ЕКСПЕРИМЕНТАЛЬНІ РОБОТИ

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RIBONUCLEASE ACTIVITY OF BUCKWHEAT PLANT (*Fagopyrum esculentum*) CULTIVARS WITH DIFFERENT SENSITIVITIES TO BUCKWHEAT BURN VIRUS

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Ribonucleases (RNases) are present in base-level amounts in intact plants, but this level is able to increase greatly under stress conditions. The possible cause for such an increase is protection against plant RNA-virus attack. Buckwheat burn virus (BBV) is a highly virulent pathogen that belongs to Rhabdoviridae family. In our study, we have analyzed the correlation between RNase activity and resistance of different buckwheat cultivars to BBV infection. Two cultivars, Kara-Dag and Roksolana, with different sensitivities to BBV have been used. Kara-Dag is a cultivar with medium sensitivity to virus and Roksolana is a tolerant cultivar. It has been shown that the base level of RNase activity in Roksolana cultivar was in most cases higher than the corresponding parameter in Kara-Dag cultivar. Both infected and uninfected plants of Roksolana cultivar demonstrated high RNase activity during two weeks. Whereas infected plants of Kara-Dag cultivar demonstrated unstable levels of RNase activity. Significant decline in RNase activity was detected on the 7th day post infection with subsequent gradual increase in RNase activity. Decline of the RNase activity during the first week could promote the virus replication and therefore more successful infection of upper leaves of plants. Unstable levels of RNase activity in infected buckwheat plants may be explained by insufficiency of virus-resistant mechanisms that determines the medium sensitivity of the cultivar to BBV. Thus, plants of buckwheat cultivar having less sensitivity to virus, displayed in general higher RNase activity.

Key words: plant defence mechanism, ribonuclease (RNase) activity, buckwheat (Fagopyrum esculentum), Rhabdoviridae family, buckwheat burn virus (BBV).

It is well known that plant virus-resistance mechanism is a complex process that includes interaction of the components at different levels [1, 2]. Research of various details and peculiarities that underlie it is a highly relevant issue, because the obtained information, may help further increase virus resistance of economically-valuable plants.

Ribonucleases (RNases) are a group of enzymes that hydrolyze both own [3] and foreign RNAs [4, 5]. RNases may have diverse functions in plants, including protection against pathogens' attack [4–7]. Some works suggest that nucleases may be a part of non-specific defence against plant viruses (phytoviruses). For example, in some experiments RNases cause aggregation or degradation of virions [8]. Plant pathogenesis-related (PR) class 10 proteins exhibit a ribonucleolytic activity and this activity may increase in plant cells after viral attack [9, 10]. RNases play an important role in RNA silencing establishment – general plant defence against viruses [2, 11]. Moreover, transgenic plants expressing bovine pancreatic RNase, *Zinnia elegans* RNase II or dsRNA-specific ribonuclease, were protected against some viruses/ viroids (tobacco mosaic virus, tomato mosaic virus, tomato spotted wilt virus, buckwheat burn virus, potato spindle tuber viroid, chrysanthe-

mum stunt viroid) or characterized by a delay in the appearance of symptoms on plants inoculated with cucumber mosaic virus or potato virus Y [12–15]. Transgenic plants of *Nicotiana benthamiana* overexpressing the cytosolic exoribonuclease Xrn4p have indicated increased level of tombusvirus RNA degradation [16]. Contrariwise the exoribonuclease Xrn4p-silenced *N. benthamiana* plants promoted tombusvirus RNA accumulation [6], thus confirming that exoribonuclease Xrn4p was involved in virus RNA degradation. Plant endoribonuclease RNase MRP is involved in tombusvirus RNA degradation as well [17].

In a study concerning correlation between virus resistance and ribonuclease activity of plants one of phytorhabdovirus has been used – buckwheat burn virus (BBV). The viral disease was found on the buckwheat fields in Khmelnytskiy region, Ukraine, and then the pathogen – BBV – was characterized [18]. This object (BBV) was chosen due to its high pathogenicity for buckwheat plants. In case of infection, plants either die or their generative organs get damaged. This may decrease the yields of buckwheat for up to 80%. Apart from buckwheat, BBV affects other valuable crops from different families – tobacco, potato, pea, pepper, tomato, cucumber and others [18]. Selection of the buckwheat cultivars resistant to BBV may greatly increase the crop production.

BBV has negative-sense RNA genome and, taking into consideration its structural organization, may be included into *Rhabdoviridae* family [19]. So another motivation for involving BBV was that mechanism of rhabdovirus reproduction differs from that of positive-sense RNA genome viruses – tobacco mosaic virus, potato virus Y, bean pod mottle virus studied earlier in a similar sphere [8, 20-22].

Materials and Methods

Plant material and infection by the pathogen. Among 120 buckwheat cultivars from the collection of Podolsky State Agricultural and Technical University (Ukraine) the two most perspective ones, but different in susceptibility to BBV, have been chosen for the current research. Kara-Dag is a medium sensitive cultivar and Roksolana is a tolerant one. Seeds of buckwheat were sown directly into the soil and next seedlings and adult plants have been cultivated in greenhouse conditions. One month old plants have been mechanically infected by rubbing the viral preparation containing 0.5 mg/ml BBV in buffer (which contained 1 M glycine, 0.01 M $MgCl_2$, pH 7.0) in the presence of carborundum powder on dorsal face of low leaves. Taking into consideration that mechanical damage may influence RNase activity, the control plants have been left intact. After inoculation plants were transferred back to the greenhouse conditions. The day of the inoculation was designated as a day zero.

Virus preparation. BBV particles have been isolated from the sap of infected tissues of *Nicotiana rustica* plants with visible mosaic symptoms and purified using polyethylene glycol 6000 precipitation followed by three cycles of differential centrifugation [23]. The purity of BBV preparation was controlled using both spectrophotometer and electron-microscopic methods. The extinction factor E 260/280 nm of BBV was about 1.2. This ratio is typical of purified buckwheat burn virus. The viral suspension was applied on the grid with formvar support film and contrasted by 2% phosphotungstic acid pH 6.8-7.0 and examined in electron microscope JEM-123 [23].

Determination of RNase activity. In the course of the experiment, every 3 days after infection undamaged mature buckwheat leaves were taken to analyze the changes in RNase activity. For current research, we have chosen spectrophotometric method for determination of RNase activity. Unlike the popular qualitative method for RNase activity detection via discoloration of band on a gel, spectrophotometric method is a quantitative one. Using of spectrophotometric method has allowed us to compare our own results with results obtained by other researchers. There are several variants of the spectrophotometric method. We selected the method described by Galiana et al. [24]. One gram of fresh leaf tissue was grinded in liquid nitrogen using a mortar and a pestle; for extraction of soluble fraction containing proteins 1 ml of 50 mM Tris-HCl buffer (pH 7.0) was added. After 10 min centrifugation at 10 000 g and 4 °C, the supernatant containing fraction of soluble proteins from leaf tissue was collected and used for analyzing the RNase activity. As our study focused on enzyme activity of RNases, we use name *protein extract* to emphasize that proteins in extracts were under our concerning, but it does not mean that extracts did not have other components. Other components have not been determined. To standardize of experimental conditions and compare amounts of total soluble proteins (TSP) in extracts obtained from uninfected and infected by BBV plants, the quantity of TSP was measured. It has been performed according to Bradford's method

[25] using bovine serum albumin as a standard. The content of TSP in leaf tissue extracts was about 0.2-0.5%. Protein extracts with 5 μg of TSP were incubated for 2 h at 37 °C in 250 μl of 50 mM Tris-HCl (pH 7.0) buffer containing bovine serum albumin (0.01%) and yeast RNA (400 μg/ml) as substrate for action of RNases. After incubation, the remaining RNA was precipitated with ethanol in the presence of 2.5 M ammonium acetate and resuspended in 500 μl of water. To make sure the purity of samples, optical density was measured at 260 and 280 nm using spectrophotometer BioPhotometer (Eppendorf). For pure sample of RNA ratio 260/280 nm should be around 2. Total RNase activity was detected by decreased absorption at 260 nm compared with negative control. Reaction mixture with buffer addition was used as a negative control. Addition of purified bovine pancreatic RNase A (5 μg, Sigma R-6148) into reaction mixture served as positive control.

All data are expressed as the mean ± standard deviation (SD). Differences in means between groups have been tested using the Mann-Whitney U test and considered to be statistically significant at $P < 0.05$.

Results and Discussion

Buckwheat burn virus particles were isolated from tissues of *N. rustica* plants with visible symptoms of infection: reduction and yellowing of upper leaves, wrinkled leaves, chlorotic spots, and partial leaf necrosis at the later stage. The purity of viral preparation was verified prior to the study of buckwheat burn virus effect upon RNase activity of different buckwheat cultivars.

Electron-microscopy method proved that viral particles correspond to BBV by their bacilli-shape morphology and the size of viral particles was 230-270×75-90 nm (Fig. 1). Any contaminations by othes viruses was not observed.

As it was reported earlier by Spivak et al. different buckwheat cultivars have dissimilar sensitivity to buckwheat burn virus [13]. Some cultivars have severe damages if infected by BBV, but others are only slightly injured by the virus. The possible cause is different RNase activity of the cultivars. So we have determined the RNase activity of uninfected and infected plants of two buckwheat cultivars with different resistance to virus. Roksolana is a tolerance to BBV buckwheat cultivar and Kara-Dag is a medium sensitive to BBV buckwheat cultivar.

Every three days during three weeks leaves from uninfected buckwheat plants of two cultivars were collected and used for extraction. Extracts obtained from leaf tissues of uninfected buckwheat plants were tested for activity of RNases (activity of uninfected plants named as base RNase activity). Total RNase activity was determined as the decrease of absorption at 260 nm relative to the control without adding the protein extract. We measured total RNA absorption at 260 nm after incubating the reaction mixture containing yeast RNA with plant extracts. So the more active plant RNases were, the less intact RNA remained in the solution and the less values of absorption at 260 nm were observed. It should be noted that the base nuclease activity observed during experimental time in the slightly-injured Roksolana cultivar was higher in most cases than the corresponding parameter in medium-injured Kara-Dag cultivar. RNase activity in leaf protein extracts from Roksolana buckwheat cultivar was close to the one of purified RNase A (Fig. 2). It should be noted that control and experimental reaction mixtures has been supplied with equal quantity of total proteins (5 μg), but the control mixture was supplied with purified RNase A only and experimental reaction mixture was supplied with extracts containing total soluble cell proteins (not only plant RNases). Thus we may conclude that some plant RNases have more enzymatic activity.

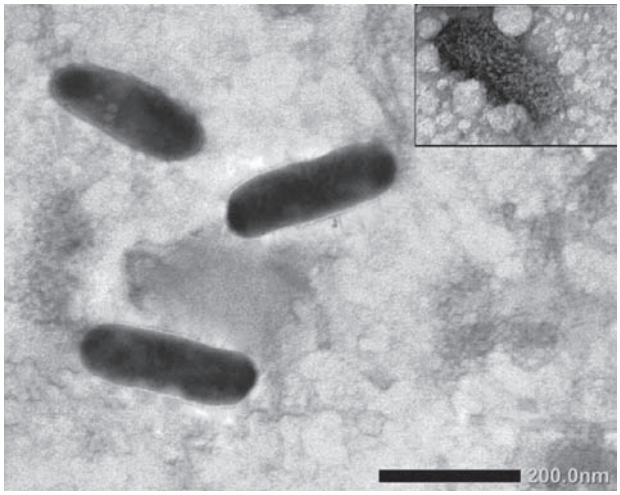

Fig. 1. Electron microscopy detection of buckwheat burn virus particles

Uninfected plants of medium-injured buckwheat cultivar Kara-Dag were characterized by decreasing of RNase activity after 10th day. Plants infected with BBV demonstrated a sharp decrease of RNase activity on the 7th day, with its slow subsequent increase. The highest level of RNase activity

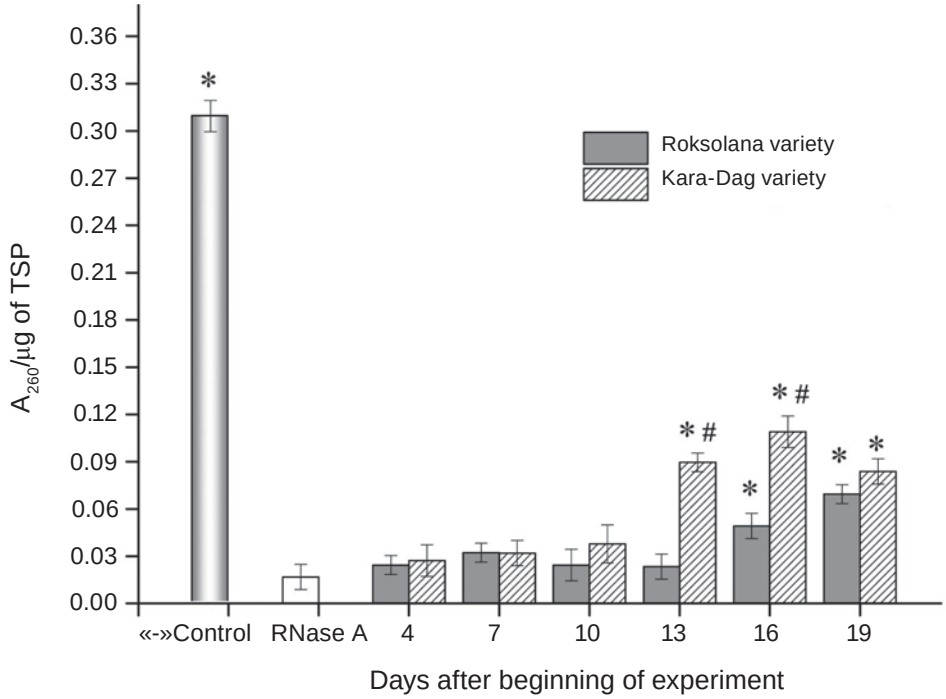

*Fig. 2. Ribonuclease activity of uninfected control buckwheat plants of Roksolana and Kara-Dag cultivars (n = 6). Each value represents M ± SD. * Significantly different compared with those RNase A control sample (P < 0.05), # Significantly different compared with those in Roksolana variety (P < 0.05)*

in leaves was detected on the 16th day after plant infection. At the first days viruses begin the replication. So decline the RNase activity during the first week could promote the virus replication and, therefore, increase the number of virus particles. Great numbers of virus particles may be more successfully spread throughout the plant and cause systemic disease in spite of subsequent increasing of RNase activity (Fig. 3). It may be related to activation of RNase synthesis in response to systemic viral infection. But this does not ensure prevention of the infection of upper leaves. At this time the symptoms of disease were visible. Unstable levels of RNase activity may contribute to viral expansion across the plant and therefore determine the medium level of injury of buckwheat cultivar itself.

Uninfected and infected plants of slightly-injured Roksolana cultivar were characterized by constant high RNase activity during first two weeks (Fig. 4). We supposed that highly active RNases promote destruction of the virus RNA, especially during the first week, and thus interfere with spreading of BBV into upper uninfected leaves. On the 16th day RNase level in infected buckwheat plants decreased relatively to base RNase level of the uninfected plants, but later reincreased back. Despite the transient decreasing of RNase activity on the 16th day it may be concluded that infectious process is very weak. Symptoms of disease were slightly visible on the upper uninfected leaves. In this way high RNase activity in the leaves of infected plants helps limit the infection process, preventing virus dissemination.

As it was shown by Šindelařova et al. [26] a hypersensitive cultivar of tobacco plants infected with of tobacco mosaic virus (TMV) demonstrated higher RNase activity than the susceptible ones. Since hypersensitive reaction is one of the variations of plant resistance these observations confirm our results.

Our data does not correspond with the results demonstrated by Burketova et al. [20]. In this work it was indicated that the higher RNase activity promoted RNA biosynthesis of beet necrotic yellow vein virus and was found in the susceptible cultivar of sugar beet. Other work reported about the same results with potato virus Y (PVY) – susceptible potato cultivar demonstrated higher level of RNase activity, but the reliability of data was confirmed not for all experimental time [20]. Testing of susceptible tobacco cultivar and transgenic breeding line producing coat protein of PVY has demonstrated that increased RNase activity was detected

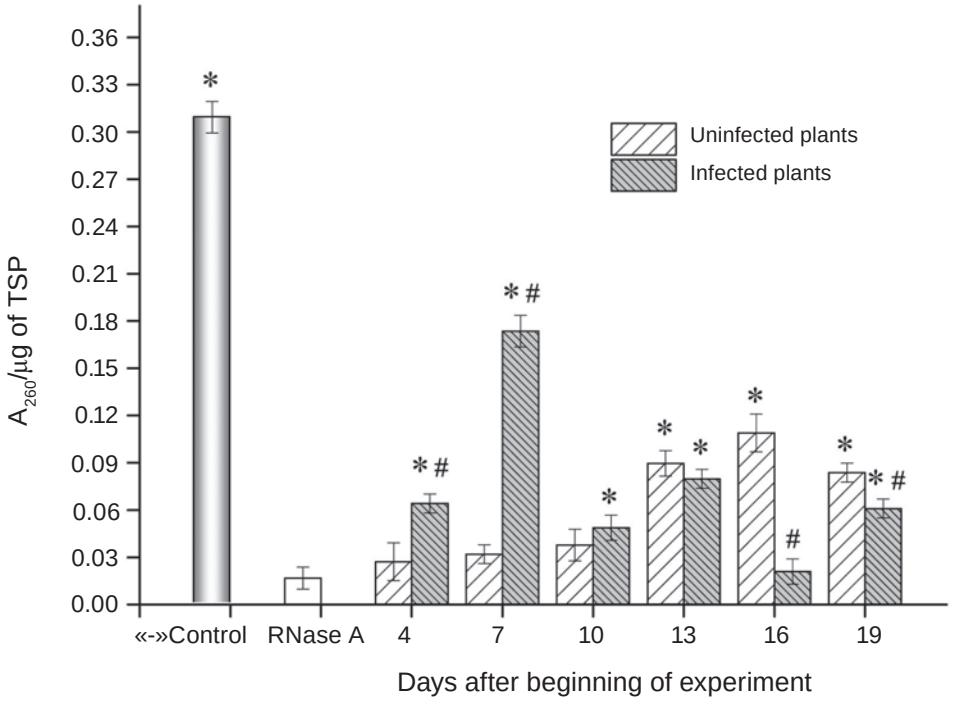

*Fig. 3. Ribonuclease activity of uninfected and infected buckwheat plants, Kara-Dag cultivar (n = 6). Each value represents M ± SD. * Significantly different compared with those RNase A control sample (P < 0.05), # Significantly different compared with those in uninfected plants (P < 0.05)*

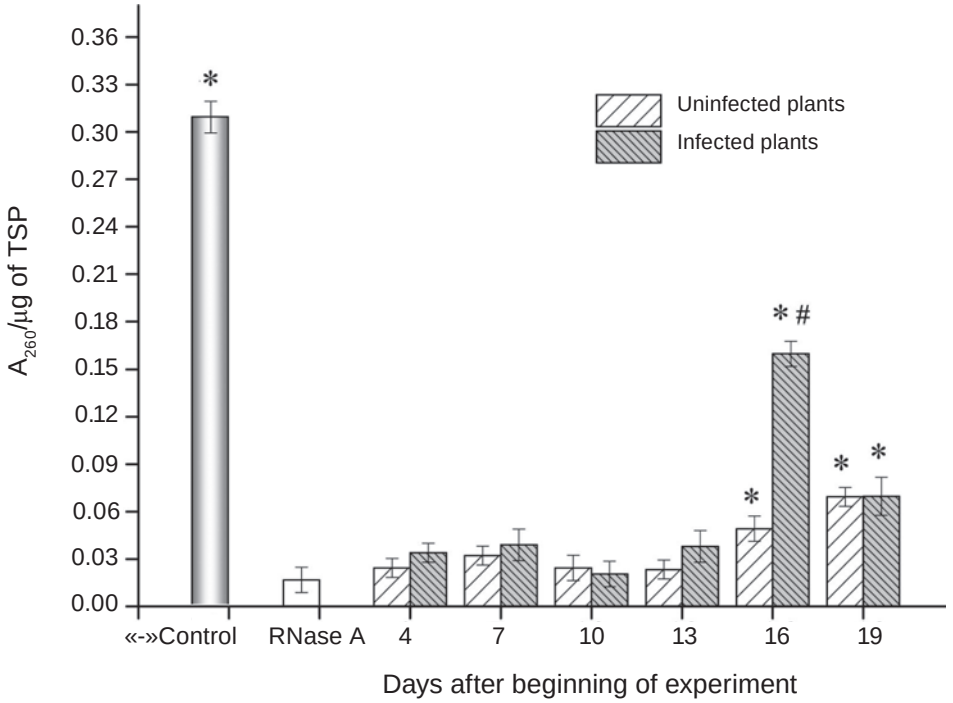

*Fig. 4. Ribonuclease activity of uninfected and infected buckwheat plants, Roksolana cultivar (n = 6). Each value represents M ± SD. * Significantly different compared with those RNase A control sample (P < 0.05), # Significantly different compared with those in uninfected plants (P < 0.05)*

for susceptible cultivar if infected by PVY whereas transgenic line had invariable level of RNase activity both in infected and healthy plants [27]. Šindelař and Šindelařova have observed positive correlation between RNase activity in tobacco and PVY reproduction as well [28]. The work of Šindelařova et al. suggested that RNase activity is positively correlated with the susceptibility of tobacco cultivars to PVY [22]. This fact is obvious for susceptible cultivars. But both RNase activity and PVY content in tolerant and resistant cultivars of infected tobacco did not differ significantly. In all mentioned works authors have used viruses with positive-sense RNA genome, so their RNA was infectious. In our research, negative-sense RNA genome virus was used, so its RNA was not infectious. Also it was supposed that using the host RNA degraded by the plant RNases for virus RNA biosynthesis is triggered only when the sources of nucleotides formed *de novo* are insufficient for the synthesis of a high virus content (as in a case TMV or for a very fast virus RNA synthesis (for example, potato virus X) [29]. But buckwheat burn virus has a slow rate of systemic infection; one of the possible reasons is slow virus RNA synthesis. The content of BBV in infected tissues is not as high as TMV one.

On the other hand, our data correlate with data demonstrated by Trifonova et al. [15]. In the mentioned article, it has been demonstrated that transgenic tobacco line expressing gene of RNase II of *Zinnia elegans* and demonstrating lower RNase activity had higher viral antigen accumulation and quicker symptoms appearance than transgenic tobacco line with high RNase activity. But in both cases transgenic plants with heterologous gene of RNase II were better protected from infection of TMV than control non-transgenic plants.

Šindelař et al. has demonstrated that the protein content had decreased since the first day after inoculation of potato plant leaves with PVY in comparison with the control non-infected plant leaves [29]. In our own research, we have not noticed decrease in the level of total soluble proteins (TSP) when 'Roksolana' cultivar infected by BBV was used, except the 16th day when such a decrease (but statistical not reliable) was observed. The levels of TSP were similar for infected and uninfected plants in the same experimental day and the means lied between 2-4.5 mg TSP/g fresh weight of leaves. The level of TSP of Kara-Dag cultivar uninfected and infected with BBV varied during the same experimental day. The means lied between 1.3-5.5 mg TSP/g fresh weight of leaves. But the most prominent decrease the level of TSP of infected plants compared with control plants was observed on the 4th and 7th days after inoculation when the level of TSP decline twice. On the 7th day the RNase activity was the lowest. The absence of changes in protein content was also reported by Šindelařova et al. [26] when PVY and TMV or mixed PVY/ TMV infections in tobacco plants or tobacco chloroplasts only were studied. The same results – absence of TSP content changes – were reported for crude homogenates from healthy and PVY infected plant tissues of susceptible and resistant breading line of tobacco [27].

Taking into consideration the obtained data, we may conclude that the level of ribonuclease activity is positively correlated with the resistance of buckwheat cultivar to buckwheat burn virus (negative-sense RNA genome virus). Thus determination of RNase activity level may facilitate screening of buckwheat cultivars more resistant to virus infection.

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РИБОНУКЛЕАЗНА АКТИВНІСТЬ СОРТІВ ГРЕЧКИ (*Fagopyrum esculentum*) З РІЗНОЮ ЧУТЛИВІСТЮ ДО ВІРУСУ ОПІКУ ГРЕЧКИ

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Рибонуклеази (РНКази) в неушкоджених рослинах присутні на певному рівні, який може різко підвищитися в умовах стресу. Можливою причиною його підвищення є захист рослин від атаки РНК-вмісних вірусів. Вірус опіку гречки (ВОГ) є високовірулентним збудником, який належить до родини *Rhabdoviridae*. У нашому дослідженні ми проаналізували кореляцію між активністю РНКази та стійкістю

різних сортів гречки до ВОГ. Були використані два сорти «Кара-Даг» і «Роксолана» з різною чутливістю до ВОГ: «Кара-Даг» – сорт із середньою чутливістю до вірусу, «Роксолана» – толерантний сорт. Показано, що базовий рівень активності РНКази в рослинах сорту «Роксолана» в більшості випадків був вищим, ніж у рослинах сорту «Кара-Даг». Інфіковані та неінфіковані рослини сорту «Роксолана» мали високу активність РНКаз протягом двох тижнів, тоді як в інфікованих рослин сорту «Кара-Даг» рівень активності РНКаз був нестабільним. Значне зниження активності РНКаз виявлено на 7-й день після зараження з подальшим поступовим збільшенням активності РНКаз. Зниження активності РНКаз протягом першого тижня може сприяти реплікації вірусу й інфікуванню верхніх листків рослин. Нестабільні рівні активності РНКаз у заражених рослинах гречки можна пояснити недостатністю механізму вірусостійкості, який визначає середній рівень чутливості сорту до ВОГ. Таким чином, рослини гречки менш чутливі до вірусу і в цілому демонструють вищу активність РНКаз.

Ключові слова: механізми захисту рослин, активність рибонуклеази (РНКази), гречка (*Fagopyrum esculentum*), родина *Rhabdoviridae*, вірус опіку гречки (ВОГ).

РИБОНУКЛЕАЗНАЯ АКТИВНОСТЬ СОРТОВ ГРЕЧИХИ (*Fagopyrum esculentum*) С РАЗНОЙ ЧУВСТВИТЕЛЬНОСТЬЮ К ВИРУСУ ОЖОГА ГРЕЧИХИ

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Рибонуклеазы (РНКазы) в неповрежденных растениях присутствуют на определенном уровне, который может резко возрасти в условиях стресса. Возможной причиной его увеличения является защита растений от атаки РНК-содержащими вирусами. Вирус ожога гречихи (ВОГ) является высоковирулентным возбудителем, который относится к семейству *Rhabdoviridae*. В нашем исследовании мы проанализировали корреляцию между активностью РНКаз и устойчивостью различных сортов гречихи к ВОГ. Были использованы два сорта – «Кара-Даг» и «Роксолана» – с различной чувствительностью к ВОГ: у сорта «Кара-Даг» – средняя чувствительность к вирусу, «Роксолана» – толерантный сорт. Показано, что базовый уровень активности РНКаз в растениях сорта «Роксолана» в большинстве случаев выше, чем в растениях сорта «Кара-Даг». Инфицированные и неинфицированные растения сорта «Роксолана» проявляют высокую активность РНКаз в течение двух недель, тогда как у инфицированных растений сорта «Кара-Даг» уровень активности РНКаз нестабильный. Значительное снижение активности РНКаз обнаружено на 7-й день после заражения с последующим постепенным ее увеличением. Снижение активности РНКаз в течение первой недели может способствовать репликации вируса и инфицированию верхних листьев растений. Нестабильные уровни активности РНКаз в зараженных растениях гречихи можно объяснить недостаточностью механизма вирусоустойчивости, который определяет средний уровень чувствительности сорта к ВОГ. Таким образом, растения гречихи, менее чувствительны к вирусу и в целом проявляют более высокую активность РНКаз.

Ключевые слова: механизмы защиты растений, активность рибонуклеазы (РНКазы), гречиха (*Fagopyrum esculentum*), семейство *Rhabdoviridae*, вирус ожога гречихи (ВОГ).

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