FINAL BIOAG REPORT: Biodegradable peptides as triggers of plant defense against pathogens and pests

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ABSTRACT

Biologically active compounds derived from plants, e.g., peptide elicitors are known as a strong inducer of defense responses against pathogens and pests. Recently, the PI's group found that several peptides from potato can induce a defense response. They also found that other peptides have a potential to be a stronger defense inducer in potatoes. To expand this study, they further synthesize candidate peptides, and evaluate those activities on plant immunity responses in potatoes. The overall objective of this proposal is to evaluate the effect of peptide elicitors as a defense inducer for potato plants. This project provides a starting point to develop a novel approach to control destructive pathogens and pests by maximizing the natural immunity of potatoes. The identified defense inducers in this proposal are comparable to natural biological compounds. All experiments in the project were performed on a lab-bench basis.

PROJECT DESCRIPTION

Potato is the world's fourth largest food crop and have a key role in agriculture, the economy, and world food security. Because potato is vegetatively propagated through tubers, it is particularly threatened by the accumulation of diseases over time. This presents a significant challenge to sustainable and profitable production. Infection often results in rejection of seed potato fields, and yield losses due to these pathogens can be as high as 70%.

Plants respond to pathogen and herbivory with rapid changes in gene expression and concomitant accumulation of defensive metabolites. It has been reported that a few of the peptides derived from damaged plant tissues are induced upon pathogen infection and insect herbivory, and can evoke a strong, effective defensive response in plants. For example, systemin and pep elicitors are well known to enhance plant resistant to a wide range of fungal, bacterial, and viral diseases during crop growth and post-harvest in Arabidopsis, tomato, and maize (Pearce and Ryan, 2003; Huffaker et al., 2006; Pearce et al., 2010). Unlike the action of most pesticide chemicals, such elicitors do not interact directly with disease pests. Therefore, these pests are not expected to develop resistance to elicitors. Most peptide elicitors offer great prospects as natural immunostimulants due to their potential of high biocompatibility and biodegradability. Therefore, peptide compounds used for pathogen control have the advantage of being considered "natural" and friendly to the environment compared to synthetic chemicals. The peptide elicitor can be a new type of pesticide by reducing usage of chemical pesticides while decreasing environmental impact, therefore is not expected to cause adverse effects to human health or the environment. The development of the natural immunity in plants is especially timely as chemical pesticides are increasingly becoming vulnerable to the resistant strains of fungal pathogens (Fisher et al., 2018). Currently, limited information is available in regard to peptide elicitors in potato plants. The elicitor specific for potato species needs to be determined. In this project, we attempted testing known peptides and yet-unidentified peptides as defense elicitors.

OUTPUTS

- **Overview of Work Completed and in Progress:**

The hypothesis is that stress-induced peptides in plants play an essential role in plant resistance as triggers of defense against pathogens and pests. To pursue this hypothesis, the PI's and Co-PI's groups have evaluated known peptides and yet-unidentified peptides as defense elicitors using several immune response assays. The synthesized peptides were assessed as a trigger of plant defense. This BIOAg grant mainly requested to support for method establishments and peptide synthesis expenses within the project.

- **Methods, Results, and Discussion (discussion for final reports only):**

Measurements of early defense responses in potato

PI's group has been specifically used a potato powdery scab disease as a test case in this study. The disease is caused by a protist pathogen, *Spongospora subterranea* f. sp. *subterranea* (Sss). A quantitative and robust method to assess defense responses is needed to study plant disease resistance, but also for the early identification of disease establishment during pre- or non-symptomatic phases. The PI's and Co-PI's groups have been working on establishing methods using potato roots, stems, and suspension culture cells to monitor early defense responses, e.g., cytosolic calcium responses, production of reactive oxygen species (ROS), extracellular pH dynamics (alkalinization), and changes in expression of defense-related genes (Figure 1). Notably, the alkalinization assay in potato was established in PI's lab (Moroz et al., 2017b; Moroz et al., 2017a). An increase in extracellular pH is considered an early plant defense responses (Pearce et al., 2001; Haruta and Constabel, 2003). We have observed alkalinization response against pathogen- and plant-derived elicitors (Figure 1). The alkalinization patterns were strongly correlated with defense responses based on defense-related gene expression (Moroz et al., 2017a), suggesting that the alkalinization assay is a rapid and reliable way to monitor plant defense

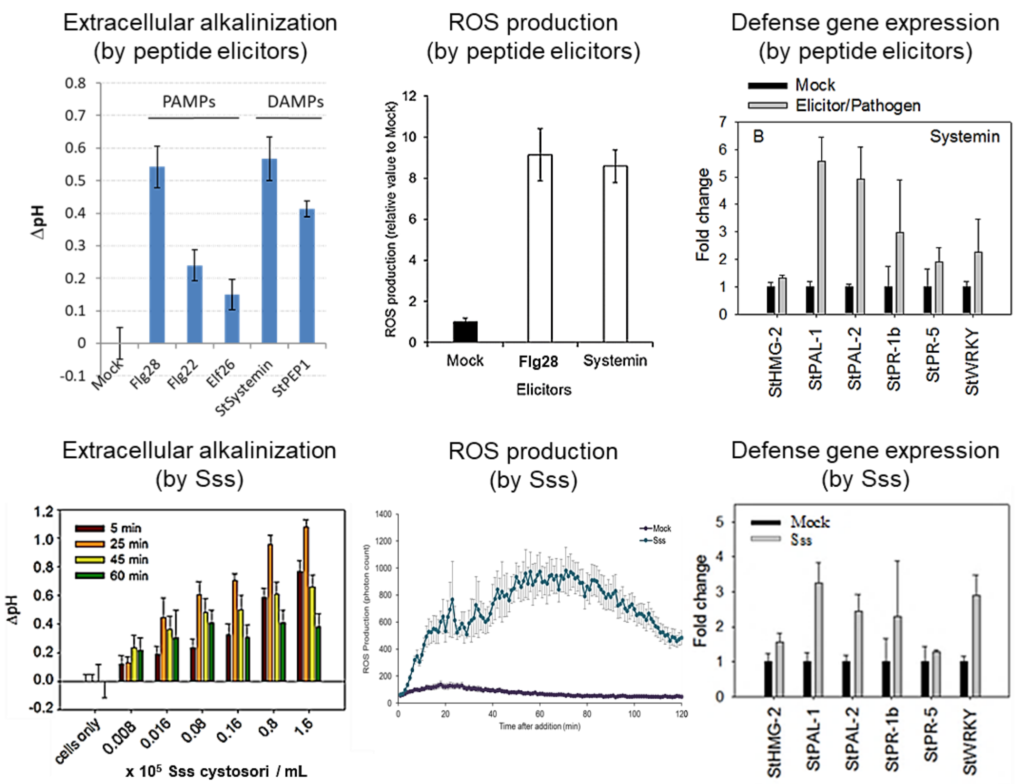

Figure 1. Early defense response in potato caused by peptide elicitors and *Spongospora subterranea* f. sp. *subterranea* (Sss). Potato cells were treated with peptide elicitor compounds (1 µM ea.) or Sss cystosori (10^4-10^5). Results of extracellular pH (left), ROS burst (center), and expression of defense-related genes at 30 min of treatment (right) are shown as early defense responses. PAMPs = pathogen-derived peptide elicitors; DAMPs = plant-derived peptide elicitors.

responses. Most notably, the early defense responses caused by Sss inoculation are similar to those caused by the peptide elicitors tested (Figure 1), suggesting that potato cells possibly recognize unknown elicitors derived from Sss or damaged plants upon Sss infection.

Method establishment for Sss infectivity assay

To evaluate effectiveness of peptide elicitors against Sss infection, a simple and rapid method for Sss infectivity assay is necessary. Unfortunately, since Sss is nonculturable, most studies have been done in fields, even though the experiments provide variable results due to uncontrolled environmental factors and microbial contaminations. Experiments can be performed in a greenhouse trial or a growth-chamber trial. However, it takes 15-18 weeks to obtain the disease symptoms. For a quick pathogen infectivity assay, we have established a hairy root culture system as a lab-based infection system for qualitative assessment or quantitative measurement of the disease symptom (Figure 2). In general, hairy roots can be readily induced from diverse plant tissues upon infection by a soil bacterium, *Rhizobium rhizogenes* (Young et al., 2001). The bacteria introduce its root-inducing transfer-DNA encoding the root locus genes into the plant genome, which induces hairy root initiation and proliferation. An important feature of hairy roots is that they are anatomically and metabolically similar to normal roots and possess intact xylem and phloem vasculature connected to the source explant (Lucas et al., 2011; Ron et al., 2013; Ron et al., 2014). We have generated potato hairy root cultures in the lab (Figure 2). When we inoculated these roots with a suspension of Sss cystosori (spore balls), we observed the presence of zoosporangia in the root hairs within two weeks after inoculation. Subsequently, we could observe the presence of sporeballs (cystosori) in the cortex area (see the microscopic image in Figure 2). These results are comparable to previously

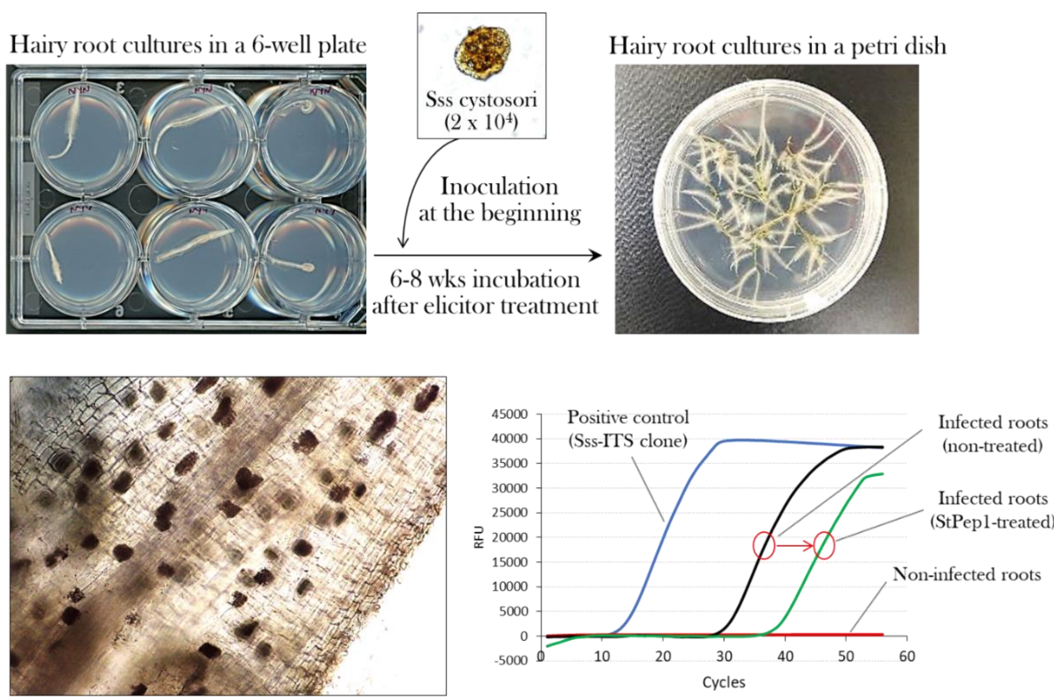

Figure 2. Sss infection using hairy root culture system and effect of a peptide elicitor on Sss infection. Purified Sss cystosori (2 x 10^4) were applied over the hairy root cultures and incubated further 6-8 weeks. Microscopic image (200x magnification) shows Sss sporosori in root cells 6 weeks after inoculation. Amplification curve (in the right-bottom graph) shows detection results of the Sss pathogen DNA in the ITS region in Sss-infected tissues with or without a peptide elicitor, StPep1. DNA from non-infected tissues (red line) was used as negative control. A Sss-ITS clone in a plasmid DNA was used as a positive control for the Sss detection (blue line). Note that StPep1 (green line) reduced Sss infection level by ~2^{10} (= ~1,000) times in comparison with mock treatment (black line).

published infection timelines (Balendres et al., 2016; Falloon et al., 2016; Balendres et al., 2018). Our results demonstrated that Sss-infected root tissues could be obtained within 4-8 weeks using the hairy root cultures. Sss was successfully detected in the infected roots using ELISA and qPCR (see the black line in the amplification curve in Figure 2).

Using the hairy root cultures, we further tested a known peptide elicitor, StPep1, and successfully observed its inhibition effect on Sss infection (compare the green line with the black line in Figure 2). StPep1 reduced Sss infection by ~1,000 times in comparison with mock treatment. The data suggest that the peptide elicitor, StPep1, acts as an immunostimulant to immunize the potato roots, which resulted in reduction of Sss infection. Our data also suggested that hairy root cultures are useful to perform a rapid, scalable, high-throughput screening of peptide elicitors against Sss infection. For the goal of elicitor screening, hairy root cultures provide an unparalleled advantage over greenhouse- and field-based testing, and provide a unique opportunity to quickly validate antimicrobial immune activities.

Isolation of novel peptide elicitors as immunostimulants

Using Sss-infected tissues, we have been working on the identification of pathogen- or plant-derived defense elicitors that evoke a specific and strong defense response. We conducted an acid-based extraction, in which trifluoroacetic acid was used for acidification and reverse-phased chromatography, using a Sep-Pak C18 column for fractionation of mixed compounds (Figure 3). We also included an HPLC step in the purification procedure to fractionate at a high resolution. Finally, several fractions containing active compounds were purified by several fractionation steps. All procedures were performed under aseptic conditions. As shown in Figure 3, we monitored defense elicitation activity using an extracellular alkalinization assay. As a result, the purified fractions, CSss30 (eluted by 30% acetonitrile from Sss-infected cells), MSCSss30 (eluted by 30% acetonitrile from supernatant of Sss-infected cells), and Csss40 (eluted by 40% of acetonitrile from Sss-infected cells) strongly induced plant defense responses based on the alkalinization assay. We submitted these purified fractions to LC-MS/MS and identified ~17,000 peptides in total. Most of them (~65%) were derived from potato proteins. The other peptides were matched to Sss proteins. In this peptide list, known peptide elicitors (e.g., systemin) were not found, suggesting that there is a potential to be able to isolate novel peptide elicitors. To narrow the list of elicitors, we further selected peptides that were enriched only in Sss-infected samples (not in Sss only or potato tissue only). As of now, ~100

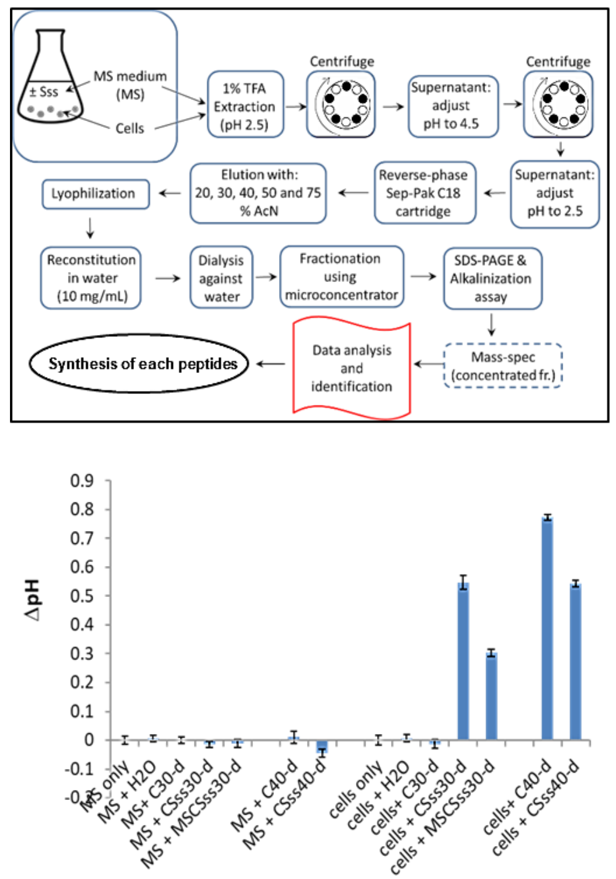

Figure 3. Peptide elicitor isolation from Sss-infected potato cells. Top panel shows a workflow of fractionation and purification steps to isolate active peptide compounds in Sss-infected potato cells. AcN = acetonitrile; TFA = trifluoroacetic acid. Bottom panel shows defense inducible activity of each fraction on extracellular alkalinization, which is an indicator of early plant defense response. The fractions showing a strong activity were subjected to LC-MS/MS for peptide identifications.

peptides are finalist candidate elicitors. To narrow the list further down, we need to test all peptide candidates for defense inducing activity using the alkalinization assay and other assays, i.e., ROS assay and measurement of PR genes.

Other novel peptide elicitors (clues from past publications)

A unique peptide called Pep13 was reported in 1994 prior to the routine commercial synthesis of peptides (Nürnberger et al., 1994). Pep13 is unique in that it arises from *Phytophthora* species some of which infect potatoes. It is now possible to design Pep13 analogs to improve its action. This action was evaluated cytologically on pea endocarp tissue responding to a true pea pathogen, *Fusarium solani* f. sp. *pisi* or metabolically as a phytoalexin inducer (Figure 4). Interestingly, the nuclear-localized type analog strongly inhibited the fungi hyphal growth in comparison to others. Further analysis is necessary regarding detail function of the elicitor analogs in plant immunity before the broader studies for any field applications.

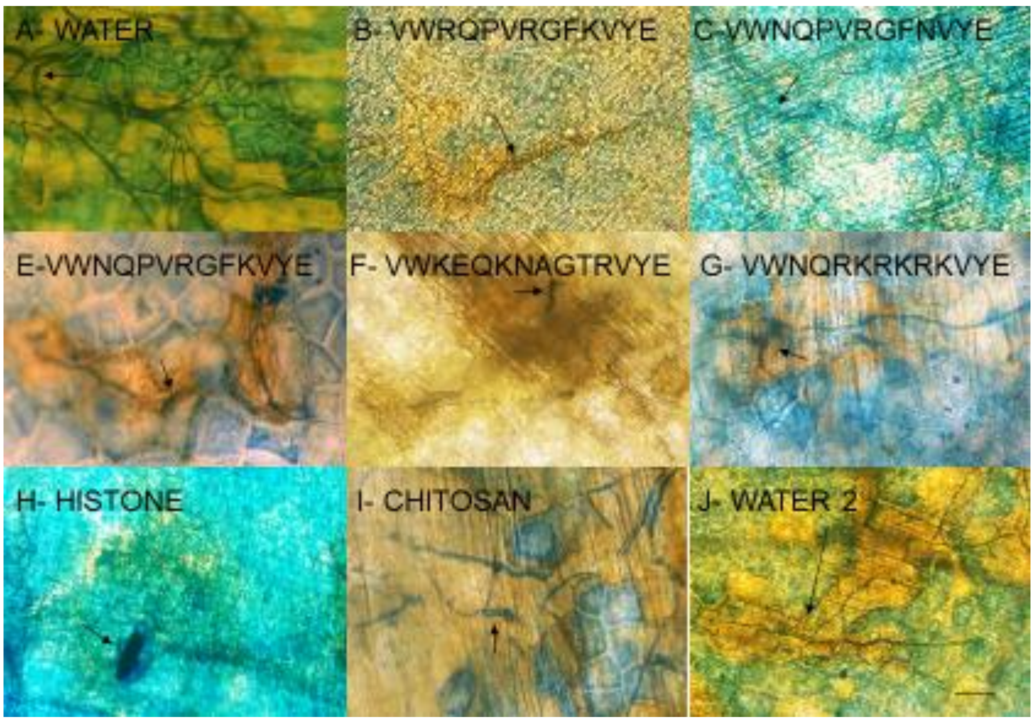

Figure 4. Effect of pep13 analogs on plant defense response against pathogen growth. Pathogen growths of *Fusarium solani* f. sp. *pisi* (48 h post inoculation) were observed in the samples pre-treated for 1 h with water (A and J), histone and chitosan (H and I), and pep13 analogs: N3R mutant (B), K10N mutant (C), native pep13 (E), nuclear-localized type analog (F), and basified analog (G).

- **Publications, Handouts, Other Text & Web Products:**

The following publication is anticipated to be published.

- Tanaka K (2019) Damage-associated molecular patterns (DAMPs): detection, signaling, and responses. *Annu. Rev. Phytopathol.* (in preparation)
- Moroz N, DeSields JB, Tanaka K (2019) Novel peptide elicitors from potato roots infected by powdery scab pathogen. *Front. Plant Sci.* (Submitted)

- **Outreach & Education Activities:**

The PI presented the concept of the project on October 2017 in Boise, ID, and answered questions from the NPRC group composed of potato industry members invited by the state potato commissions (ID, OR, and WA) who represent growers, processors, chemical registrants and distributors, consultants, etc. They showed an understanding of the importance of using the peptide elicitors for environmentally-friendly management for potato disease control.

Two high school students, Frank Hamel from Columbia High School, Hunters, WA (July 2018) and Jasmine Abrams from Eisenhower High School, Yakima, WA (July 2017), were involved in the project as summer interns, along with the Upward Bound Program. Both were learned the pathogen infectivity assay using the potato cells and contributed the peptide screening from the elicitor candidates.

IMPACTS

- Short-Term: This project has trained one graduate student, one postdoc, and two high school students through direct involvement in several activities. The PI's and Co-PI's groups obtained some peptide elicitors as a trigger of potato defense against pathogens and pests. Outcome form this research is currently prepared for publication in scientific journals.
- Intermediate-Term: The data obtained in this research provide a foundation for comprehensive investigations of mechanisms of the plant's damaged-self recognition that improves resistance to fungal infections and the damage caused by herbivores. It has a potential to develop a strategy to control various potato diseases.
- Long-Term: The ultimate goal is to obtain public awareness regarding the benefits of using the peptide elicitors, i.e., biologically natural compounds derived from potatoes. It is expected to reduce usage of pesticides, eventually decrease environmental impact and retain agro-ecosystem.

ADDITIONAL FUNDING APPLIED FOR / SECURED

This BIOAg grant has been supported this project as seed funding to generate preliminary data to submit the following proposals.

Secured

- Northwest Potato Research Consortium ($40,573). Identification and characterization of elicitors to maximize defense system against powdery scab in potato roots. PI: Tanaka K; Co-PIs: Johnson DA and Brown CR. 7/1/17 – 6/31/18
- Northwest Potato Research Consortium – Renewed ($40,603). Identification and characterization of elicitors to maximize defense system against powdery scab in potato roots. PI: Tanaka K; Co-PIs: Johnson DA and Brown CR. 7/1/18 – 6/31/19

Applied

- USDA-AFRI ($499,021) Rhizospheric delivery system of biodegradable immunostimulants to control powdery scab disease in potato. PI: Tanaka K; Co-PI: Gleason C (submitted in 2018)
- WSDA-SCBGP ($248,470) Improved disease control strategies of potato powdery scab. PI: Tanaka K (submitted in 2018)
- Northwest Potato Research Consortium ($10,000) Defense responses signaled in potato tissue. PI: Hadwiger LA; Co-PI: Tanaka K (submitted in 2018)

GRADUATE STUDENTS FUNDED

Joseph B. DeShields, MS. was supported in the project. He contributed developing the pathogen detection method that enable quantitative evaluation of the pathogen infection during testing the peptide elicitors.

RECOMMENDATIONS FOR FUTURE RESEARCH

To control pathogens and pests. the most common tools are synthetic chemical fungicides/pesticides (Pimentel, 2009) and resistant crop genotypes (Green and Owen, 2011; Nelson et al., 2018). However, classical chemical pesticides cause environmental pollution. Moreover, pathogens and pests can evolve to become resistant to pesticides. Similarly, resistance traits in crops that are based on a single gene or a small number of genes are quickly overcome by pathogens and pests that evolve counter-resistance. Alternative strategies should be developed that employ non-toxic compounds and natural plant traits for crop protection. Beneficial microbes (act as pathogens of pathogens and pests and act as plant resistance elicitors) and beneficial insects and other arthropods (act as predators and parasitoids) has received increasing interest over the recent years.

The main concept to use peptide elicitors is to vaccinate plants by stimulating the plant's own immune system. Our ultimate goal is to develop a new system for the control of potato diseases (currently powdery scab infestation and an indirect prevention of PMTV infection) by modifying the immune system in the potato roots. Our expectation is that an effective plant immunostimulant can evoke a strong defensive response in potato roots against the powdery scab disease. Given their function in plant basal resistance to diverse pathogens, the identified elicitors might also be useful for other potato diseases, including Pythium leak, pink rot, Fusarium dry rot, silver scurf, and soft rot, which are important to be tested in the future before commercializing the immunostimulant as a novel pesticide. In other respects, the elicitors will be a valuable tool to help us study and identify key genes underlying plant innate immunity involved in plant defense. Such works will be crucial for revealing the mechanisms of plant-protist interactions, which are still largely unknown.

Identified peptide elicitors from the proposed project are beneficial for growers and potato industry since they should be useful for developing new types of chemicals, i.e., a nontoxic biodegradable pesticide, to control potato diseases. The results from this proposal are a starting point for broader studies addressing field applications and commercial product development.

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