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The Activity Of Hydrolases And Their Protein Inhibitors In Potato Tissues At Phytophthora infestans infection And Stability Inductors Processing.

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ABSTRACT

We studied the effect of processing stability inductors – salicylic (SA) and jasmonic (JA) acids on the activity of proteolytic and amylolytic enzymes and their protein inhibitors in calli and in-vitro potato plants Solanumtuberosum L. upon infection of late blight pathogen Phytophthorainfestans Mont. de Bary. It has been shown that treatment of SA and JA reduces the penetrating ability of the Ph. infestansmycelium in plant tissues and exerts a high stimulatory effect on the transcriptional activity of genes of amylases and proteases inhibitors in potato. Discusses the potato resistance to Ph. infestansregulation under the influence of SA and JA involving complex "hydrolase - inhibitor".

Keywords: Solanumtuberosum, Phytophthorainfestans, hydrolases, amylases and proteases inhibitors, salicylic and jasmonicacids, induced resistance.

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INTRODUCTION

One of aggressive and pathogenic factors of microbial plant pathogens are hydrolytic enzymes which provide efficient cleavage of vegetable polymers [1, 2]. It is known that an important component of plant defense response to pathogens action is the activation of molecules inhibiting the hydrolytic activity of microorganisms [3]. Numerous data indicate the involvement of a hydrolase inhibitor in plant defense reactions [4, 5], a number of studies shows the change in the balance of proteinase inhibitors in the tissues under the influence of biotic and abiotic stressors [6, 7, and 8]. Probably reducing the hydrolytic enzymes activity is an important part of the adaptive mechanisms of a plant, primarily, upon infection with pathogenic organisms.

The induction and the implementation of protective response in plants involve various signaling systems. The salicylic and jasmonicacids are important signaling molecules (mediators) in the development of plant defense reactions [9]. Since both the SA and JAhave positive effect on plant resistance to a number of harmful diseases and enhance productivity, these compounds have wide application in the plant [10, 11]. Therefore, research of enzymatic processes occurring in tissues treated with SA and JA on the background of plant infection by pathogenic microorganisms is of great scientific and practical interest.

Resistance of the potato to late blight pathogen Phytophthorainfestans Mont. de Bary many researchers associated with hypersensitivity reaction of host cells in a zone of contact with the intruding hyphae [12].

A purpose of this work was a comparative study of changes in the activity of amylolytic and proteolytic enzymes and their protein inhibitors in calluses and leaves of potato plants while infecting with pathogen Phytophthorainfestans on background of salicylic and jasmonic acids effect.

MATERIALS AND METHODS

The object of research was calluses and test tube potato plants (Solanumtuberosum L).

As explants for callus used green leaves of tube plants. Leaves were incised along veins and cultured on a Murashige and Skoog (MS) agar medium in the dark at 26 °C. After 10 days of culture, the explants were losing green color and started the formation of callus tissue on the edges of the cuts. After 30 days formed a well-developed callus tissue on the leaves. The resulting calli were placed into Petri dishes onto the surface of Murashige and Skoogagar medium containing salicylic or jasmonicacid at final concentrations of 10^{-6} M and 10^{-7} M, respectively. The controls were calluses growing on MS medium without regulators. At 3rd day after the first passage, the calli were infected with 2 µl of a suspension of Ph. infestanszoospores (10^5 spores per ml).

To obtain zoospores, the late blight pathogen Ph.infestans were grown in test tubes on a potato-glucose medium for 11 days. Then oomycetes zoosporangia were filled with distilled water and incubated for 45 min at 4 °C and then 45 min at room temperature. The concentration of zoospores was determined in the hemocytometer.

Test-tube potato plants cultured in-vitro for 30 days on MS agar medium [13] containing SA or JA at final concentrations of 10^{-6} M and 10^{-7} M, respectively [14]. Plants were infected by applying 5 µl of a zoospore suspension (10^5 spores per ml) on the leaf. The controls were uninfected and infected plants growing on MS medium. The degree of Ph. infestansgrowth on potato leaves was assessed at 7th day after inoculation as the area on the leaf surface.

After 6, 48 and 72 hours, the plants were fixed to assess transcriptional activity of amylase XM006351484 and protease JX683427 genes.

EXPERIMENTAL

The activity of proteases determined with spectrophotometer by hydrolysis of a chromogenic synthetic substrate BAPNA and by gelatin hydrolysis [15].

Amylase activity was evaluated by immobilized in agarose gel starch hydrolysis [16].

The activity of trypsin inhibitorsdetermined by the Hoffmann – Vaysblaymethod [17] with some modifications. In 0.5 ml of 0.05 M Tris-HCl buffer, pH 8.2, was added 1.0 ml of extract and 0.5 ml of enzyme (1 mg / ml). To the mixture was added 1.0 ml (1 mg / ml) of BAPNA and incubated in a water bath for 10 minutes at 37 °C. The reaction was stopped with 0.5 ml of 30% acetic acid. As a control used mixture not containing the enzyme, which was added after the reaction was stopped. The absorbance of the resulting solutions was measured on a spectrophotometer Biospek-Mini (Shimadzu, Japan) at 405 nm. Activity of inhibitor was expressed as inhibitory units (IU). Under standard conditions, the unit of inhibitory activity is equal to the amount which is necessary for full suppressing the unit of trypsin activity.

Amylase inhibitors measured usingagarose gel plates [17].

For staining mycelium gentian violet dye, prepared with aniline,was used [18].

Isolation of total RNAof potato leaves fixed in liquid nitrogen were performed using Trizol according to the protocol of the supplier company (Sigma, Germany). Nucleic acids content was measured on spectrophotometer Smart Spec Plus (Bio-Rad, USA), after dissolving the samples in Tris-EDTA buffer. For obtaining cDNAbased on studied samples RNA,the reverse transcription reaction were performed using reverse transcriptase according to the protocol of the supplier company (Synthol, Russia). Analysis of accumulation of amylase inhibitor XM006351484 and proteinase inhibitor JX683427 genes transcripts performed by quantitative real time PCR on the "iCycler iQ5 Real-Time PCR Detection System" (Bio-Rad, USA) using the intercalating dye SYBR Green I (Synthol, Russia). Changes in the transcriptional activity of genes (i.e. evaluation of mRNA copies for each gene) were determined with reference St_act gene (housekeeping gene).

Statistical analysis was carried out using StatSoftStatistica 6.0software. Experiments included at least 3 biological repeats in the analysis of biochemical parameters and at least 15 repeats in the analysis of transcriptional activity. Figures show the average results of repeats and their standard errors.

RESULTS AND DISCUSSION

Potato callus tissue, where is able to observe the development of oomycetes on ecto- and endophytic stages of development, are a convenient model for research of the development ofplantsprotective response mechanismsto Ph. infestans. Our observations of the growth of the late blight pathogen on potato calluses revealed that at 24 h after Ph. infestans inoculation, penetrating of oomyceteshyphae in calli was deep enough (reached 1/3 of callus tissue volume) (Table 1). After 48 hours, the depth of penetration of the fungus in calli was already half of their diameter. Hyphae of the pathogen grew abundantly on the callus tissue, while most of them were thickened and intensely colored.

Table 1: Effect ofSA and JA on Ph. infestans growth in calluses of potato.

Timeafterinoculation, h	The size of the infected areas to callus diameter, %		
	Control	SA	JA
24	34.2 + 2.2	26.1 + 2.1	24.3 + 2.3
48	56.7 + 4.0	51.0 + 4.2	49.5 + 4.2

The cultivation of calli in media containing SC and JA inhibits the growth of fungi. Apparently, SA and JA influenced the growth rate of the Ph. infestans indirectly through a potato callus.

Important weapon of pathogens attack are produced hydrolytic enzymes, especially amylases, ensuring the introduction of the fungus in the tissue [19]. The response plant protection reaction is accompanied by synthesis of inhibitors of these enzymes [20, 21].

According to our research, the defeat of potato callus withPh. infestans changes theactivity of hydrolytic enzymes (Table. 2) and their protein inhibitors (Fig. 1).

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Table 2: Effect of SA and JA on the activity of hydrolytic enzymes in the potato calliduring infection of Ph. Infestans

No	variant	Proteolytic Activity (gelatin) units / ml		Amylolyticactivity, units / ml	
		I	II	I	II
1	Control	1.9 ± 0.01	9.75 ± 0.8	4.2 ± 0.03	6.3 ± 0.04
2	Ph. infestans	3.4 ± 0.03	19.5 ± 1.2	0	0
3	SAprocessing	2.3 ± 0.02	9.75 ± 0.7	6.2 ± 0.04	9.6 ± 0.5
4	SA + Ph. infestans	3.6 ± 0.02	18.25 ± 1.3	0	0
5	JAprocessing	1.9 ± 0.01	12.5 ± 0.9	4.2 ± 0.02	19.6 ± 1.3
6	JA + Ph. infestans	3.6 ± 0.03	13.75 ± 1.1	0	0

Note: I - the 3rd day after infection; II - 10th day after infection

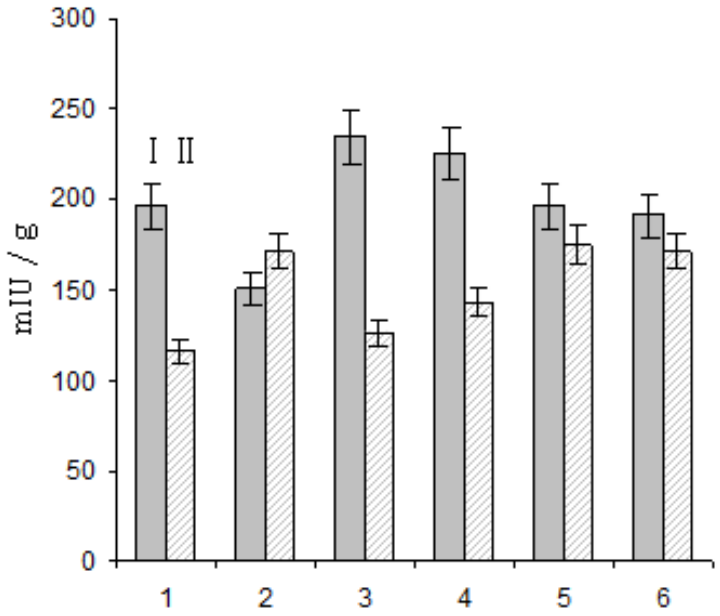

Fig 1: Activity of proteinase inhibitors in potato calli treated with inducers of resistance and infected withPh. infestans.

1- control; 2 - Ph. infestans; 3 - SA processing; 4 - SA + Ph. infestans;5 - JA processing; 6 - JA + Ph. infestans. I - 3 days, II - 10 days after inoculation.

Moreover, proteolytic andamylolytic enzymes and their inhibitors activity during infection variesboth byPh. infestans infection and the influence of treatment of calluses with resistance inductors. Pathogen infection leads to a sharp increase in the activity of proteases that hydrolyze gelatin. In infected calli activity of these enzymes is several times higher than in the control variant (Table 1). The high proteolytic activity in infected tissues provides not only amino acids for pathogenic microorganism growth and development, but also can simultaneously neutralize potato protective proteins such as lectins and hydrolase inhibitors. It has been shown that extracellular metalloproteinase of phytopathogenic bacterium E. carotovora (Jones) Waldee splits potato lectin which takes part in the plant protection [22].

Interestingly, in our experiments on the initial stages of a pathogen infection (day 3) was observed decrease in activity of inhibitors that suppress the action of trypsin proteases (Fig. 1). However, at day 10 of infection activity of proteinase inhibitors in potato calli in all experiments becomes higher than the control. It should be noted that the processing with SA and JA stimulated callus and antiproteolytic activity in both

infected and in uninfected tissues (Fig. 1). Moreover, in all experiments with processing calliwith SA and JA proteinase inhibitors activity was significantly higher than in uninfected and infected control experiments.

The research of amylases activity in infected calluses revealed a very different picture compared with the dynamics of proteolytic activity. It turned out that the contamination of potato callus with Ph. infestans results in complete inhibition of amylolytic activity in the tissues (Table 2). In all experiments with no inoculation, amylolytic enzymes activity is detected in calli. Moreover, treatment of uninfected calliwith resistance inductors significantly improves amylases activity in uninfected tissues. On the contrary, in the calliinfected with Ph. infestans, including processedwith resistance inductors, amylases activity could not be detected (Table 2).

It is known that the amylolytic activity is characteristic of representatives of a majority of taxonomic groups of fungi and almost always these enzymes are constitutive proteins, even in wood-destroying fungi [23]. However, the amylases is absent in the oomycetes, in particular in fungi of the genus Phytophthora. Representatives of this type of fungi usethe potato enzymes for its starch degradation, activating theirbiosynthesis in the diseased tubers [24]. It can be assumed that the absence of amylolytic activity in infected calluses can be explained by the high level of activity of highly specific inhibitors of amylases produced by plant tissues in response to the introduction of pathogens.

In connection with this hypothesis, we carried out a measurement of the expression level of amylase inhibitor gene XM006351484in potato leaves infected with Ph. infestans and processed withSA and JA.

Research has shown that during the first day after infection in potato plants starts increasing of the levels of amylase inhibitor gene transcription (Fig. 2).

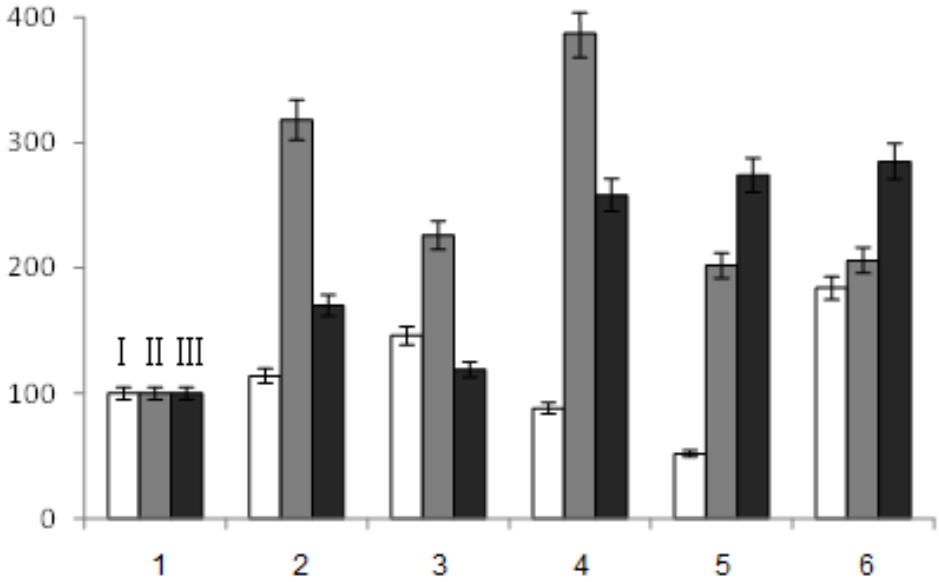

Fig 2: Changing the transcriptional activity of XM006351484 amylase inhibitor gene in potato leaves influenced by SA, JA, and Ph. infestans infection.1- control; 2 - Ph. infestans; 3 - SA processing; 4 - SA + Ph. infestans; 5 - JA processing; 6 - JA + Ph. infestans. I - 6 h, II - 24 h, III - 48 h after inoculation.

Thus, at 24 hours after infection,transcription activity of the gene was 3 times greater than that of control plants. In plants pretreated with SA and JA during infection also provides a high level of transcriptional activity of the amylase gene that persisted for 48 h (Fig. 2). Probably in response to Ph. infestans infection, potato plants can synthesize de novo inhibitors of enzymes which are able to completely inhibit the activity of its own amylase. Our results indicate that pretreatment with SA and JA stimulates the synthesis of these inhibitors in plants.

We obtained similar results in the study of transcriptional activity of a proteinase inhibitor JX683427gene. Thus, Ph. infestansinfection of potato leaves led to 2 times increase in the level of transcriptional activity of a

proteinase inhibitor gene within 6 h after inoculation (Fig. 3). Moreover, such a high transcriptional activity level of proteinase inhibitor gene in infected plants was maintained for 48 hours.

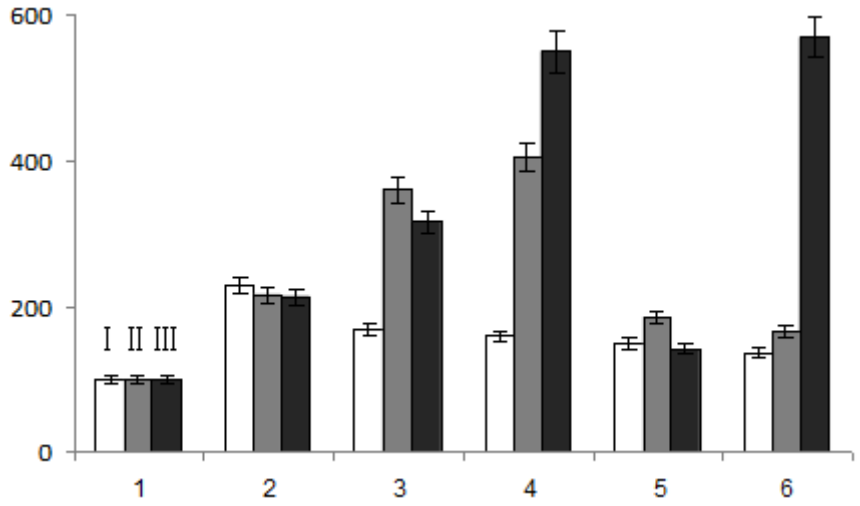

Fig 3: ChangeJX683427 proteinase inhibitor gene transcriptional activity in leaves of potato withSA and JA and Ph. infestans infection.1- control; 2 - Ph. infestans; 3 - SA processing; 4 - SA + Ph. infestans; 5 - JA processing; 6 - JA + Ph. infestans. I - 6 h, II - 24 h, III - 48 h after inoculation.

Interestingly, pretreatment the plants with SA and JA had a significant stimulatory effect on the transcription activity of a proteinase inhibitor gene in non-infected plants, but especially during infection (Fig. 3). Thus, at pretreatment with SA and JA and subsequent infection of the Ph. infestans, transcriptional activity of proteinase inhibitor gene at 48 h after infection was 6 times morethan in control experiment (Fig. 3).

It can be assumed that a JX683427 proteinase inhibitor has no specificity to gelatin-hydrolyzing potato enzymes, since the activity of these hydrolases increased significantly in potato plant tissues during Ph. infestansinfection (Table 2).

Protease inhibitors synthesis occurs in plants in response to infection by pathogens and pests. A key role in the initiation of protective response plays systemin membrane receptor, a protein with a molecular mass of approximately 160 kDa. In response to damage, it induces membrane depolarization that leads to the opening of ion channels and a sharp increase in the level of intracellular calcium ions. This activatesMAP kinase and phospholipase, and a series of reactions synthesize a jasmonic acid which probably serves as an activator of protective proteins genes transcription [25, 26].

CONCLUSION

Our studies on the potato callus and plants support this hypothesis and suggest that SA and JA have an impact on the development of Ph.infestans. It is expressed in the reduction of the pathogen mycelial penetrating ability and in high stimulative effect of these compounds on transcriptional activity of hydrolase inhibitors gene at infection.

The obtained data suggest that activation of the synthesis of hydrolase inhibitors in tissues of potato plants under the influence of the SA and the JA can be a factor in the formation of resistance to Ph. infestans.

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