

Article

Phenylalanine Ammonia-Lyase-Mediated Differential Response of Tomato (*Solanum lycopersicum* L.) Cultivars with Different Stress Tolerance to Treatment with Low-Molecular-Weight Chitosan

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Abstract: The latest research has shown that chitosan acts as a growth stimulator and elicitor in plants, including resistance to biotic and abiotic factors. However, increasing concentrations could possibly make chitosan a source of stress for plants. In this study, we investigated the effect of low-molecular-weight chitosan hydrolysate on the root development of tomato (*Solanum lycopersicum*) cultivars Red Cherry, Lel, and Tytan. The growth rate change, together with total phenolic content, phenylalanine ammonia-lyase (PAL) activity, and gene expression, were studied in relation to tomatoes. High concentrations of chitosan negatively affect the growth of tomato seedlings and contribute to changes in the tropism of the seedling roots. After the addition of chitosan hydrolysate, the PAL activity and the total phenolic content decreased 24 h later. PAL is a key enzyme in the biosynthesis of many plant stress factors. An analysis of the tomato PAL gene family was carried out. The *SIPAL* gene expression in the seedlings of cv. Cherry increased 1.5 times after 48 h, while in cv. Lel, the expression stably decreased in the presence of chitosan. The obtained results are supposed to aid our understanding of the mechanisms underlying the effects of chitosan on plant development and further its successful application in agriculture as well as in research on plant stress.

Keywords: *Solanum lycopersicum*; chitosan hydrolysate; phenylalanine ammonia-lyase; PAL gene family



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1. Introduction

Tomato (*Solanum lycopersicum*) is considered to be one of the world's most popular vegetable crops, which is cultivated both outdoors and indoors, including hydroponic modules, for all-year-round consumption. The yield and quality of tomatoes directly depend on the proper usage of crop protection methods [1]. Because of the very short time between tomato harvest and its sale, it is very important to consider the safety requirements and use the least toxic crop protection. In this case, chitosan, being a biodegradable, nontoxic natural compound, fully satisfies the safety standards [2,3].

Chitosan, being a deacetylated form of chitin, is considered to be the second most common natural polymer after cellulose. The fungicidal activity of chitosan has been confirmed both in vitro and in vivo [4–7]. Studies have revealed the high efficiency of chitosan as an inhibitor of fungal pathogen growth, causing morphological and structural changes and molecular disorganization in fungal cells [8]. Therefore, chitosan is considered to be a potent biological fungicide that could reduce the chemical load in agroecosystems [9]. In addition, chitosan can enhance physiological responses in plants, functioning as a stimulator of plant growth and resistance to pathogens. In a lot of studies, the positive influence of chitosan on germination and seedling growth rates, root length, and the volume of the root system has been proven [9–13]. It has been suggested that the stimulatory effects of chitosan are due to the activation of the plant immune system, including the biosynthesis of phytohormones and defense enzymes [12,14–16]. Chitosan is recognized

by plants as a microbe-associated molecular pattern (MAMP). Similar to other MAMPs, such as flagellin, glycoproteins, and lipopolysaccharides, chitosan triggers a cascade of immune responses [9,17–19], the production of phytohormones, and other components of plant immune signaling, including phenylpropanoids [20,21]. The effects of chitosan on tomato plants have also been studied [22–28]. Researchers noted that the presence of chitosan alters the levels of primary and secondary metabolites in tomato tissues [22,23,29]. Additionally, an increase in the activity of oxidative stress enzymes and chitinase was observed [23,24,26,28,30]. Treating young plants led to growth retardation [29]. Meanwhile, investigations involving more mature tomato plants showed a positive influence on plant development and enhanced tolerance to various stresses by chitosan [22,23,30].

Thus, numerous studies have focused on chitosan's involvement in plant defense against phytopathogens due to its fungicidal activity. The ability to suppress fungal development increases with the concentration of the active substance. On the other hand, researchers need to explore how useful chitosan is as a stimulator of plant growth and development. However, finding a balance between chitosan's stimulating and fungicidal activities remains unclear. Moreover, the differences between the characteristics of chitosan used in different studies complicate the search for a universal answer, necessitating the selection of optimal parameters for each new preparation.

Here, we would like to point out the effect of chitosan on the biosynthesis of phenolic compounds in tomatoes. Phenolic compounds play an important role in plant metabolism. Analysis of their activity can become one of the essential elements for finding the optimal mode of use of chitosan. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is a key enzyme in the phenylpropanoid pathway (which catalyzes the loss of ammonia and the formation of cinnamic acid), which is directly involved in both the biosynthesis of phenolic compounds and the synthesis of salicylic acid [31–33]. All of these compounds play an important role in plant responses to biotic and abiotic stresses [34]. Several subfamilies of PAL genes have been identified in tomatoes [35,36]. Their activity varies with the type of stress present, and phylogenetic analyses suggest a common ancestor with monocots [35]. The phenylalanine pathway of salicylic acid (SA) biosynthesis is activated in plants in response to stress. Silencing the PAL genes leads to the accumulation of reactive oxygen species, the disruption of SA biosynthesis, and increased susceptibility to pathogens [37]. In many studies, it has been shown that the activity of the PAL involved in SA biosynthesis and plant protective responses to stress [33] is upregulated by chitosan [11,16,21,38–40]. Here, we investigated the effect of low-molecular-weight chitosan hydrolysate on PAL activity in tomato cultivars.

Chitosan is essentially a stress-stimulating factor for plants; there is also evidence of the negative impact of this polymer on their development. It has been shown that the efficiency of chitosan depends on its molecular weight, chemical structure (degrees of deacetylation, polymerization, and protonation), source of chitin, and polymer concentration [41–44]. In our study, we used low-molecular-weight chitosan hydrolysate obtained from crab shells. The results of this study make it possible to determine the parameters and limitations of the use of this type of chitosan, which will contribute to its successful use in agronomy. The results obtained under conditions of elevated concentrations are supposed to help our understanding of the molecular mechanisms of chitosan–tomato plant interactions.

2. Materials and Methods

2.1. Chitosan Hydrolysate Preparation

High-molecular-weight crab shell chitosan (molecular weight 1040 kDa, deacetylation degree 85%) was obtained from Bioprogress (Shchelkovo, Russia). Chitosan hydrolysate (CH) was prepared by depolymerizing high-molecular-weight chitosan with nitric acid (Merck, Darmstadt, Germany), as described previously [45]. Briefly, 10 g of chitosan were dispersed in 200 mL of 6.5% nitric acid, incubated for 7 h with stirring at 70 °C, cooled, and kept at room temperature for 16 h without stirring. The pH of the reaction mixture was adjusted to 5.2 with 25% ammonium hydroxide (Helicon, Moscow, Russia) before diluting

with distilled water to a final volume of 400 mL. The final chitosan concentration in the CH-stock solution was 10 mg/mL.

The molecular weight (MW) of chitosan in the main fraction was 33 kDa, with a polydispersity index of 2.1 and a deacetylation degree (DD) of 95%. For the determination of the MW and DD of chitosan in the main fraction of CH, CH was adjusted to pH 10 with 25% ammonium hydroxide for chitosan precipitation, after which the precipitate was dialyzed and freeze-dried. The MW of the chitosan's main fraction was determined by high-performance gel permeation chromatography in a S 2100 Sykam chromatograph (Sykam, Eresing, Germany) using a separation column of 8 mm × 300 mm and a precolumn of 8.0 mm × 50 mm (PSS NOVEMA Max analytical 1000 A, PSS, Mainz, Germany). The 0.1 M NH₄-acetate buffer + 0.2 M NaCl at 30 °C, pH 4.5, was used for elution, with an elution rate of 1.0 mL/min. The analysis of chromatograms was carried out using the MultiChrom software version 1.6 (LLC Ampersand, Moscow, Russia). Pullulans (MW: 342, 1260, 6600, 9900, 23,000, 48,800, 113,000, 200,000, 348,000, and 805,000 Da) (PSS, Mainz, Germany) were used as calibration standards. The DD was determined by the method of proton nuclear magnetic resonance (H-NMR). The chitosan sample was prepared in deuterated water. The proton spectrum was recorded on a Bruker AMX 400 spectrometer (Bruker, Billerica, MA, USA); 4,4-dimethyl-4-silapentane-sulfonic acid was used as a standard.

2.2. Plant Cultivation

Tomato plants were grown in an experimental climate control facility of the Institute of Bioengineering (Research Center of Biotechnology, Russian Academy of Sciences). Seeds of *S. lycopersicum* L. Red Cherry, Titan, and Lel cultivars were kindly provided by the Federal Research Center of Horticulture (Russia).

Seeds were sterilized by successive soaking in 70% ethanol (1 min) and 2% sodium hypochlorite solution (15 min), washed three times with sterile distilled water, and germinated in Petri dishes on moistened filter paper in the dark at 20–22 °C.

2.3. Effect of Chitosan Hydrolysate Concentration and Exposition on Tomato Seedlings

Following sterilization, the seeds were induced to germinate within Petri dishes for 4 days. After that, these seedlings were carefully transferred into comparable Petri dishes containing low-molecular-weight CH and sterile distilled water as a control. Chitosan hydrolysate was at concentrations of 5.0, 2.0, 0.2, 0.02, and 0.002 mg/mL. Distilled water was used to lower the concentration. The seedlings were stored according to the exposure time: 4 h, 24 h, and 48 h (a table describing this experiment is available in Supplementary—Table S1). Following this period, the seedlings were transferred to Petri dishes with distilled water, maintaining their growth for a cumulative period of 8 days. After the end of the experiment, the root length of the seedlings was measured. Each experimental variant involved the utilization of 3 distinct Petri dishes, each containing 25 seeds. Every Petri dish was assessed individually to ensure biological replication.

2.4. Chitosan Hydrolysate Effect on the Tomato Seedlings Development

Following sterilization, the seeds were induced to germinate within Petri dishes for 7 days. After that, these seedlings were carefully transferred into comparable Petri dishes containing a low-molecular-weight CH or sterile distilled water (control) and incubated for 4 h, 24 h, and 48 h. In a series of experiments, a concentration of low-molecular-weight CH of 2 mg/mL was used. The experiments were carried out in five biological replicates; each Petri dish contained 30 tomato seedlings.

After incubation, every plant root was separated from hypocotyl and cotyledon, collected in 1.5 mL tubes (Eppendorf AG, Hamburg, Germany), and instantly frozen in liquid nitrogen. Frozen sample tubes were weighed and stored in a freezer at −80 °C for use in RNA and metabolite extraction. For gene expression analysis, seedlings were grown on the medium for 30 min, 4 h, 24 h, and 48 h and processed as described above.

2.5. Total Phenolic Content Analysis

The content of total phenols in tomato roots was measured according to the previously described method [12], with some modifications. The extraction solution was 96% EtOH with 0.1 M HCl in a ratio of 2:1 (final pH 4.0). Moreover, 50 μ L of the solution was added to 50 mg of tomato root tissue (from 2.4.) and kept in a freezer at -80 °C for 48–72 h. The samples were centrifuged for 15 min at maximum speed in a $+4$ °C refrigerated centrifuge (Eppendorf 5418 R, Eppendorf AG, Hamburg, Germany). After centrifugation, the extract was taken and stored in a freezer.

To 25 μ L of the extract were added 25 μ L of ethanol, 125 μ L of sterile water, and 12.5 μ L of 1 N Folin–Ciocalteu reagent (Sigma Chemical Co., Burlington, MA, USA), incubated for 5 min. Then, 25 μ L of a 5% sodium carbonate solution (Helicon, Moscow, Russia) was added to the sample and incubated in the dark for 1 h. The absorbance was then measured at 725 nm on a spectrophotometer (Eppendorf BioSpectrometer, Eppendorf AG, Hamburg, Germany). A calibration curve for analysis was prepared with gallic acid (Helicon, Moscow, Russia) (5–100 mg/mL), and the results were expressed as mg of gallic acid per g of fresh weight.

2.6. Determination of Phenylalanine Ammonia-Lyase Activity

Phenylalanine ammonia-lyase activity in tomato roots was measured according to [12], with some modifications. Moreover, 50 mg of frozen tomato tissue (from 2.4.) was homogenized in 50 μ L of 0.05 M PBS (pH 7.4) containing polyvinylpyrrolidone (13 mg/mL) (Merck, Darmstadt, Germany) and 2-mercaptoethanol (5 mM) (Merck, Darmstadt, Germany). The homogenate was centrifuged at maximum speed for 15 min (Eppendorf 5418 R, Eppendorf AG, Hamburg, Germany). The extract was transferred to a new tube and stored at -80 °C. Moreover, 90 μ L of borate buffer (0.1 M, pH 8.5) and 900 μ L of a 2 mg/mL phenylalanine solution in borate buffer were added to the 10 μ L of the extract. The reaction mixture was incubated for 30 min at 40 °C. The reaction was stopped by adding 0.25 mL of 1 N HCl. The optical density was measured at 290 nm (Eppendorf BioSpectrometer, Eppendorf AG, Hamburg, Germany). A solution of phenylalanine in borate buffer, with the addition of 0.25 mL of HCl, was used as a reference solution.

2.7. PAL Gene Family Analysis

The search for genes in the *PAL* family was performed using the Sol Genomics Network database [46]. Analysis of the expression level of the identified genes in different tomato tissues was carried out using the TomExpress platform [47]. Gene alignment was performed using ClustalW [48]. The subsequent protein homology analysis was performed by aligning the amino acid sequences in Clustal W. The search for cis-regulatory sequences was carried out using Plant Care [49].

Root samples (~0.1 g) were minced in liquid nitrogen, and RNA was extracted using the ExtractRNA kit (Evrogen, Moscow, Russia), treated with DNase (QIAGEN, Hilden, Germany), purified on CleanRNA Standard columns (Evrogen, Moscow, Russia), and used for first-strand cDNA synthesis with an oligo-dT primer and the MMLV RT kit (Evrogen, Moscow, Russia). RNA and cDNA concentrations were determined by fluorometry (Eppendorf BioSpectrometer, Eppendorf AG, Hamburg, Germany). Real-time quantitative PCR (RT-qPCR) was performed in a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Each sample of the PCR reaction mixture contained 12.5 ng of cDNA, gene primers (Table 1), and the SYBR Green RTqPCR mixture (Evrogen, Moscow, Russia). The RT-qPCR reaction was performed under the following cycling conditions: initial denaturation at 95 °C for 3 min, 44 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 65 °C for 60 s. Additional information about *SIPAL* primers is available in the Supplementary Materials (Figures S1 and S2).

Table 1. Primer pairs for q-PCR analysis.

Gene Name	Primer Pair
<i>SIPAL</i>	5'-GGTGTGACTACTGGATTTGGTGC-3' 5'-GAGTAGCCTTGAAGCAAAGTGTGA-3'
<i>SIEF1β2</i>	5'-AAGGTATATGGGGCAATTTTGGAAAC-3' 5'-GCAGCCTTTTCCTCCTCTGTCTCGA-3'

2.8. Data Analysis

The initial data were analyzed and visualized using Microsoft Excel 2019 (Microsoft Corporation, Redmond, WA, USA), and statistical analysis was performed with the Analysis Tool Pack (Microsoft Corporation, Redmond, WA, USA). The data were subjected to an analysis of variance (one-way ANOVA). Results with a significant F-value were compared at a 95% probability level by the unequal variance (Welch's) *t*-test; $p < 0.05$ was considered to indicate statistical significance.

Relative mRNA expression was calculated after normalization to the mRNA levels of a tomato reference gene (*EF1β2*) [50] using the $2^{-\Delta\Delta C_t}$ method [51,52]. The data were expressed as the mean \pm standard error (SE) based on three technical replicates of three biological replicates for each combination of cDNA and primer pairs. The unequal variance (Welch's) *t*-test was applied to assess differences in gene expression; $p < 0.01$ was considered to indicate statistical significance.

3. Results

3.1. Identification of the Chitosan Hydrolysate Stress Effect on Tomato Seedlings

Increasing the concentration of CH and the incubation time affects the development of Cherry tomato seedlings (Figure 1). Incubation for 48 h at concentrations of 2 mg/mL and 5 mg/mL reduced the length of the roots of tomato seedlings by almost half compared to the control. Incubation for 24 h also had a negative effect on the growth rate; the length of the roots was reduced compared to the control. Tomato seedlings grown in a medium with chitosan hydrolysate of various concentrations for 4 h had no effect on the further growth of Cherry tomato roots.

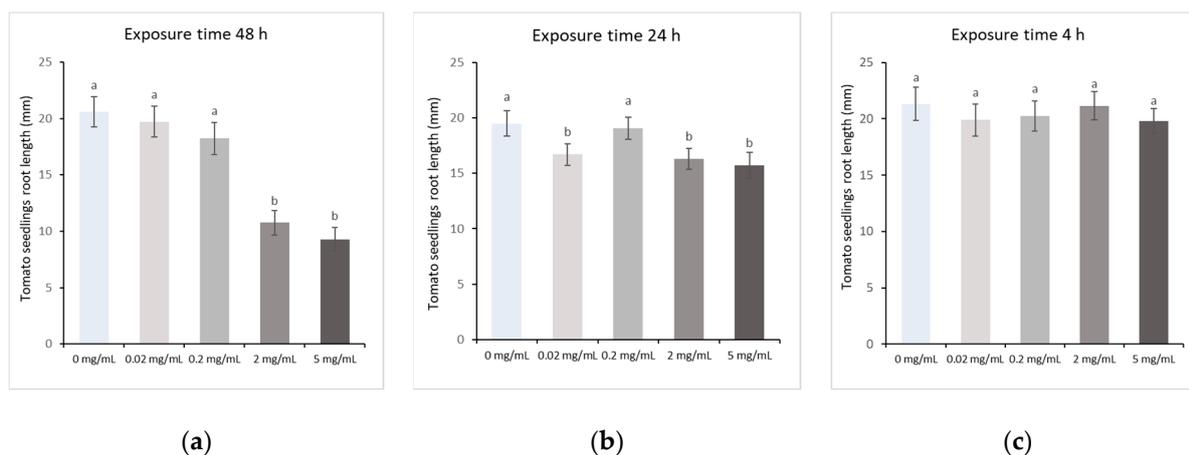


Figure 1. Changes in the seedling root length of tomato (cv. Red Cherry). Seedlings were incubated for (a) 48 h, (b) 24 h, and (c) 4 h in chitosan hydrolysate at concentrations of 2 mg/mL. Following this period, the seedlings were transferred to distilled water, maintaining their growth for a cumulative period of 8 days. Results are represented as mean values \pm SD for each variant. Different letters indicate variants with significant differences, $p < 0.05$.

We have observed that chitosan hydrolysate at a high concentration (2–5 mg/mL) causes changes in the root tropism of tomato seedlings. We analyzed the frequency of the

occurrence of morphological changes in seedlings after treatment with lower concentrations of chitosan hydrolysate. The presence of low-molecular-weight (33 kDa) chitosan hydrolysate in the cultivation medium at a concentration of 2 mg/mL and higher affected the morphology of the roots of tomato seedlings (Figure 2). Thus, a change in the direction of root growth was observed in 14 out of 25 seedlings (56%) at 2 mg/mL after 4 h of exposure to chitosan hydrolysate. Additionally, at a concentration of 5 mg/mL in 22 out of 25 seedlings (88%), root tropism was observed. However, at a lower concentration (0.2–0.002 mg/mL), chitosan hydrolysate did not produce such a significant effect on root growth direction after 4 h.

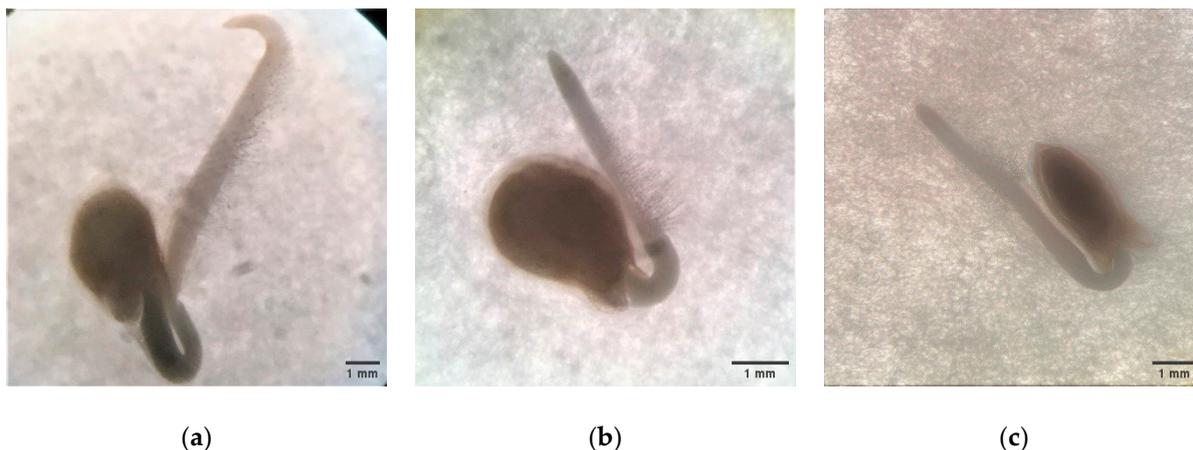


Figure 2. Changes in the tropism of tomato cv. Red Cherry seedling roots after cultivation with chitosan hydrolysate. Seedlings were incubated for 4 h in the presence of chitosan hydrolysate at concentrations of 2 mg/mL (a) or 0.2 mg/mL (b) or distilled water as a control (c) and observed under a Leica MZ FL III fluorescence stereomicroscope (Leica Microsystems Ltd., Wetzlar, Germany).

According to the obtained results, we decided to pay attention to chitosan hydrolysate at a concentration of 2 mg/mL. This concentration significantly affects the development of Cherry tomato seedlings. In addition, it was the minimum concentration with a visual effect on 4-day-old tomato seedlings. We used 2 mg/mL as a standard in the following studies. In our opinion, the use of this chitosan hydrolysate concentration allows us to most fully study its effect on tomatoes.

3.2. Effect of Low-Molecular-Weight Chitosan Hydrolysate on the Phenylalanine Ammonia-Lyase Activity in Tomato Roots

In contrast to the experiment in Section 3.1, where the seedlings were grown in distilled water after treatment for 4 h, 24 h, and 48 h, here onwards, we examined the effect of low-molecular-weight chitosan hydrolysate immediately after the treatment period finished.

Chitosan hydrolysate affects the activity of phenylalanine ammonia-lyase in tomato roots. This part explored the influences of chitosan hydrolysate on PAL activity in three distinct cultivars of tomato, namely Lel, Cherry, and Titan. The PAL activity was measured in the root tissues of 7-day-old seedlings after being placed in a medium containing CH. A standardized concentration of 2 mg/mL was employed to understand the effects of the low-molecular-weight hydrolysate of chitosan. In all three tomato cultivars, a significant decrease in enzyme activity compared to the control was found after 24 h of cultivation (Figure 3).

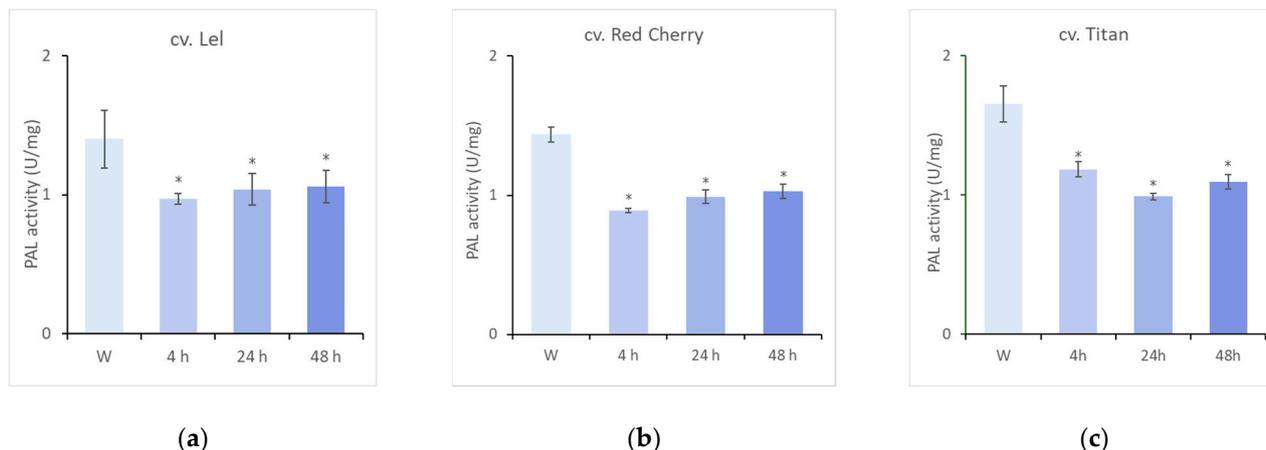


Figure 3. Changes in PAL activity in tomato roots after 4 h, 24 h, and 48 h treatment with 2 mg/mL chitosan hydrolysate and water (W). Results are represented for (a) the Lel cultivar, (b) the Red Cherry cultivar, and (c) the Titan cultivar as mean values \pm SD for each variant. (*) indicates a significant difference compared to the control (W), $p < 0.05$.

The total phenolic content decreased by almost two times compared to the control after 4 h in the roots of the Red Cherry tomato cultivar (Figure 4). In tomatoes from the other two cultivars, the decrease in phenols was not so low; however, compared with the control, the difference was significant. The total phenolic content in the roots of the cv. Titan after 48 h had no difference from the control. The medium with chitosan hydrolysate at a concentration of 2 mg/mL was also used here.

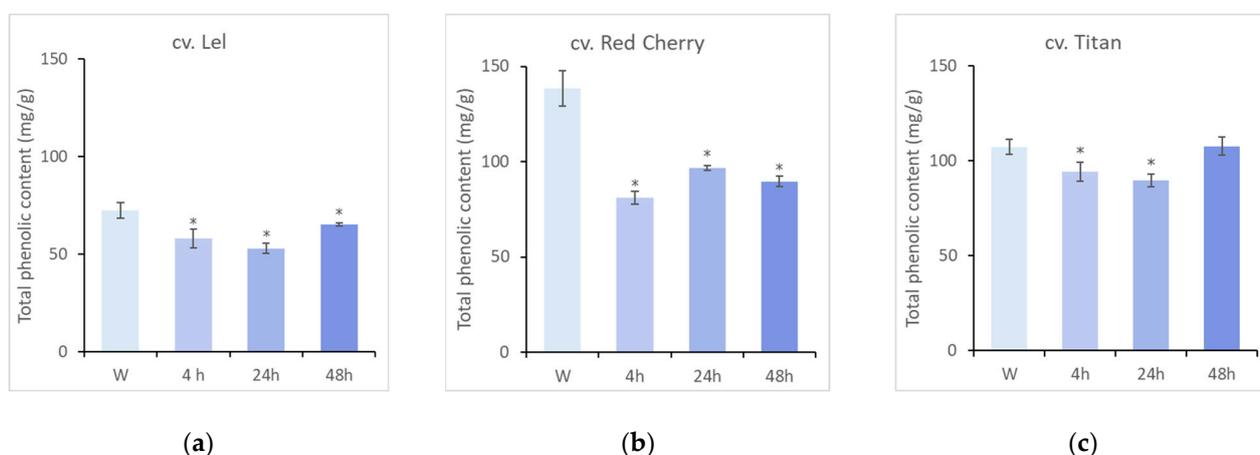


Figure 4. Changes in the total phenolic content in tomato roots after 4 h, 24 h, and 48 h treatment with 2 mg/mL chitosan hydrolysate and water (W). Results are represented for (a) the Lel cultivar, (b) the Red Cherry cultivar, and (c) the Titan cultivar as mean values \pm SD for each variant. (*) indicates a significant difference compared to the control (W), $p < 0.05$.

Interestingly, in comparison between cultivars, cv. Red Cherry showed the lowest total phenolic content of compounds per mg of tissue.

3.3. In Silico SIPAL Gene Analysis

As a result of the search for genes encoding the phenylalanine ammonia-lyase enzyme in the genome of *Solanum lycopersicum*, 16 genes responsible for the synthesis of PAL were identified by the SolGenom database (Table 2). These genes are located in different parts of the genome and are grouped mainly on chromosomes 3, 9, and 10. An analysis of the expression level of the detected genes in cv. MicroTom showed that the activity of these genes varies in different organs and tissues. The *Solyc09g007920* and *Solyc09g007900*

sequences showed the highest level of expression in many tissue types. A noticeably high level of expression relative to other genes is also observed in *Solyc10g086180* in flowers and in *Solyc09g007890* in the meristem (Figure 5).

Table 2. Phenylalanine ammonia-lyase gene family in *Solanum lycopersicum* by SolGenom.

Gene ID	Uniprot ID	Locus Name	Gene ID	Uniprot ID	Locus Name
851	K4AS30_SOLLC	Solyc00g282510	16996	K4C2U1_SOLLC	Solyc05g056170
9277	K4BFW8_SOLLC	Solyc03g036470	24995	K4CQH8_SOLLC	Solyc09g007890
9278	K4BFW9_SOLLC	Solyc03g036480	24996	K4CQH9_SOLLC	Solyc09g007900
9286	K4BFX7_SOLLC	Solyc03g042560	24997	K4CQI0_SOLLC	Solyc09g007910
9879	K4BHL5_SOLLC	Solyc03g071860	24998	K4CQI1_SOLLC	Solyc09g007920
9880	K4BHL6_SOLLC	Solyc03g071870	27806	K4CYG9_SOLLC	Solyc10g011920
9920	K4BHQ6_SOLLC	Solyc03g078270	27807	K4CYH0_SOLLC	Solyc10g011930
9921	K4BHQ7_SOLLC	Solyc03g078280	29798	K4D451_SOLLC	Solyc10g086180

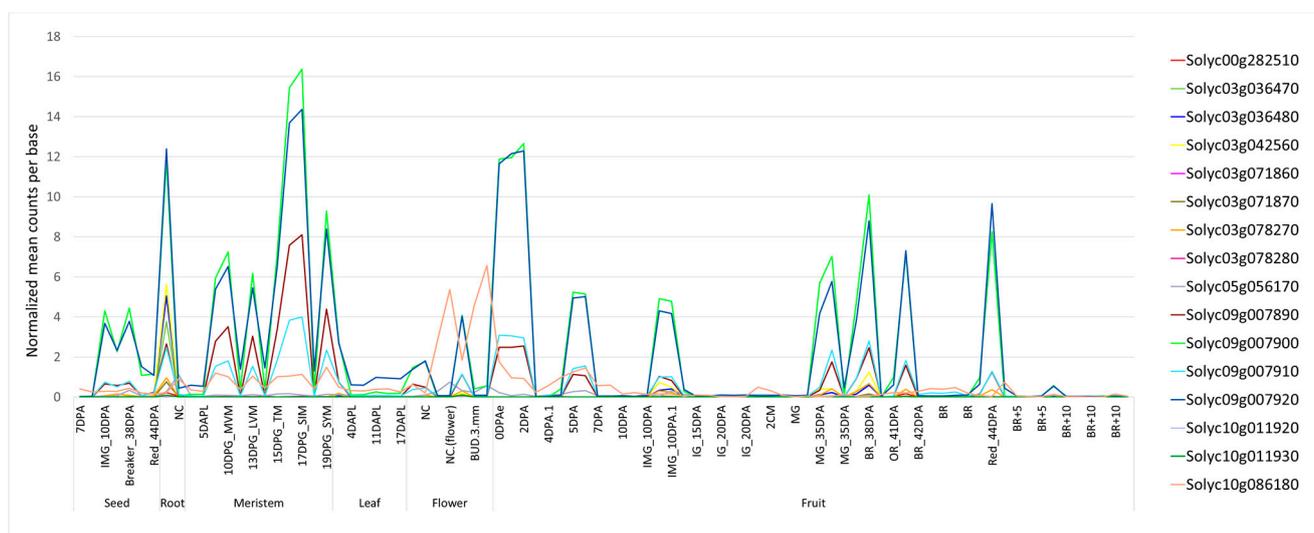


Figure 5. Relative PAL family gene expression in tomato tissues by TomExpress. Expression levels are represented for *Solanum lycopersicum* cv. MicroTom in seed, root, meristem, leaf, flower, and fruit tissues in different growing stages.

Figure 6 shows the expression levels of PAL family genes in tomato roots based on TomExpress data. To better understand the processes, we compared the expression levels of these genes in MicroTom and Heinz 1709 cultivars. The average expression level of PAL family genes differs tenfold between the cultivars. Despite this, it is easy to note the genes with the highest expression level. These are *Solyc09g007920* and *Solyc09g007900* for the MicroTom, and *Solyc10g086180* and *Solyc05g056170* for the Heinz 1709 cultivar. *Solyc09g007920* also has relatively high expression in the root data for the Heinz 1709 cultivar.

We further decided to restrict our interest to the following five genes: *Solyc10g086180*, *Solyc05g056170*, *Solyc09g007900*, *Solyc09g007910*, and *Solyc09g007920*.

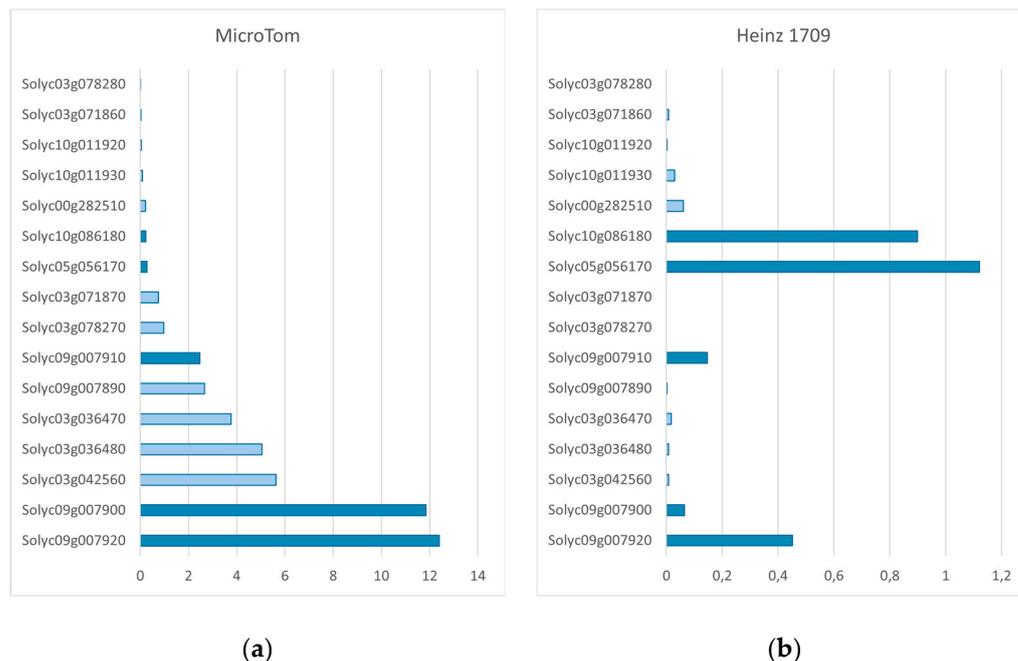


Figure 6. Relative PAL family gene expression in tomato roots by TomExpress. Expression levels represented for *Solanum lycopersicum* cv. MicroTom (a) and cv. Heinz 1709 (b).

We also performed comparative alignments in the NCBI database to find matches with the characterized genes. It was found that *Solyc09g007920* in NCBI is validated as *PAL3* (Gene ID 101243922), and *Solyc05g056170* is validated as *PAL2* (Gene ID 101249824). Additionally, *Solyc09g007910* is validated as *PAL5* (Gene ID 101244220). It should be noticed that the *PAL5* gene is frequently mentioned in studies related to plant stress and is the most popular among researchers [35,53]. However, its expression level remains at an average level in both MicroTom and Heinz 1709 cultivars (Figure 6). Out of 16 genes, the remaining ones have no confirmed annotation in the NCBI database so far.

To compare the protein sequences of the selected genes, an alignment was performed using Clustal W. The most significant fact is that genes *Solyc09g007900* and *Solyc09g007920* have only one amino acid substitution in position #634 (in the alignment). *Solyc09g007900* has an alanine in this position of the protein sequence, while *Solyc09g007920* has a valine. The other genes we selected have more protein sequence differences. *Solyc10g086180* and *Solyc05g056170* have 128 positions with different amino acids in the protein structure. *Solyc09g007900* and *Solyc09g007920* have 16 and 15 amino acid substitutions, respectively, compared to *Solyc09g007910* (*SIPAL5*), and all three protein sequences have the same length (722 amino acids). When comparing *Solyc09g007910* with the amino acid sequence of *Solyc10g086180* (total length 712 amino acids) and *Solyc05g056170* (total length 710 amino acids), 79 and 127 different positions were found, respectively.

For functional characterization of the selected genes, we also searched for cis-regulatory elements of the promoter region of these genes using the Plant Care tool (Figure 7). The figure shows the number of elements found. The region –1000 bp before the start of transcription initiation was selected for the search.

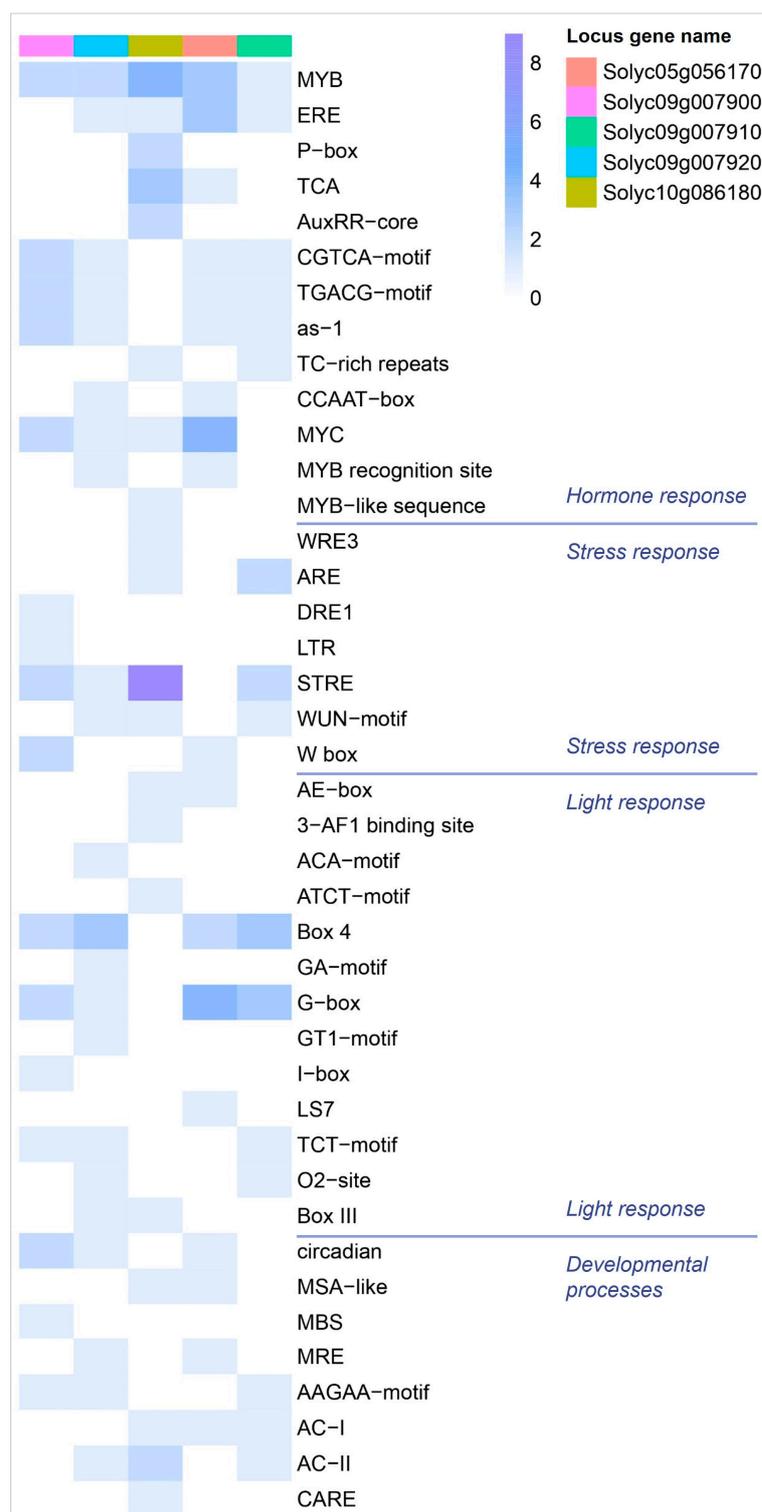


Figure 7. Frequency of occurrence of cis-regulatory elements of promoters in genes *Solyc05g056170*, *Solyc09g007900*, *Solyc09g007910*, *Solyc09g007920*, and *Solyc10g086180* in the -1000 bp region before the transcription start region. Cis-regulatory elements had been sorted according to the plant metabolism processes.

Despite the similarity in protein sequence, the selected genes showed high variability in cis-regulatory elements. For example, the AuxRR-core involved in the response to auxin [54] is present only in the promoter region of the *Solyc10g086180* gene. Additionally,

only this gene has the gibberellin-responsive element P-box [54]. The groups of elements involved in hormonal regulation by abscisic acid (MYB motifs [55]) and jasmonic acid (CGTCA-motif, TGACG-motif, as-1, and TCA [56]) are present in the promoter regions of all five genes. Additionally, important is the presence of STRE sites and WUN motifs [57], which also characterize PAL family genes as stress-inducible. Regarding the processes of plant response to different stresses, it can be noted that cis-regulatory elements associated with drought (DRE1) and low-temperature stress (LTR) [58] are present only in *Solyc09g007900*. Additionally, motifs associated with biotic stress (WRE and W-box) are absent in *Solyc09g007910* and *Solyc09g007920*.

PAL gene family analysis revealed varied gene activity influenced by the plant tissue, genotype (i.e., exhibiting variety specificity), and external stress factors triggering cis-regulatory element activation. In this study, we investigate the stress impact of chitosan hydrolysate on the roots of three tomato varieties. For this purpose, we selected five genes highly expressed in the roots of both tomato varieties, with data available in TomExpress. All five genes have distinct cis-regulatory element profiles, each regulated uniquely. Considering this, we decided to observe the cumulative expression activity of these genes.

3.4. *SIPAL* Gene Expression Analysis by RT-qPCR

We analyzed the overall expression level of selected PAL family genes in tomatoes by RT-qPCR. Here, we provide the *SIPAL* expression after incubation with chitosan hydrolysate for 30 min, 4 h, 24 h, and 48 h. We included the 30-min point to detect the minimum period of chitosan treatment for a tomato physiological reaction. Expression was analyzed by RT-qPCR in the roots of Lel, Red Cherry, and Titan cultivar seedlings (Figure 8).

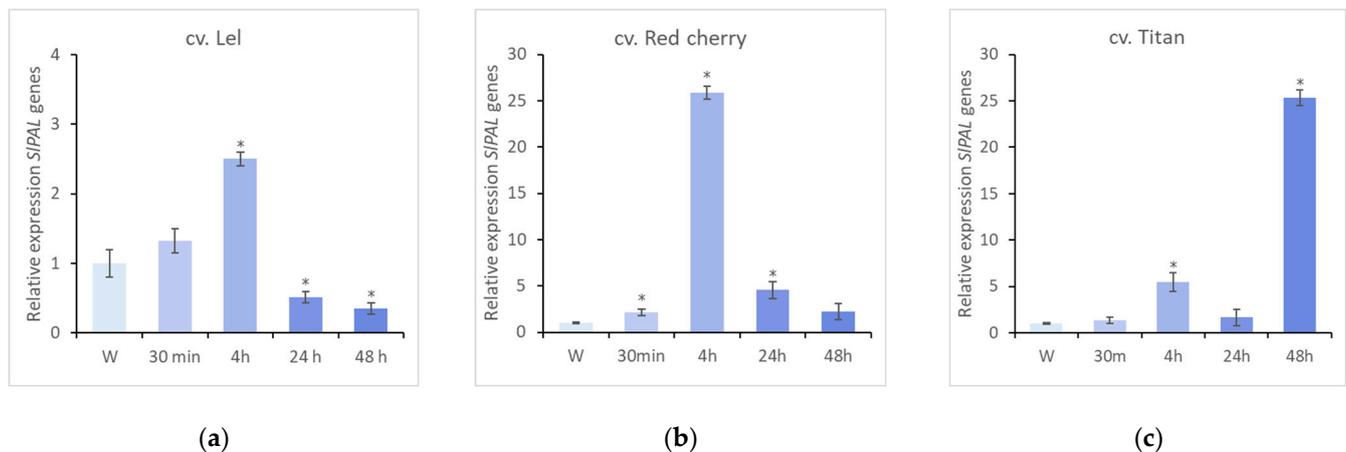


Figure 8. Relative expression of *SIPAL* in tomato roots after 30 min, 4 h, 24 h, and 48 h of treatment with 2 mg/mL chitosan hydrolysate and water (W), by RT-qPCR. Expression was analyzed for (a) Lel cultivar, (b) Red Cherry cultivar, and (c) Titan cultivar roots. Results are represented as mean values \pm SD ($n = 9$) for each variant. (*) indicates a significant difference compared to the control (W), $p < 0.01$.

In the roots of all tomato cultivars, the expression of the *SIPAL* gene was significantly increased at 4 h of chitosan hydrolysate treatment. In cv. Red Cherry, it was increased more than 25 times compared with the control variant. At 30 min after treatment, the expression of *SIPAL* was significantly increased only for cv. Red Cherry; it was decreased back to the control level at 48 h, whereas in cv. Lel, there was a steady and significant decrease in *SIPAL* expression, which at 24 h was more than two times lower than in control and more at 48 h. In cv. Titan, the expression of *SIPAL* was increased by 25 times compared with control at the 48-h point, though after 24 h, the expression level did not differ from control.

4. Discussion

Previous studies have shown that low-molecular-weight chitosan hydrolysate acts as a growth stimulant for tomatoes, increasing the length of seedlings that emerge from chitosan-soaked seeds [59]. We also conducted studies on lettuce (*Lactuca sativa*). Treatment of lettuce seeds with chitosan hydrolysate revealed better conditions for the seedlings compared to the control [60]. Similar results were reported in soybean (*Glycine max*) [39], cucumber (*Cucumis sativus*) [12], sunflower (*Helianthus annuus*) [41], and other plants [10,13,38,61–63].

Despite the extensive research supporting the positive effect of chitosan on plant growth, some studies have reported adverse results. Positive effects are frequently noted when priming plant seeds before germination, while negative results are observed in young plants. Notably, the response of young plant roots to chitosan presence differs [38,59,64,65].

For instance, the study [29] of the negative effect of chitosan (70 kDa, 85% deacetylation) on *Arabidopsis thaliana* root development. This effect could be linked to the repression of gene transcription involved in the quiescent center's functioning and stress hormone synthesis. The other study with *Arabidopsis thaliana* [64] shows that seedling growth slowed under the influence of a 0.1 mg/mL chitosan solution. Molecular weight, chemical structure, and chitin source are influential factors in processing results [42].

In our study, seedlings also showed a negative response to the presence of chitosan hydrolysate. Increasing the concentration amplified this effect further (Figure 1). The negative effect manifested itself in suppressed root development, affecting growth rate and length. Additionally, an impact on gravitropism was observed, which is the subject of further discussion.

It could be supposed that more mature plants might not present such pronounced reactions to the chitosan hydrolysate. However, to confirm that hypothesis, some additional research is required. In the study [65], leaf treatment in another context did not yield visible effects, underscoring the complexity of the interactions between different plant growth stages and chitosan.

In this research, we observed a change in the tropism of tomato seedlings under the influence of chitosan hydrolysate. Figure 2 showcases images of 4-day-old tomato seedlings that underwent a 4-hour chitosan hydrolysate treatment. There, one can clearly observe the change in the seedling root tropism in the variant with 2 mg/mL of chitosan hydrolysate. Likewise, treatment with chitosan also altered the architecture of *Cannabis sativa* roots [65] and decreased their growth rate, activating genes associated with pathogenesis-related (PR) in roots. However, this treatment had no effect on the development of aboveground biomass in these plants.

To understand deeper the effect of tomato root tropism induced by the chitosan hydrolysate, attention should be directed towards plant hormones. Firstly, the influence of chitosan on the activity of the auxin biosynthesis pathway may be reflected in the rate of plant root growth [66]. It is possible that the decrease in auxin concentration, together with the blocking of auxin translocation in the root tip, leads to the disruption of the root tip architecture. However, some reports indicate that the accumulation of auxins in roots increases after the application of chitosan hydrolysate [12,29]. Other hormones, such as abscisic acid and ethylene, also influence the tropism of plant roots. The presence of chitosan hydrolysate might affect the activity of these hormones [38].

It is possible that the presence of chitosan oligomer itself creates an artificial biotic stress for tomato seedling roots. Since chitosan is a derivative of chitin found in the cell walls of fungi, we can hypothesize that high concentrations of chitosan are perceived by the plant as a pathogen attack, triggering the activation of plant defense mechanisms. This includes alterations in hormonal homeostasis, leading to changes in PAL activity and the accumulation of phenolic compounds.

The accumulation of phenols in plant roots is also a frequently observed effect induced by chitosan. Treatment of *Hypericum perforatum* L. with chitosan resulted in the hypertrophy of the root tip [67]. Three days after chitosan treatment, the production of phenolic compounds increased in the plant's roots, and root development slowed down.

Browning of the root tip also indirectly indicates the accumulation of phenolic compounds, as mentioned by many researchers [29,39,64,68]. The reduction in plant growth rate upon application of the chitosan is further corroborated by our study on cv. Rad Cherry. However, analysis of the total phenolic content revealed a decrease in their level in the presence of chitosan hydrolysate in all three tomato cultivars compared to the control. (Figure 4). Negative effects on the accumulation of the secondary metabolites have also been observed in *Calendula officinalis* [69].

Phenylalanine ammonia-lyase (PAL) is a widely studied enzyme used as a marker for the physiological state of plants [31]. On one hand, this enzyme regulates the biosynthesis of numerous phenolic compounds, while on the other hand, it is involved in the synthesis of salicylic acid [37,70]. Despite the isochromatic pathway being considered the main biosynthesis pathway for salicylic acid in *Arabidopsis*, it has been shown that PAL significantly influences the amount of salicylic acid in soybean and potato (*Solanum tuberosum*) plants [32,71].

The researchers demonstrated that the biosynthesis of PAL increased under the influence of the chitosan oligomers in soybean. The total phenolic content and PAL activity in soybean leaves increased after chitosan treatment, and a correlation between enzyme activity and total phenolic content was observed. The highest PAL activity in leaves was observed after 36 h of treatment, decreasing at 48 h [42]. In addition, in hydroponic tomatoes, the presence of chitosan in the culture medium increased the total phenolic content and PAL activity in roots after 48 h [30]. We also observed a correlation between the accumulation of total phenolics and PAL activity (Figures 3 and 4) in all three cultivars. However, in our case, the values decreased both at the 24 h and 48 h marks. Perhaps in subsequent studies, it would be prudent to include several additional time points to gain a more comprehensive understanding.

The negative effect of low-molecular-weight chitosan hydrolysate is partially explained by the early stage of plant development and its high concentration. It is worth mentioning that the difference between the control and the 4 h point in the Cherry tomato samples is the highest. There are varietal differences in tomato plants, with Cherry varieties being highly susceptible to environmental stressors, while Titan and Lel are characterized as drought-tolerant and resistant to some tomato diseases.

The SolGenom database contains 16 characterized *Solanum lycopersicum* genes encoding PAL (Table 2). In previous studies, researchers were able to detect and characterize 14 PAL genes in the *Solanum lycopersicum* genome by aligning motifs of *Arabidopsis thaliana* PAL genes on the tomato genome. However, not all the genes in the tomato genome exhibit the same activity. Moreover, in response to stress, the expression of some PAL genes is activated while others are suppressed [72].

We had ranked the genes of the PAL family according to the level of expression in the roots of tomato cv. MicroTom and Heinz (1709). Additionally, we decided to pay attention to the five genes with the highest expression levels in roots. We also selected *Solyc09g007910* (*PAL5*) due to its high popularity in plant stress studies as well as the effects of chitosan on plants. The expression level of *PAL5* increased both when tomato plants were treated with chitosan [73] and when plants were damaged by insect pests [53].

Although the sequences of *Solyc09g007900* and *Solyc09g007920* are annotated as different genes, their aminoacidic sequences are almost identical. It would be logical to assume that proteins with the same amino acid sequences have the same functions. In this connection, we can conclude that we are observing two genes encoding the same protein. It is also obvious that these genes were annotated by nucleotide sequence, so they could be identified as different genes. Additionally, this difference is mainly displayed in the literature data because *Solyc09g007900* and *Solyc09g007920* are generally considered two different genes [72]. Apparently, the same amino acid sequence and the location of genes on the same chromosome may indicate the result of a recent gene duplication. We speculate that the genes of the PAL family could be duplicated due to the high demand of the organism for the proteins encoded by these genes.

The study of cis-regulatory elements of the promoter region of selected genes revealed their high variability. It is worth noting the great diversity of elements involved in hormonal regulation and plant stress response [55]. When comparing *Solyc09g007900* and *Solyc09g007920*, significant differences in both the composition of regulatory elements and the number of detected motifs are noticed. In general, it can be assumed that transcription activation of selected genes occurs with different intensities depending on the type of impact on the plant.

To fully analyze the effect of chitosan on tomato seedlings, gene expression in tomato root samples was analyzed by qRT-PCR. The primers were chosen to anneal the transcripts of all five selected genes. Additional information about *SIPAL* primers is available in the Supplementary (Figures S1 and S2). Of course, this cannot reflect the total transcription activity of all PAL family genes. However, the in silico data revealed five genes that may contribute most of the total *PAL* activity in the plant root. Thus, it may be assumed that the summary activity of these five genes may be used to characterize the overall effect of chitosan hydrolysate on plant development. In our study, the *SIPAL* expression pattern showed dependence on tomato cultivar (Figure 8), including its response to chitosan treatment. The correlation between gene and enzyme activity was observed only in cv. Lel, while in the other two cultivars, the *PAL* activity did not correspond to the expression level of the analyzed *SIPAL*s. Based on the results obtained, we can assume that low-molecular-weight chitosan may contribute to the regulation of *PAL* activity in the roots. The absence of a correlation between *PAL* activity and the expression of five analyzed genes may indicate the involvement of other known *SIPAL* genes, which normally do not express highly in the roots. Thus, the response of each of the *PAL* family genes to chitosan treatment will be one of the aims of our future research.

5. Conclusions

Our research confirms the influence of chitosan on plant development. Prolonged exposure to a high concentration of low-molecular-weight chitosan has a negative impact on the further growth of tomato seedlings, contributing to a decrease in *PAL* activity and the content of phenolic compounds in roots. This effect can be applied to studying plant stress responses triggered by chitosan treatment. A chitosan concentration of 0.2 mg/mL, at which plant development parameters do not change in comparison with an untreated plant (this study), can at the same time be effective in suppressing the growth of fungal pathogens (according to the previous study [7]) and therefore may be optimal for chitosan pretreatment.

The tomato genome contains 16 genes encoding *PAL* isoenzymes, but not all of them are equally active. In this study, we did not observe the dependence of *PAL* enzyme activity on the cultivar in response to chitosan treatment, but the *SIPAL* gene expression changed in a genotype-specific pattern.

The results obtained in this study provide the foundation for further, more extensive transcriptional profiling (including in response to chitosan) and functional analysis of the *SIPAL* gene family and could contribute to the breeding of stress-resistant tomato cultivars.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy14020386/s1>. **Table S1.** The experimental scheme for studying low-molecular-weight chitosan hydrolysate on cv. Cherry tomato seedlings. **Figure S1.** cDNA sequence alignment of the *Solyc10g086180*, *Solyc05g056170*, *Solyc09g007900*, *Solyc09g007910*, *Solyc09g007920* genes and *SIPAL* primers. **Figure S2.** Schematic representation of primer alignment on exons of selected genes of *PAL* family.

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