inoculated with *P. ramorum*. Second, we tested the ability of culture filtrates and purified, recombinant *P. ramorum* elicitins (i.e., the major proteins secreted by *P. ramorum* grown *in vitro*) to induce physiological changes in incompatible *Nicotiana tabacum* (tobacco) and compatible tanoak, rhododendron, and *Umbellularia californica* (California bay laurel) host species.

To determine whether toxins secreted from *P. ramorum* contribute to physiological injury in the host, two stems (ca. 2.5 cm dia) from each of 12 three-year-old rhododendron plants were artificially inoculated with a 5 mm dia hyphal plug cut from ca. 2 week-old *P. ramorum* starter cultures (2 percent cornmeal agar) or uninoculated control plates, which was secured under the bark, ca. 15 cm below the lowest leaf, using dH_2O-saturated gauze. The *P. ramorum* isolate used for inoculation was a North American mating type (A2) obtained from infected native plants growing in Curry County, Oregon. On a weekly basis (1-4 weeks after inoculation), A/C_i curves (net CO_2 assimilation over a range of CO_2 concentrations), stem-specific hydraulic conductivity, and stem lesion lengths were monitored.

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Phytophthora ramorum elicitins purified from culture filtrates or obtained from a prokaryotic expression system (pET SUMO expression system, Invitrogen, Carlsbad, CA) were tested for their ability to cause physiological damage when applied to the four host species listed above. Measured responses included H^+ uptake, ethylene production, and chlorophyll fluorescence.

A search of the *P. ramorum* genome project (DOE Joint Genome Project, http://genome.jgi-psf.org/ramorum/) revealed five sequences coding for recognizable elicitin proteins (protein ID: 47381, 47386, 47376, 71636, and 78569). Based on these sequences, two conserved primer sets were designed to amplify full-length elicitin genes. High homology between the gene sequences prevented the design of individual primer sets for all five sequences. Primer sets were as follows: ram-α1 5'-GAACTTCCGCGCCCTG and 5'-ACAGCGACGCGCACGT) and ram-α2 (5'-ATGCAGTTCGCCGCTCTC and 5'-TACAGCGACGCACACGT). The two primer sets were tested on six different *P. ramorum* isolates, producing two unique elicitin proteins common to all six isolates. Full-length ram- α1 and ram- α2 genes were cloned into the pET SUMO vector, induced with 1 mM IPTG for 6 h at 37°C, and purified by affinity chromatography (ProBond Resin, Invitrogen, Carlsbad, CA). The purity of the recombinant elicitins was verified visually by SDS-PAGE.

All artificial inoculations of rhododendron were successful, resulting in an average lesion length of 6.9 $\pm$ 0.9 cm by the end of the four week study. Reisolation of *P. ramorum* was 100 percent successful from all symptomatic stem tissues, but not from any of the asymptomatic stem or leaf tissues. Physiological changes developed rapidly in leaves of the inoculated stems, despite the lack of visible symptoms in the leaves or petiole. Three weeks after inoculation, when stem lesion lengths were 4.4 $\pm$ 0.6 cm, V_{cmax} (maximum rate of carboxylation limited by the amount, activity, and kinetics of rubisco) was reduced by ca. 21 percent. Additional declines occured during the fourth week, after the development of significant impacts on plant-water-relations.

The functionality of *P. ramorum* infected stems to supply water to host leaves and maintain photosynthetic rates was assessed from K_S (stem-specific hyrdaulic conductivity) and g_s (stomatal conductance) measurements. Four weeks after inoculation, but not before, both measures declined; g_s, a measure of stomatal openness, declined by 36 percent, and K_S, a measure of xylem water supply capacity, declined by 64 percent. A culture filtrate derived elicitin from *P. ramorum* was purified and tested for its ability to influence leaf processes. Similar to the artificial inoculation experiment, the CF-elicitin caused a significant decline of 23.4 percent in photosynthetic capacity and 14.8 percent in the efficiency of open PSII centers (F_v/F_m). Two components often associated with the hypersensitive response (HR), H^+ uptake and ethylene production, were also influenced by elicitin uptake changing by 78.8 and 92.4 percent, respectively.

The two purified, recombinant elicitins (ram-α1 and ram- α2) were tested for biological activity in both compatible and incompatible hosts. Both recombinant elicitins produced a visible hypersensitive response and developed necrotic areas when infiltrated into leaves of the incompatible host, tobacco; however, no macroscopically visible necrosis was observed in any of the three compatible hosts. Independent of the development of visible necrosis, the recombinant elicitins significantly affected a variety of physiological characteristics of all four host species. In all species, exposure to recombinant elicitins caused a decline in the maximum efficiency of PSII centers or F_v/F_m, while enhancing H^+ uptake and ethylene production, relative to the controls. Thus, for all treatment combinations (elicitin and host species) the decline in F_v/F_m was strongly and positively correlated to H+ uptake ($R^2 = 0.801$) and ethylene production ($R^2 = 0.884$). Like the culture filtrate tests, tobacco exhibited the greatest responses, followed by tanoak, myrtle, and rhododendron. For all three measures, ram-α1 triggered significantly greater responses compared to ram-α2, except in rhododendron, and in tanoak F_v/F_m.

While toxins have been suggested to play a role in *Phytophthora* spp. pathosystems, previous efforts to document elicitin toxicity in compatible hosts have met with varying degrees of success. For example, elicitin exposure did not influence stomatal conductance in chestnut (Maurel and others 2004) or net photosynthesis in beech (Fleischmann and others 2005). However, ultrastructural changes in oak (Brummer and others 2002) and varying degrees of necrosis or cell apoptosis have been observed in several Solanaceae plants (Vleeshouwers and others 2000). Based on these observations and those of the current study, a wide range of host responsiveness to elictins is possible. Although the mechanistic basis for the observed photosynthetic declines was not fully explored in this study we hypothesize that it is associated with an incomplete or hypersensitive-like response. In part, this hypothesis is based on the strong correlation between the decline in F_v/F_m and two processes typically associated with HR: H^+ uptake and ethylene production. To date, the vast majority of work with elicitins has focused on their ability to induce the HR and systemic acquired resistance in incompatible hosts such as tobacco (Bonnet and others 1996). Furthermore, both artificial inoculation (Scharte and others 2005) and elicitin exposure (Matsumura and others 2003) result in photosynthetic declines in incompatible hosts. Part of this decline surely arises from the death of functional mesophyll cells during a successful HR. However, Scharte and others (2005) recently showed that a successful HR requires the suppression of photosynthesis, associated with callose depositon and/or sugar accumulation, before HR cell death can be initiated. Thus, it follows that host differences in the degree of the HR response to elicitins (i.e., highest in resistant species) could be the source of the observed photosynthetic declines (i.e. highest in resistant species) in response to elicitin infiltration. Consistent with this hypothesis, Vleeshouwers and others (2000) examined HR cell death in several *Solanum* clones and found a high degree of variation in the timing and degree of HR cell death, which was correlated with resistance to *P. infestans*. Finally, the notion of an effector triggering HR-like processes in both compatible and incompatible hosts is supported by other studies. For example, the NPP1 effector from *Phytophthora* species induces typical HR-associated (ethylene accumulation, callose deposition, and necrosis) and SAR-associated (pathogenesis-related gene accumulation) processes in both compatible and incompatible host species (Fellbrich and others 2002).

In conclusion, we have shown that exogenous application of elicitins results in photosynthetic declines in both compatible and incompatible hosts. The mechanism responsible for the declines is unknown but may be associated with quantitative differences in the timing and degree of a hypersensitive-like response. Previous studies have shown that elicitins are avirulence factors in some nonhosts, such as tobacco (Kamoun 2006). In this study, elicitin sensitivity was inversely related to *P. ramorum* susceptibility (tobacco > tanoak > myrtle > rhododendron). It is unknown if host sensitivity to elicitins directly contribute to quantitative differences in host susceptibility to *P. ramorum*; however, elicitins appear to contribute to virulence by directly reducing the photosynthetic performance of its host.

Key words: Chlorophyll fluorescence, elicitin, photosynthesis, sudden oak death, toxin.

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