Expression of PR genes and genes of heat shock proteins in potato plants in vitro under infection with ring rot and heat stress

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Aim. The changes in expression of the HSP101, HSP60, and HSP17.8 genes in tissues of potato plants of varieties Lukyanovsky under in vitro heat treatment and infection with a ring rot pathogen Clavibacter michiganensis ssp. sepedonicus (Cms) were investigated. Methods. These changes were assessed at the transcript and protein levels. Results. It was shown that under heat treatment at 39 °C for 2 h, the maximum accumulation of HSP101 was observed. In control experiments, the plants of the two varieties showed neither synthesis of HSP101, HSP60 and HSP17.8 proteins nor expression of the HSP101, HSP60 and HSP17.8 genes. Infection without heat treatment induced HSP60 expression. Infection suppressed activation of HSP genes upon heat stress. Additionally, infection of potato plants by Cms caused an increase in transcription of PR-2 and PR-4 genes. Conclusions. Potato plants under biotic and abiotic stress, both independently and combined, activate the expression of a wide range of the protective proteins, including HSP and PR families.

Keywords: heat shock proteins, PR, potato, Clavibacter michiganensis ssp. sepedonicus

Introduction

Plants are exposed to various stress factors of both abiotic and biotic nature. To maintain homeostasis in plants under stress conditions, nutrient and energy redistribution between growth, development processes and protective reactions takes place [1].

Heat shock proteins (HSPs) are synthesized in plants in response to the increase in temperature to protect from damages induced by extremely high temperature. HSPs act as chaperones, preventing from protein denaturation and aggregation, and promoting the restoration of protein activity after the exposure to high temperature [2, 3]. HSPs (i) provide for temporary binding and folding facilitation of immature peptides in course of translation, (ii) disassemble oligomeric protein complexes, (iii) control biological activity of regulatory proteins (including the transcription factors), (iv) facilitate protein transport through plant cell membranes, (v) prevent against aggregation of partially denatured proteins due to intermolecular
interactions [4]. It is known that HSPs are synthesized not only in response to high temperature, but also to a wide range of stress factors, e.g., salinity and dehydration [2, 5].

HSPs are divided into several classes depending on molecular weight: HSP100, HSP90, HSP70, HSP60 and low molecular weight HSP (sHSP) [6]. They differ by the functions and capability to accumulate under stress and normal conditions. Protein HSP101 plays a leading role in the development of plant thermotolerance [7], therefore, investigation of the HSP101 synthesis level in plants is of interest. A number of HSPs accumulate in plant cell in the absence of stress, and their expressions only slightly changed under stress, for example, HSP60 involved in the folding of newly synthesized proteins [8]. In some cases, the HSP accumulation, in particular sHSPs, is observed under biotic stress [9–11].

The expression of the HSP101 gene is sensitive to various stresses [7]. Therefore, the protein is convenient for monitoring the influence of different stress factors on the HSP expression. HSP60 may be used as a control variant. HSP17.8 belongs to the sHSP family, so it can be induced under pathologies [6].

The available literary data on the influence of thermal action on the plants subjected to biotic stress are highly controversial. In some cases, a preliminary thermal treatment led to an increase in susceptibility of plants to infection. For example, the temperature rise suppressed the defense responses of Arabidopsis thaliana seedlings against Pseudomonas syringae pathogen infection [12]. An increased temperature at tobacco cultivation suppressed the hypersensitivity reaction (HR) and promoted the enhanced penetration of mosaic virus into the plants [13]. The preliminary thermal impact at 36 °C for 30–120 min intensified the infection of barley with the powdery mildew (fungus Erysiphe graminis f. sp. Hordei) [14] and with the pathogen Blumeria graminis f. sp. Hordei [15]. Short-term heat treatment of soybeans at 44 °C induced their susceptibility to Phytophthora [16]. Similar situation was observed in case of infecting Coffea arabica L. with the pathogens Colletotrichum kahawae and C. gloeosporioides, and the development of susceptibility in this case correlated with the induction of HSP70 synthesis [17]. On the contrary, the 50 °C heat treatment for 30–60 s suppressed infecting barley seedlings with powdery mildew. Similarly, the heat treatment of potato plants at 40 °C for 48 h inhibited their infecting with powdery mildew [11].

Currently, various infection diseases of cultivated crops, including potato, are widely spread. Most of such infections are represented by pathogenic fungi and bacteria [18]. For example, in the Nordic countries and Canada, up to 50 % of the potato crops are regularly lost owing to the ring rot potato disease caused by gram-positive bacterium Clavibacter michiganensis ssp. sepedonicus (Cms). There is no information about the conditions provoking HSP synthesis in potato under heat stress and pathogenesis, or in case of superposition of the two stress factors. The data available are either indirect or quite contradictory. The tomato plant cultivation at 15 °C suppressed manifestation of the symptoms of C. michiganensis infecting [19]. In contrast, planting the wild potato species S. acaule at 15 °C stimulated colonization of the plants with Cms, and only at 21 °C – made the plants resistant to the pathogen [20]. However, in these experiments, the plants were grown (i) all the time at elevated
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temperature, (ii) in the temperature conditions (21–23 °C), when there was no synthesis of HSPs in plants, and (iii) the species peculiarities were not taken into account [2].

So, a role of HSPs in a cell under combined effect of the two stress factors on plant – infection and heat shock – is not clear. The aim of our investigation was to study the character of variations in the content of different HSP families (HSP101, HSP60, and HSP17.8) and PR proteins in potato tissues in vitro under heat stress and infection with the ring rot pathogen.

Materials and Methods

Our investigations were conducted with the potato plants Solanum tuberosum L. of species Lukyanovsky in vitro. The plants of this species are characterized by susceptibility to a number of pathogens, including ring rot [21]. That is why this species was chosen to obtain a vivid picture of the gene expressions changes. Microcloning of in vitro plants was performed by grafting. The seed pieces (grafts) were placed into the agar-based nutrient Murashige and Skoog (MS) medium with the addition of sucrose 30 g/l, pyridoxine 1 mg/l, thiamine 1 mg/l, ferulic acid 1 mg/l, pH 5.8–6.0, at the depth of the internodes. The seed pieces were cultivated under factorostatic conditions at 26±1 °C, illumination 32W/m², photoperiod duration – 16 h.

Potato tissues were infected with Cms, strain Ac 14 05, obtained from All-Russian collection of microorganisms (Moscow). The bacterial culture was grown in agar-based medium placed in Petri dishes and containing dialysate solution of yeast extract (Sigma-Aldrich, Inc., USA) 10 g/l, glucose 15 g/l, agar-agar (Biotechnovatsiya, Inc., Russia) 10 g/l, CaCO₃ (Reachem, Inc., Russia) 5 g/l, pH 7.0. The bacteria were cultivated in a thermostat at 25 °C, in darkness.

To reveal the temperature of maximum HSP synthesis the potato plants in vitro were heated in an air-drying thermostat during 2 h at 26, 35, 37, 39, 42, 45, 50 °C. Next, the total protein was isolated, and the content of HSP101 in the samples was determined by PAGE, Western blotting and staining nitrocellulose membranes with antibodies.

A series of the following experiments was conducted to investigate the effect of the ring rot infection upon the accumulation of the HSP gene transcripts and content of HSPs in potato plants. The potato plants were infected with Cms and after 48 h of incubation at 26 °C underwent heat stress (39 °C, 2 h). The changes in expression of the genes were registered at both levels – transcript accumulation and protein synthesis. The time of coincubation was chosen due to our previous observations [22], which showed that after 24 h of coincubation the bacteria penetrated into the root and stem zones of potato plants.

We also investigated the process of accumulation of the studied genes’ transcripts under the conditions of heat treatment and infection with the pathogen using reverse transcription real-time PCR. Earlier, in order to exclude possible false positive results, PCR was conducted with primers of the potato HSP genes on the matrix of plasmid and chromosomal DNA of Cms. As known, in the process of co-evolution of the pathogen and the host plant, acquisition and transfer of genes from one organism to the other (horizontal gene transfer) may occur [23]. As a result of the PCR test conducted on the Cms DNA matrix, no products of amplification were found, that evidenced for the absence of
genetic sequences, to which the primers were selected, in the bacteria.

In experiments on the influence of Cms bacterium infection (in combination with heat shock) upon HSP expression, the suspension with Cms (titer = 1×10⁹ CFU/ml) was introduced into the growth medium of potato plants in vitro. The plants were incubated under factorostatic conditions for 48 h. Next, the plants underwent heat treatment in an air-drying thermostat at 26 or 39 °C for 2 h, afterwards the total protein and RNA were separated. The time of coincubation was chosen according to our previous experiments on the intensity of potato plant colonization with Cms by plating tissue homogenates of root, stem and apical zones. It has been found [22] that 2 days after infecting the potato plants in vitro were completely colonized with the pathogen.

For isolation of the total protein we used 0.5 g sample of the total plant (leaves, stems and roots). A buffer was added to the sample to extract the protein (0.1 M Tris-HCl, 0.003 M DDS-Na, 0.001 M β-mercaptoethanol, 4 % polyvinylpyrrolidone, pH 7.4–7.6) and a 1 mM solution of phenylmethylsulfonyl fluoride (PMSF) for inhibiting proteases. A sample with quartz sand was thoroughly ground to powder in a mortar with liquid nitrogen. Coarse cellular components of the sample were removed by centrifugation at 15,000 rpm (Centrifuge Allegra 64 R, USA) for 15 min. The protein from the supernatant was precipitated with a three-fold volume of cooled acetone. The protein pellet was dissolved in the buffer prepared for the sample (0.625 M Tris-HCl, 0.008 M DDS-Na, 0.1 M β-mercaptoethanol, 10% glycerol, pH 6.8), incubated at 100 °C for 5 min and centrifuged at 5000 rpm (Centrifuge MiniSpin, Germany) for 15 min. The samples obtained were boiled in the water bath at 95 °C for 3 min. The protein was diluted in the sample buffer with bromophenol blue (0.625 M Tris-HCl, 0.008 M DDS-Na, 10 % glycerol, 0.001 % bromophenol blue, pH 6.8) and used for electrophoresis fractionation of 10 μg of protein per track [24]. Electrophoresis in PAAG was conducted according to the modified Laemmli system, on the Mini-PRONEAN III Electrophoretic Cell, Bio-Rad (USA). 13 % PAAG was used to the end of simultaneous fractionation of high-molecular weight and low-molecular weight proteins. The protein molecular weight was determined by the path length of the corresponding protein in the gel with the aid of a standard set of protein markers (SibEnzyme). In order to determine the protein load upon the track and to equalize the volume of application of the samples, a special program Gel Analysis (Russia) was used.

The transfer of proteins onto the nitrocellulose membrane (Sigma, USA) was carried out by a “wet” technique in a special blotting device (Bio-Rad, USA). In this work, antibodies against HSP101 (Agrisera AS 07253, Sweden), HSP60 (US Biological H1830-77B, USA), HSP17.6 TTP 2 (Agrisera As 07255, Sweden), class I, were used. The antibodies against HSP17.6 specific for Arabidopsis thaliana were used because HSP17.8 (present in potato tissues) is homologous to HSP17.6 of Arabidopsis. Visualization of antibodies was conducted with the use of secondary antibodies conjugated with alkaline phosphatase (Sigma, USA), in the presence of BCIP and NBT (Sigma, USA). After that, the nitrocellulose membranes were dried at indoor temperature and scanned.
Isolation of plasmid DNA (miniprep) from bacteria was carried out by the technique of alkaline lysis using polyethylene glycol (PEG 6000). Chromosomal DNA of the bacteria was obtained with commercial kits GenFlute Bacterial Genomic DNA Kit (USA). In PCR on the matrices of plasmid and chromosomal DNA we used ReadyMix Tag PCR Reaction Mix kits (Sigma, USA). PCR was conducted on the GENE CYCLER (Bio­Rad, USA) according to the following program: 1 cycle 94 °C – 5 min; 25 cycles of 94 °C – 1 min; 56 °C – 1 min; 72 °C – 1 min; 1 cycle 72 °C – 7 min. For electrophoretic separation of the amplification products, 1.2 % agarose gel in TAE buffer (242.2 g/L TRIS, 89.6 ml/L glacial acetic acid, 18.62 g/L disodium EDTA pH 7.6) was used with the addition of 5 μg/ml ethidium bromide. The voltage was 80 to 110 V. The gel was photographed in the ultraviolet spectrum with the gel-documentation system (Bio­Rad, USA).

Isolation of RNA from plant tissues was carried out with TRI-Reagent (Sigma-Aldrich, USA) according to the manufacturer’s protocol. Since small volumes of the samples were used in the experiments, for RNA isolation the tissues of leaves were taken from the middle tier of the potato plants. Homogenization of the TRI-Reagent material was carried out in a TissueLyser II homogenizer (Qiagen, USA) for 2 min at the frequency of 30 cycles per second. The proteins were denatured with bromochloropropene (Sigma-Aldrich, USA). The nucleic acids were precipitated with 2.5 volumes of 96 % ethanol at – 20 °C during the night. Next, the samples were centrifuged at 14,000 g and +4 °C for 10 min, the residual nucleic acid pellet was dried at the room temperature, resuspended in 25–40 μl of deionized sterilized water and used to synthesize the first cDNA strand. The amount (normalized multiplicity of expression) and purity of the isolated RNA were evaluated spectrophotometrically (NanoPhotometer NP80 spectrophotometer, Implen GmbH, Germany) via the optical density indicator at 260/280 nm. Furthermore, the quality of isolated RNA was monitored by the technique of electrophoresis in 1 % agarose gel under non-denaturing conditions.

In case of cDNA synthesis, 1 μg of RNA was taken for one reaction. When the first cDNA strand was created, RNA previously treated with DNA-ase I (Fermentas, Lithuania) was used as the matrix. Synthesis was conducted using oligo (dT) 15 primer and reverse-transcriptase Rever-tAid H M-MuLV (Fermentas, Lithuania) according to the manufacturer’s protocol (with minor modifications).

The sequences of potato genes HSP17.8, HSP101, HSP60 and EF-1α (the reference gene) were taken from the Spud DB database (http://solanaceae.plantbiology.msu.edu/) and tested in Blast NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The following primers for the analyzed genes were chosen. For the HSP17.8 gene, the pair HSP17.8L: TCCAAAGGAAGAGGTGAAGAAACC and HSP17.8R: CGACTCAGCATAAGACACAGGCA. For the HSP101 gene, the pair HSP101L: AGGAGGTGGTTGGAGAAGAAAGT and HSP101R: CCCAGTAGCAGCATTCACAAGC. For the HSP60 gene, the pair HSP60L: GTAGAGGGTGCTGTCGTGGT and HSP60R: GCTGTCCTAATCACTTTCACTGGAT. For the EF-1α gene, the pair EF-1αL: TCCAAAGGAAGAGGTGAAGAAACC and EF-1αR: CCCAGTAGCAGCATTCACAAGC. For the PR4 gene, the pair PR4L: GGCTGGACCGCTTTTTG
TGG and PR4R: CTGTTTCTTGTTCTCCTGT TCCTGT. For the PR2 gene, the pair PR2L: GCTGCGATGGAACGAACAGGA and PR2R: CCAGGCTTTCTCGGACTACCT.

The reverse-transcriptase PCR was conducted in real time using a ready-made mixture of qPCR-mix-HS SYBR reagents (Eurogen, Russia) and the proper equipment C 1000 Thermal Cycler CFX 96 Real-Time System (Bio-Rad, USA). The volume of the reaction mixture was 10 μl. PCR was conducted according to the following protocol: warming up to 50 °C for 2 min, one denaturing cycle (95 °C, 5 min), 36 amplification cycles (95 °C, 20 sec – 60 °C, 30 sec – 72 °C, 30 sec).

Software MS Excel and Statistica was used for statistical analysis of the data obtained.

Results and Discussion

It is known that HSP101 plays a leading role in thermotolerance in plants [7], therefore, in the first series of experiments we have chosen the conditions, under which maximum synthesis of HSPs in vitro under heat treatment is observed in potato plants. The maximum amount of HSP101 in potato plants in vitro was found under heat treatment at 39 °C, 2 h (Fig. 1).

Then we have investigated the process of accumulation of transcripts of the studied genes under the conditions of heat treatment and infection with the pathogen. It was revealed that the greatest accumulation of HSP101 (Fig. 1) and a high content of the HSP101 gene transcripts (Fig. 2) were observed at 39 °C. As far as the genes HSP17.8 and HSP60 are concerned, a multiple increase in the level of transcripts after heat treatment of the plants was revealed with respect to the control plants (without heat treatment). The smallest changes in the level of transcripts (dozens-fold) were observed for the gene HSP60, the largest changes (thousands-fold) – for the gene HSP17.8 (Fig. 2). No valid expression level for all these genes was observed in the control plants and in case of infecting the plants with the phytopathogen. In the case of superposition of the two stress factors (heat treatment of the plants infected with Cms) a 4-fold decrease in the number of the HSP101 gene transcripts was observed. Noteworthy, a level of the HSP60 gene expression in potato tissues at heat stress and infection was substantially lower (18 c.u. (conditional units)) than that at heat treatment only (35 c.u.). The combined treatment of potato plants resulted in an increase of the number of the HSP17.8 gene transcripts by 1.5 times as compared to heat treatment only.

At the next stage of the research the content of tested HSPs was measured. The data are presented in Fig. 3. Synthesis of HSP60 and HSP101 in the control plants was observed in trace amounts. Heat treatment of potato plants at 39 °C induced the synthesis of HSP101, HSP60 and HSP17.8. Infecting potato plants with Cms significantly induced the synthesis of HSP60, and insignificantly – HSP101 (Fig. 3). However, infecting potato plants under heat...
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In present work both the largest content of HSP101 in potato plants in vitro (Fig. 1, 3), and a large number of the HSP101 gene transcript (Fig. 2) were revealed under heat treatment at 39 °C (2 h). All the available data only indirectly indicate the temperature range influencing the HSP synthesis in potato plants. Meanwhile, there are no clear data on the time and temperature ranges of thermal exposure inducing the HSP synthesis in potato plants [25–27]. So, it was revealed for the first time that the heat treatment of potato plants in vitro at 39 °C for 2 h induced maximum synthesis of HSPs. The temperature of maximum synthesis of HSPs in A. thaliana is known to be 37–38 °C [28], in Sorghum bicolor – 45 °C [29], in yeast S. cerevisiae – 37–39 °C [30].

Infecting potato plants in vitro with Cms actually did not influence the HSP expression at both the HSP gene transcript level (Fig. 2) and the protein level (Fig. 3). Infection induced the HSP synthesis only in trace amounts (Fig. 3). It is likely that, because of the lack of specific receptors to Cms pathogen, the pathogen susceptible potato plants react to Cms bacteria like to any other stress factors, and respond to this pathogen with a such nonspecific reaction as the HSP synthesis. Owing to the presence of specific Cms receptors, recognition of the pathogen takes place in the cells of resistant variety plants, and a cascade of signaling reactions is initiated [31]. This process leads to the regulation of gene expression and probably to obtaining synthesized proteins of direct antimicrobial activity.

However, the results of measuring the studied HSP gene transcripts indicate that the
combination of \textit{Cms} infecting and heat treatment of potato plants inhibits the thermal activation of the \textit{HSP101} and \textit{HSP60} gene expression. Meanwhile, heat stress stimulates the \textit{HSP17.8} gene expression.

These results evidence that biotic stress is able to initiate and transform the protective response of plants to heat influence. The fact of such response correlates with the data related to variations in the expression level of the genes encoding pathogen-related (PR) proteins. In this work, it was shown that the expression levels of the genes \textit{PR2} (1,3-\(\beta\)-glucosidase) and \textit{PR4} (hevein-like protein) in tissues of the susceptible potato variety Lukyanovsky were amplified under the \textit{Cms} infection, and the expression level of the \textit{PR2} gene under biotic stress was twice higher than that of \textit{PR4} (Fig. 4). The growth of the number of transcripts of these genes by 2–3 times is considered to be substantial. According to literary data, the infecting of tomato plants with \textit{Clavibacter michiganensis ssp. michiganensis} (tomato pathogen) is known to amplify the contents of such proteins as 1,3-\(\beta\)-glucosidase (\textit{PR2}), endochitinase (\textit{PR3}), hevein-like protein (\textit{PR4}), thaumatin/osmotin (\textit{PR5}), cucumisin-like serine protease [32]. The induction of expression of the \textit{PR} genes (\textit{PR1, PR2, PR3, PR4, PR5, PR6}) was also shown in the tomato plants infected with bacteria \textit{Pectobacterium carotovorum} [33].

Noteworthy, in our investigation, when potato plants were heat treated, the levels of transcripts of the \textit{PR2} and \textit{PR4} genes were also increased in comparison with control samples. The \textit{PR4} gene expression was 3-fold higher under heat stress compared to the control sample. There are the reports that expression of this gene in tissues of other plants can increase

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image1}
\caption{Results of Western blotting of the HSP101, HSP60 and HSP17.8 proteins of potato plants under the conditions of heat exposure and infecting with \textit{Cms}.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image2}
\caption{Variations in the level of transcripts of the \textit{PR-2} and \textit{PR-4} genes in potato leaf tissues \textit{in vitro} under heat stress and \textit{Cms} infection.}
\end{figure}
under abiotic stresses. For example, it was observed in rice under ultraviolet treatment, temperature effect (4 °C, 12 °C), salinity, exposure to abscisic and jasmonic acids [34], and in arabidopsis – when treated with ethylene [35].

Under the combined influence of the two stress factors (Cms infection and heat treatment), there was a 4-fold growth of the PR2 and PR4 gene transcripts number (Fig. 4). The changes in the number of transcripts of the HSP genes may be explained by changes in the PR-protein genes expression. For example, under biotic stress, one can observe an increase in the PR gene expression (Fig. 4), while the HSP gene expression is not intensive. Under heat treatment, it is possible to observe some increase in the HSP and PR gene expression, which may be explained by intensification of the protective proteins synthesis as a non-specific protective response of the plant to stress factors. In case of superposition of two stress factors, the number of transcripts of the majority of analyzed HSP genes is smaller than that under only heat treatment; furthermore, on the contrary, the number of transcripts of the PR genes grows.

Conclusions

Therefore, for the first time we have shown the following. (1) High-temperature exposure (39 °C for 2 h) of potato plants cultivated in vitro provokes the greatest increase of the HSP101 gene transcript level and maximum induction of the HSP101 synthesis. (2) Cms infecting does not affect the expression of HSPs and activates the expression of PR2 and PR4 genes in potato plants. (3) Heat stress combined with infection leads to an increase in the expression of all the genes studied, whereas the maximum expression of the HSP17.8 and PR2 genes is observed. The obtained results indicate that the plants under stressful influences activate the protective programs, including a wide range of the protective proteins.

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Експресія генів PR та білків теплового шоку в картоплі in vitro при інокуляції кільцевою гниллю й тепловому впливі

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Мета. Дослідити зміну експресії білків теплового шоку BTШ101, BTШ60 і BTШ17.8 в тканинах картоплі сорту Лук’яновський in vitro при тепловому впливі та зараженні збудником кільцевої гнилі Clavibacter michiganensis ssp. sepedonicus (Cms). Методи. Зміну експресії вивчені на двох рівнях: кількості транскриптів та вмісту протеїну. Результати. Показано, що при тепловому впливі 39 °C протягом 2 годин in vitro спостерігається максимальне накопичення HSP101. В контрольних варіантах у рослин було не було відміченого збільшення синтезу білків HSP101, HSP60 й HSP17.8, але утворення транскриптів генів HSP101, HSP60 та HSP17.8. Зараження без теплового впливу не вплинуло на експресію білку HSP60. Зараження пригнічувало активна експресії BTШ при тепловому стресі. При зараженні рослин картоплі Cms підвищувалась кількість транскриптів генів PR-2 та PR-4. Висновки. Рослини картоплі при біотичних і абіотичних стресах, як окремо, так і спільно, активують експресію широкого спектру захисних білків, включаючи білки сімейства HSP і PR.

Ключові слова: білки теплового шоку, PR, картопля, Clavibacter michiganensis ssp. sepedonicus.

Ціль. Ісследувати зміну експресії білків теплового шоку в картоплі in vitro при зараженні кільцевою гниллю і тепловим впливом

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