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# Cellulose synthase gene expression profile and physiological responses of tomato cultivars exposed to virus and salt stresses

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**Summary.** Plants are exposed to adverse growth conditions, and have developed mechanisms to adapt and survive under abiotic and biotic stresses. The plant's response to the combined effects of biotic and abiotic stress represents a highly complex phenomenon, involving intricate interactions between the host plant and associated pathogens, further modulated by the intensity, duration, and type of environmental stressors. Tomato production can be severely affected by tomato yellow leaf curl virus (TYLCV) and tomato chlorosis virus (ToCV), and salt stress inhibits tomato crop productivity, although molecular regulation controlling tomato resistance to salt stress remains unclear. The cellulose synthase (*Ces*) and cellulose synthase-like (*Csl*) gene families control biosynthesis of cellulose and hemicellulose in plant cell walls, and *Ces/Csl* genes are also involved in resistance against abiotic and biotic stresses, including those from viruses and salt. To gain understanding of the molecular basis of combined viruses (TYLCV/ToCV) and salt stresses on the tomato cultivars Money Maker and Yegana, comparative analyses of four cellulose synthase genes (*CesA/Csl*) were carried out using Quantitative Reverse Transcription Polymerase Chain Reaction (*RT -qPCR*). Tomato physiological parameters, including relative water content, specific leaf weight, leaf area, and dry biomass, were also assessed. *CesA/Csl* genes (*Ces-A2, Csl-D3,2, Csl-D3,1, Csl-H1*) were up-regulated in virus-infected plants. These genes, associated with the biosynthesis of *CesA/Csl* genes are probably pivotal in defense mechanisms against TYLCV/ToCV. Relative water content in plants subjected to combined ToCV and salt stresses were similar to those observed in non-inoculated controls. Congruence between the outcomes of these analyses and physiological studies indicates that the Yegana tomato cultivar may be as sensitive to these stresses as the Money Maker cultivar. This research emphasizes the importance of up-regulating specific genes, namely *Csl-D3,1*, *Csl-D3,2*, and *Ces-A2*, to confer host resistance to the complex effects of salt and virus stresses. This study will facilitate development of stress-resistant tomato plants, and contribute to elucidating the molecular mechanisms of *CesA/Csl* genes in abiotic and biotic stress situations.

**Keywords.** *Solanum lycopersicum* L., tomato yellow leaf curl virus, tomato chlorosis virus, cellulose synthase genes, RT-qPCR.

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## INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown food crops, and is consumed as fresh or processed food products (Li *et al*., 2021; Roșca *et al*., 2023). World tomato production in 2021 was 189.1 million metric tons (FAOSTAT, 2022), and diseases caused by viruses cause reductions in crop yields and impair fruit quality, causing important economic losses (Jones and Naidu, 2019).

Tomato yellow leaf curl virus (TYLCV) and tomato chlorosis virus (ToCV) are pathogens that can cause serious losses in tomato production (Chinnaraja *et al*., 2016; Jin *et al*., 2020) which continue to spread throughout many countries (Abd El Rahman *et al*., 2024).

TYLCV (*Begomovirus*, *Geminiviridae*) affects more than 20 tomato cultivars, leading to severe productivity reducing host symptoms including yellowing, curling size reduction of leaves, stunted plant growth and early flower shedding shedding (Moriones and Navas-Castillo, 2000; Huang *et al*., 2016; Desbiez *et al*., 2018; Verdin *et al*., 2018). TYLCV ranks third among viruses reducing tomato production (Ong *et al*., 2020), and most tomato cultivars are very susceptible to this virus (Mugiira *et al*., 2011). ToCV (*Crinivirus*, *Closteroviridae*) has also emerged as an important pathogen, now recorded in 35 countries. Besides tomatoes, ToCV can infect other economically important vegetable crop plantss and many wild hosts (Fiallo-Olivé and Jesús Navas-Castillo, 2019; Louro *et al*., 2000; Elsharkawy *et al*., 2022). The first symptoms of ToCV infections are formation of interveinal chloroses on lower leaves, which then progress to upper leaves of infected plants (Kwon *et al*., 2024). Despite substantial impacts of ToCV on crop yields, resistant or tolerant tomato germplasms have not been well-documented, and the genetic basis of resistance to ToCV remains poorly understood (Gao *et al*., 2024).

The early detection of TYLCV and ToCV is critical for their effective management. Several approaches have been developed to control these two pathogens, including physical barriers and the applications of insecticide chemicals. Genetic engineering strategies have also been investigated (Tabein, 2021), with the most effective strategy for managing TYLCV and ToCV involving transfer of virus resistance genes from wild *Solanum* species into susceptible tomato cultivars.

Among abiotic stressors, high soil salinity is an increasing concern, with more than a third of irrigated areas already affected, and estimates suggesting that by 2050, more than half of the world's cropland will be affected by high salinity (FAO, 2011; Zelm *et al*., 2020; Zhao *et al*., 2021). Besides affecting morphological and physiological status of crop plants, many studies have shown that high salt concentrations cause biochemical and molecular imbalances, resulting in low plant productivity (Kusvuran *et al*., 2016). In general, salinity stress determines changes in gene expression in tomato plants, but information on these effects is still limited, and most investigations have been on changes in genes associated with transcription factors (Devkar *et al*., 2020), and studies on effects of salinity on tomato gene expression have been conducted on particular cultivars (Roșca *et al*., 2023). These results suggested changes in the expressions of genes involved in transport activity, cell wall construction, secondary metabolites, and protein synthesis. Most tomato cultivars are known to have the genetic potential to tolerate mild to moderate salt stress (Ibrahim, 2018; Alam *et al*., 2021; Guo *et al*., 2022), and knowledge of salinity effects on tomato plants is an asset in selection of appropriate crop practices to fulfill demands of tomato markets (Roșca *et al*., 2023).

The cellulose synthase (*Ces*) and cellulose synthase-like (*Csl*) gene families within the *Ces* gene superfamily are central to the biosynthesis of cellulose and hemicellulose in plant cell walls (Cao *et al*., 2019). *CesA* and *Csl* genes are key regulators in the synthesis of plant cell wall polysaccharides, which are essential for plant adaptation to abiotic stresses (Wang *et al*., 2022). Recent research has indicated that salt stress negatively affects cell wall synthesis, including *Ces* complexes (*CSCs*), *CesA*, and *Csl* genes (Maksup *et al*., 2020), leading to alterations in the expression patterns of related genes (Shafi *et al.,* 2019). In TYLCV-infected tomato plants, however, the availability of *Csl* genes to strengthen host immune systems and maintain crop productivity has been reported (Huang *et al*., 2022). Nevertheless, the *CesA/Csl* family genes have not been fully characterized in *Solanaceae* species, particularly tomatoes. Song *et al*. (2019) identified a total of 38 *CesA/Csl* protein-encoding genes in tomatoes, and characterized these based on phylogenetic, gene structure, chromosome distribution, and localization, and then deduced protein sequences.

Given the growing concern about global climate variability, there is urgent need to expand knowledge of the interactions of combined biotic and abiotic stresses in plants. Plants are exposed to many biotic and abiotic stressors throughout their life cycles, and these factors activate physiological and molecular defense mechanisms that provide viability withstand these stressors (Zhang and Sonnewald, 2017). The primary objective of the present study was to analyze expression profiles of four *cellulose synthase* (*CesA*) and *cellulose synthase-like* (*Csl*) genes in the virus sensitive tomato cultivar Money Maker (MK, UK) and virus-susceptible/unknown culti-

var Yegana (YG, AZ), which were exposed to combined salt and TYLCV and ToCV stresses, using Quantitative Reverse Transcription Polymerase Chain Reaction (*RT-qPCR*) assessments. To increase understanding of the defense responses of tomato plants to salinity stress during TYLCV and ToCV infections, effects on four key physiological parameters (relative water content, specific leaf weight, leaf area, dry biomass) were also assessed.

## MATERIALS AND METHODS

### *Plant material and single-leaflet grafting of TYLCV and ToCV*

Seeds of the virus-susceptible cultivar Money Maker (MK, UK, Milc *et al.,* 2019) were sourced from the Institute of Biotechnology at Ankara University, Türkiye, while seeds of the virus/salt tolerant or sensitive cultivar Yegana (YG, Azerbaijan) were obtained from the seed bank of the Research Institute of Crop Husbandry, Ministry of Agriculture of the Azerbaijan Republic. Tomato seeds were germinated in plastic vials containing a mixture of peat (90%), perlite (10%), and vermiculite (70%). Germination took place in an insect-free growth chamber maintained at a 26°C 16 h light and 20°C 8 h dark cycle, and relative humidity ranging between 60–70% (Çevik *et al*., 2019). The plants were watered daily at the same time each day according to the moisture condition of the plant gowth medium.

Each experiment was conducted with three technical and biological replicates. Tomato leaf samples which were infected with isolates of TYLCV (GenBank accession number MK238543) or ToCV (MK248741) (Fidan and Sarıkaya, 2020), and were showing characteristic symptoms, were collected from greenhouses in the Kumluca region of Antalya (Türkiye) in June 2022. Presence of these viruses in the tomato leaf samples was confirmed using the polymerase chain reaction (PCR) method with specific primers (BC-36 and BC-37/ BC-40 and BC-41 for nested PCR; AV632, AC950, and AC1048 for duplex PCR) (Martinéz-Culebras *et al*., 2001; Dovas *et al*., 2007), and were subsequently utilized as inoculation material for single-leaflet grafting (ToCV by nested PCR and TYLCV by duplex PCR). Single-leaflet grafting involved small incisions on the stem of each recipient seedling by removing the first leaf from the node. Leaflets obtained from diseased plant samples containing TYLCV and ToCV were used as inocula for grafting, and also as positive inoculation controls for further grafting confirmation reactions, as described by Lee *et al.* (2017). The grafted leaflets were misted with steril distiled water multiple times each day to prevent wilting. After 21 d the success grafting transmission rates were 97.6% for TYLCV and 89.3% for ToCV (Çevik *et al*., 2019). Grafting leaflets collected from virus-free tomato plants were included as experimental controls. Four weeks after inoculation (4 w.p.i.), grafting transmission was confirmed through molecular detection of TYLCV using a duplex PCR assay (Martinéz-Culebras *et al*., 2001), and of ToCV using nested PCR reactions (Dovas *et al*., 2007).

**Table 1.** Tomato cultivars and treatment abbreviations.

| No. | Cultivar | Treatment | Abbreviation |
|---|---|---|---|
| 1 | Money Maker (MK) | TYLCV, Salt | MK-TYLCV-S |
| 2 | Money Maker (MK) | ToCV, Salt | MK-ToCV-S |
| 3 | Money Maker (MK) | Control | MK-C |
| 4 | Yegana (YG) | TYLCV, Salt | YG-TYLCV-S |
| 5 | Yegana (YG) | ToCV, Salt | YG- ToCV-S |
| 6 | Yegana (YG) | Control | YG-C |

The plants were subsequently, divided into three groups: one group remained as the experimental controls (non-inoculated, healthy); the second group consisted only of virus-infected plants (MK-ToCV-1st d or MK-TYLCV-1st d, YG-ToCV-1st d or YG-TYLCV-1st d; d = day); the third group was infected with the viruses and was also subjected to salt stress (MK-ToCV-S-21std or MK-TYLCV-S-21std, YG-ToCV-S-21std or YG-TYLCV-S-21std) (Table 1). Each biological replicate consisted of a group of 15 tomato seedlings per treatment. For molecular analyses (including RT-qPCR), leaf tissue samples were collected from these groups of plants at 4 w.p.i. Five to six severely infected leaves were harvested from multiple individual seedlings within each 15-plant group, and were pooled to constitute a single biological replicate. After virus inoculations, at 4 w.p.i., the tomato plants were exposed to salt stress.

### *TYLCV and ToCV grafting confirmation with duplex and nested PCR*

*Detection of ToCV.* For confirmation of ToCV, two -step PCR assays were carried out, including the reverse transcription polymerase chain reaction (RT-PCR) and nested PCR. The first step of the RT-PCR was conducted using BC-36 and BC-37 primer pairs. Additionally, a primer designed for *heat shock protein 70 (HSP70*), the highly conserved gene region, was used for PCR amplification. For nested PCR, specific primer pairs designed by Dovas *et al*. (2007) (BC-40 and BC-41) were utilized (Table 2).

Total RNA was extracted from 30 to 50 mg of fresh leaf tissue using the Tri-Reagent solution (Biorad). RNA

**Table 2.** BC-36, BC-37/BC-40 and BC-41 primers used in nested PCRs, and AV632, AC950, and AC1048 primers used in duplex PCR reactions (Dovas *et al*., 2007; Aboul-Maaty and Oraby, 2019)

| ToCV | | | | |
|---|---|---|---|---|
| Type of PCR | Primer | Sequence (5'.....3') | Product size (bp) | Reference |
| First Step Nested PCR | BC 36-F | 5'GG(G/T)TT(A/G)GA(G/T)TT(C/T)GGTACTAC-3' | 587 | Dovas *et al*., 2007 |
| | BC 37-R | 5'-CC(G/T) CCACCAAA(A/G)TCGTA-3' | | |
| Second Step Nested PCR | BC 40-F | 5'-GG TTTGGATTTTGGTACTACTAGT-3' | 463 | Dovas *et al*., 2007 |
| | BC41-R | 5'- AAACTGCCTGCATAAAGTCT C- 3' | | |
| TYLCV | | | | |
| Type of PCR | Primer | Sequence (5'.....3') | Product size (bp) | Reference |
| Duplex PCR | AV632-F | 5′-CCG GTG TTG TGC GTT GTG TTA G-3′ | 462 | Aboul-Maaty and Oraby, 2019 |
| | AC950-F | 5′-TGA AGG AGC AGT GTY TGY TG-3′ | | |
| | AC1048-R | 5′- GGA TTA GAG GCA TGC GTA CAT-3′ | 135 | |

quality and amounts were measured using 1% agarose gel electrophoresis and a spectrophotometer (ND-1000, NanoDrop Technologies). Isolated RNA samples were stored at −80°C until the nested PCR step. In nested PCR reactions, ToCV-infected (GenBank accession number: MK248741) (Fidan and Sarıkaya, 2020) samples from the Kumluca region of Antalya (Türkiye) were used as positive controls. The PCR reactions were each conducted with three technical replicates, and sterile distilled water was used as the negative control. PCR products were separated on a 1% agarose gel containing ethidium bromide (0.5 μg $mL^{-1}$) alongside a 100-bp (basepair) DNA ladder (Invitrogen), and were visualized under an ultraviolet light using a gel documentation system (Uvitek).

*Detection of TYLCV.* Typical symptoms of TYLCV infections in the plants appeared 10 to 14 d post-infection (Çevik *et al*., 2019), and grafting transmission was confirmed 4 w.p.i. using specific primers in a duplex PCR. The duplex PCR was carried out in accordance to the method described by Martinéz-Culebras *et al.* (2001).

Primers AV632, AC950, and AC1048 were used for detection of TYLCV (Table 1) (Brown *et al*., 1996; Martinéz-Culebras *et al*., 2001). Total DNA was extracted from 50 mg of fresh leaf tissue using CTAB solution (Aboul-Maaty and Oraby, 2019). DNA quality and concentration were assessed utilizing 1% agarose gel electrophoresis and a spectrophotometer (NanoDrop ND-1000). Isolated DNA samples were stored at −80°C until the duplex PCR step.

In duplex PCR reactions, TYLCV-infected samples (GenBank accession number MK238543) (Fidan and Sarıkaya, 2020) from the Kumluca region of Antalya (Türkiye) were used instead of DNA as a positive control, while sterile distiled water was used as a reaction negative control. The PCR reactions were each carried out with three technical replicates.

PCR products were controlled within 1% agarose gel electrophoresis with a 100 bp DNA ladder (Invitrogen), and were visualized under an ultraviolet light using a gel documentation imaging device (Uvitek).

*Salt stress treatments*

Salt effects on tomato cultivars were examined using the method developed by Gharsallah *et al*., (2016). Electrical conductivity (EC) of NaCl solutions was measured with a conductivity meter (Thermo Corporation). Salt stress was applied for 21 d, with the salt treatment initiated on day one with 50 mM of NaCl solution (6 dS $m^{-1}$), then later increased to 100 mM (12 dS $m^{-1}$) on day 2, and then to 150 mM (15 dS $m^{-1}$) on day three. Three biological replicates were used for each of the two tomato cultivars, and each replicate comprised 15 plants. Control plants of each cultivar were grown under identical conditions (non-inoculated, healthy), and were irrigated with Hoagland 's nutrient solution (Hoagland and Arnon 1950) at the same time each day, as for the salt-stressed tomato cultivars.

*Primer design and RT-qPCR analyses*

The National Center for Biotechnology Information (NCBL) Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast) was used to design the *CesA/Csl* primers and designed gene-specific primers that were used to produce a PCR product of approx. 200–300 bp in tomato cultivars. The designed primer information

(gene name, gene ID, NCBI reference sequence and primer sequence) is outlined in Table 3.

Three biological replications were used for RNA extraction. Initially, five to six severely infected leaves from salt-stressed tomato cultivars were collected for each biological replication and were frozen in liquid nitrogen. The sample tissues were then homogenized in sterilized porcelain mortars and a Tri-Reagent solution (Biorad) protocol was used. Concentrations of RNA were measured using a NanoDrop spectrophotometer and 1% agarose gel electrophoresis. The RT-qPCR reactions were carried out in a Light Cycler 480 (Roche), for the genes *Ces-A2, Csl-D3,2, Csl-D3,1,* and *Csl-H1,* with three biological and three technical replicates.

The RT-qPCR method was carried out in two steps. In the first, cDNAs were synthesized using the first-strand cDNA synthesis kit (Roche, Cat no: 04897030001), following the manufacturer's protocol. Standard curves (with respective efficiency and slope values close to 2.2 and –3.2) for each primer were generated using six serial dilutions (ranging from 1/10 to 1/100,000) of a control (non-inoculated, healthy) cDNA. cDNA samples were then used as templates to quantify target gene expression levels. Non-inoculated, healthy samples were used as a control group in the reactions.

In the second step, the RT-qPCR reactions were each carried out in a 12 µL mixture containing 1.8 to 2.0 µL of forward and reverse primer (10 pmol), 3 µL cDNA (500 ng $\mu L^{-1}$), 5 µL LightCycler® 480 SYBR Green I Master (Roche) and $ddH_2O$. The amplification reaction commenced with an initial denaturation at 95°C for 10 min. This was followed by denaturation (10 s at 95°C), annealing (1 min at 52-58°C, according to the optimized annealing temperature (Tm) of the primer), and elongation (1 min at 72°C) steps conducted through 45 cycles. The specificity of amplification was examined through a melting curve analysis after the last cycle. For each gene, cycle threshold (Ct) values were obtained for infected-salt stressed and control samples.

Gene expression values were normalized to the expression of *Solanum lycopersicum* housekeeping gene actin-7-like (Gene ID: LOC101262163; Klay *et al*., 2014) in all samples. Relative expression levels were calculated using the REST 2009 software program, according to the delta delta-Ct ($2^{-\Delta\Delta CT}$) algorithm as described by Livak and Schmittgen (2001).

**Table 3.** Primer information for the RT-qPCR.

| No | Gene ID | Gene name/gene name abbreviation in this study | NCBI Reference Sequence | Forward Primer Sequence (5'...3') | Reverse Primer Sequence (5'...3') |
|---|---|---|---|---|---|
| 1 | Solyc07g051820 | XM_004243439.4, **Csl-H1** | *Solanum lycopersicum cellulose synthase-like* protein H1(LOC101259456), mRNA | ACCACCGTATACCGACTCCA | TCGATGCACCGTCGTCTGAG |
| 2 | Solyc06g097050 | XM_00423523.4, **Csl-D3,1** | *Solanum lycopersicum cellulose synthase-like* protein D3 (LOC101247596), mRNA | TGCGACGAGGTGATTCAGAC | GAGGCCGTCCATTCTTCACA |
| 3 | Solyc08g076320 | XM_004245868.4, **Csl-D3,2** | *Solanum lycopersicum cellulose synthase-like* protein D3 (LOC101249747), transcript variant X1, mRNA | ACAACTCCGAGGCAATCAAG | CGGAAGAGACAACCAGTCCC |
| 4 | Solyc12g056580 | XM_004252522.4, **Ces-A2** | *Solanum lycopersicum cellulose synthase* A catalytic subunit 2 [UDP-forming] (LOC101260024), mRNA | ATGGATCCTGCTGCCCTTGG | TGGGGCGAGGAGGAAAAGAC |
| 5 | *Solyc11g005330* | Q96483m **Actin** | *Solanum lycopersicum actin-7-like* gene (ID: LOC101262163 | TGTCCCTATTTACGAGGGTTATGC | CAGTTAAATCACGACCAGCAAGAT |

*Measurements of plant physiological parameters*

Physiological measurements were made for three groups of plants: virus (TYLCV and ToCV) infected plants, salt stressed and virus (TYLCV-S and ToCV-S)

infected plants, and healthy plants (controls). All physiological measurements were carried out on three replicates, including both biological and technical replicates. Relative water contents (RWC) of virus-infected and healthy tomato samples were determined using the method outlined by Tambussi *et al.* (2005).

To measure dry biomass (DB) of tomato leaves, samples of uniform size were collected from infected and healthy (non-inoculated) leaf samples. The samples were then weighed on an electronic balance, using the method described by Grünzweig *et al.* (1999).

Leaf area (LA) measurements were carried out for diseased and non-inoculated samples. These were determined from each leaf length and diameter (widest part), as described by Grünzweig *et al.* (1999).

Leaf water deficits were assessed through a series of steps. Initially, leaf primary weights were recorded. The leaves were then soaked in water for 1 h, and leaf wet weights were measured. The change in weight before and after soaking provided measures of water deficit in the leaves, indicating each plant's ability to retain water.

For all physiological measurements, statistical analyses were carried out to assess the differences between the experimental controls and treatment groups, using a two-way analyses of variance (ANOVA), with mean separation at $P \leq 0.005$.

## RESULTS

### *ToCV and TYLCV detection in virus inoculated plants*

*ToCV detection.* From the first step of nested PCR, a 587 bp band was amplified in all samples, and a 463 bp band of the *HSP70* gene was also amplified as a result of nested PCR in all samples and the positive controls (Figure 1, a and b).

*TYLCV detection.* In agarose gel electrophoresis, 462 bp bands were observed in all tomato samples. However, no band was observed in the healthy (non-inoculated) samples and negative controls. Tomato samples from Kumluca (Türkiye) used as positive controls also exhibited bands of 462 bp (Figure 1, c).

### *RT-qPCR analyses*

These results indicated different patterns of increases or decreases in gene expression under salt stress conditions. The expression profiles of the four assessed *CesA/Csl* genes were all low. Greatest statistically significant ($P \leq 0.05$) up-regulation (64 fold change) was

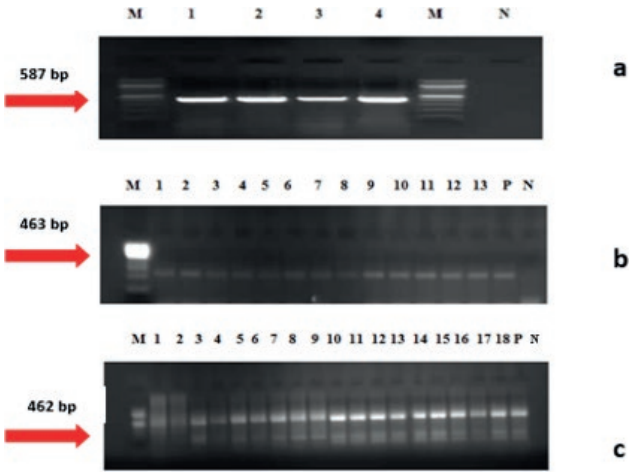


**Figure 1.** Molecular detection of tomato chlorosis virus and tomato yellow leaf curl virus. 1 to 4, ToCV infected MK tomato cultivar (first step of nested PCR). b, 1 to 8, ToCV infected YG cultivar, 9 to 13, ToCV infected MK cultivar (second step of nested PCR). c, 1 to 9 TYLCV, infected YG cultivars, 10 to 18, TYLCV infected MK cultivar (duplex PCR). In each gel, M is the 100 bp DNA ladder (Biolabs), N is the negative control of the PCR reaction, and P is the positive control.

detected for the MK-ToCV-S-21st d cultivar in the *Csl-D3,1* gene, and the greatest down-regulation (-23 fold change) was detected for the MK-ToCV-1st d cultivar in the *Ces-A2* gene.

For the *Csl-H1* gene, a comparative analysis between the 1st and 21st days showed no statistically significant changes in gene expression among the tomato cultivars. However, the YG-ToCV-21st d cultivar exhibited down-regulation at all time points, except for the 1st day of gene expression, where a 2-fold change was observed (Figure 2).

For the *Ces-A2* gene, the YG-TYLCV-1st d cultivar had down-regulation (-3.67 fold change) on the 1st day, and up-regulation (3-fold change) on the 21st day. Aside from this result, there were no statistically significant gene expression differences between the different time points for the *Ces-A2* gene. Similar to the *Csl-H1* gene, the greatest down-regulation (-23 fold change) in the *Ces-A2* gene occurred at the 1st day, specifically in the MK-ToCV-1st d cultivar (Figure 2).

The *Csl-D3,1* gene exhibited noteworthy expression variations between the 1st and 21st days in both tomato cultivars. This gene was down-regulated in both cultivars on the 1st day, but was up-regulated on the 21st day. The MK-ToCV-1st d cultivar was down-regulated (-2 fold change) on the 1st day, and was strongly up-regulated (64 fold change on the 21st day. This could have been linked to heightened sensitivity of the MK tomato cul-

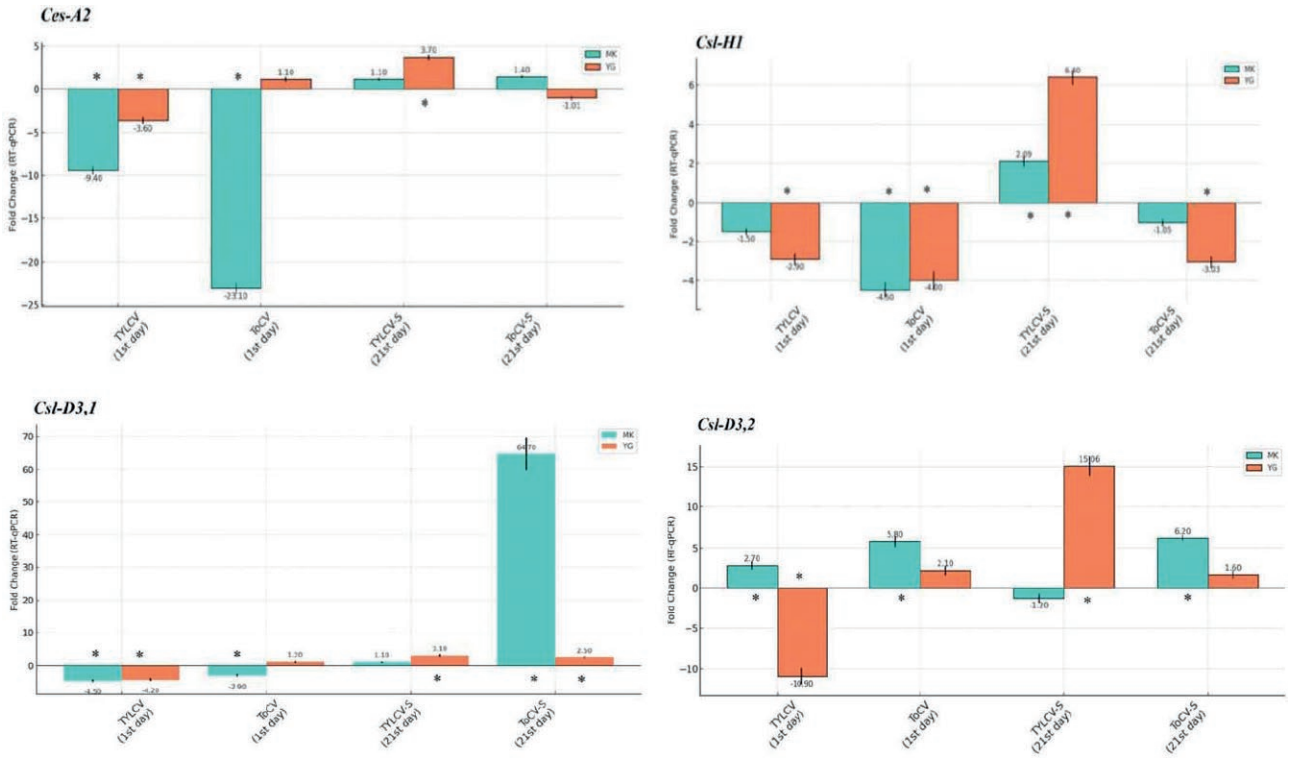


**Figure 2.** Mean relative gene expression (fold changes) of *CesA/Csl* genes (t-test; $P \leq 0.05$). Bars indicate standard errors of means. Asterisks indicate significant differences (t-tests; $P \leq 0.05$) in gene expressions.

tivar on the 21st day compared to the 1st day, implying that the *Csl-D3,1* gene was an indicator for sensitivity in this cultivar.

For the *Csl-D3,2* gene, the YG-TYLCV-1st d cultivar was down-regulated (-10 fold change) on the 1st day, and up-regulated (15 fold) on the 21st day (YG-TYLCV-S-21st d). This increased gene expression was greatest in the YG cultivar exhibiting on the 21st day in comparison to the 1st day. In contrast, observed other gene expression changes (*Csl-H1* and *Ces-A2*) did not show clear correlations with the sensitive/tolerant differentiation that was observed in both the MK-ToCV-S-21st d or MK-TYLCV-S-21st d, and the YG-ToCV-S-21st d or YG-TYLCV-S-21st d cultivars (Figure 2).

*Physiological measurements*

In the MK and YG cultivars, reductions in RWC were observed in leaves infected with TYLCV and ToCV compared to non-inoculated (healthy) samples. Although there were slight increases in the salt/virus samples compared to non-inoculated ones, these were slight. Specifically, the mean RWC was 74% in the non-inoculated group (MK-C), 70% in MK -TYLCV-S-21st d plants, and 65% in the MK -TYLCV-S-1st d plants. In the MK cultivar infected with ToCV, RWC were 79% in MK-C plants, 72% in MK -ToCV-S-21st d group plants, and 61% in MK -ToCV-S-1st d group plants. Similar results were also recorded in the YG cultivar. In that case, RWC was 59% in the YG-C variant, 53% in YG -TYLCV-S-21st d group plants, and 44% in YG-TYLCV-S-1st d. Compared to YG-C (63%), YG -ToCV-S-21st d exhibited 59%, and YG -ToCV-1st d plants showed 47% RWC (Figure 3).

When the DB percentages were evaluated, they increased by approx. 1.3-fold (20%) in the MK -TYLCV-S-1st d cultivar compared to the MK-C plants, and by approx. 1.6-fold (25%) in MK -TYLCV-S-21st d. In MK -ToCV-S-1st d, this indicator exhibited an approx. 1.2-fold increase (25%), and in the MK-ToCV-S-21st d group an approx. 1.5-fold increase (31%). DB percentage was also assessed in the Yegana cultivar, where YG-TYLCV-S-21st d samples showed an approx. 1.1-fold increase (19%) compared to YG-C samples, while an approx. 1.4-fold increase (26%) was observed only in YG-TYLCV-1st d plants (Figure 4).

In the water deficit analyses, an increase was observed both in the virus-infected and dual stressed plants, compared to non-inoculated plants. The water

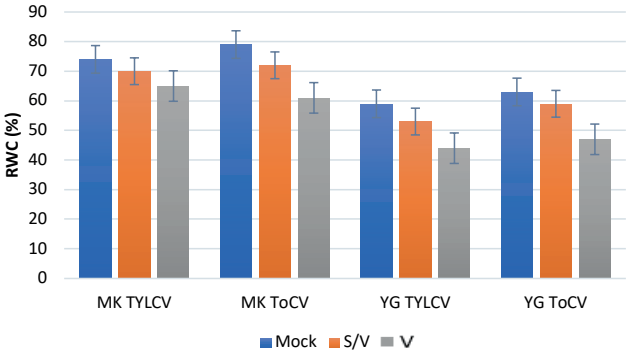


**Figure 3.** Mean relative water content (%) in MK and YG tomato cultivars exposed to combined virus (TYLCV and ToCV) and salt stress: Non-inoculated, ("Mock") controls, S/V = salt plus virus treatment, V = virus only (TYLCV or ToCV) treatment. Bars indicate standard errors of means.

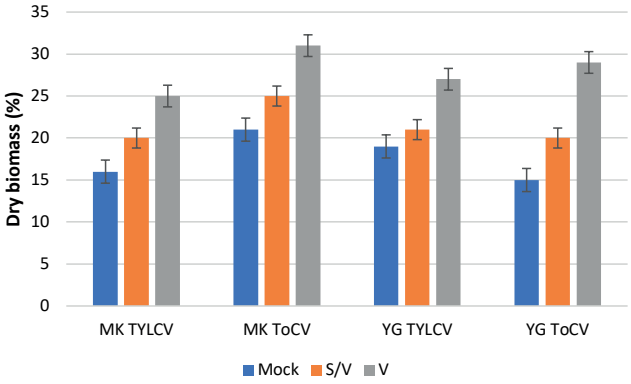


**Figure 4.** Mean dry biomass (%) of MK and YG tomato cultivars exposed to combined virus (TYLCV and ToCV) and salt stress. Non-inoculated ("Mock") healthy controls, S/V = salt plus virus treatment, V = virus only (TYLCV or ToCV) treatment. Bars indicate standard errors of means.

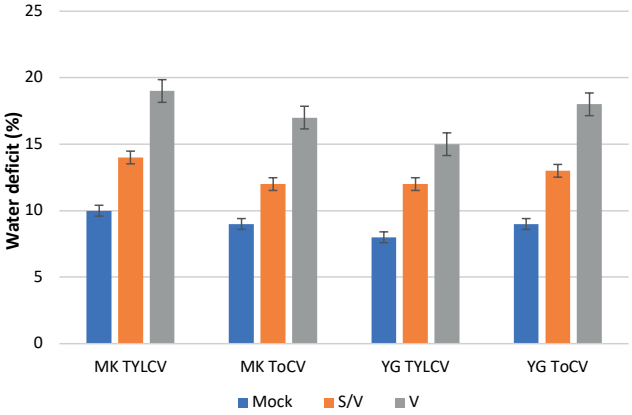


**Figure 5.** Mean water deficit (%) in MK and YG tomato cultivars exposed to combined virus (TYLCV or ToCV) and salt stress. Non-inoculated ("Mock") healthy controls, S/V = salt plus virus treatment, V = virus only (TYLCV or ToCV) treatment. Bars indicate standard errors of means.

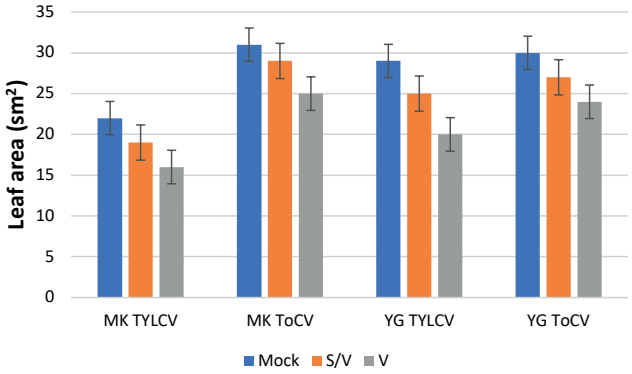


**Figure 6.** Mean leaf area (sm2) of MK and YG tomato cultivars exposed to virus (TYLCV or ToCV) and salt stress. Non-inoculated "Mock" healthy controls, S/V =salt plus virus treatment, V = virus only (TYLCV or ToCV) treatment. Bars indicate standard errors of means.

deficit percentage was 10% in the MK-C cultivar, 14% in MK-TYLCV-S-21st d plants, and 19% in MK-ToCV-S-1st d samples. Water deficit was 15% in MK -ToCV-S-1st d samples, and 12% in MK-ToCV-S-21st plants. The water deficit in YG-TYLCV-1st d plants was 12% and, and 15% in YG-TYLCV-S-21st d samples. As well, in YG -ToCV-1st d and YG-ToCV-S-21st d samples, these parameters were, respectively, 13% and 18%. Accordingly, the equivalent control plants gave, respectively, 8% and 9% water deficits (Figure 5).

For both cultivars, LA decreases were observed in samples in comparison to the non-inoculated control. These reductions were particularly noticeable in the combined stress group of plants compared to the healthy plants. Specifically, in the MK cultivar, LA was 22 $cm^2$ and 31 $cm^2$ in non-inoculated plant leaves. This parameter decreased to 19 $cm^2$ for MK-TYLCV-S-21st d, and 29 $cm^2$ for the MK-ToCV-S-21st d plants, and was 16 $cm^2$ in MK-TYLCV-S-1st d plants and 25 $cm^2$ in the MK-ToCV-S-1st d plants. During the experiments, the LAs of the YG cultivar in different groups of plants changed as follows: 29 $cm^2$ and 30 $cm^2$ in control plant leaves, 20 $cm^2$ YG-TYLCV-1st d plants and 24 $cm^2$ in the YG-ToCV-1st d plants, 25 $cm^2$ in YG-TYLCV-S-21st d plants, and 27 $cm^2$ YG-ToCV-S-21st d plants (Figure 6).

## DISCUSSION

Abiotic and biotic stresses have been shown to stimulate the expression of *CesA/Csl* genes in plants (Sha-

rif *et al*., 2021). However, there has been an absence of research on *CesA/Csl* genes in response to combined salt and virus stresses in the *Solanaceae* plants, and particularly in tomatoes.

It is well-established that salt (abiotic) stress can alter plant cell wall polysaccharide synthesis (Peng *et al*., 2019). Many reports have shown that cell walls have prominent roles in sensing salt stress (Zheng *et al*. 2019). Additionally, disruptions to cell wall polysaccharide synthesis enzymes have been linked to reduced salt tolerance (Zhang *et al*., 2016; Kesten *et al*., 2019). For example, mutations in the *CesA6* gene in *Arabidopsis* disrupt cellulose synthesis and lead to reduced salt tolerance (Endler *et al*., 2014, 2015; Kesten *et al*., 2019). Similarly, *CesA* genes have been shown to be differentially expressed under salt stress in barley, highlighting the important role of cellulose in maintaining cell wall integrity against salt permeability (Ueda *et al*., 2007).

In previous studies of TYLCV-infected tomato plants, *Csl* and *CesA* genes were found to be up-regulated (Chen *et al*., 2013; Choe *et al*., 2021), while *CesA* genes were highly down-regulated in response to TYLCV infection (Seo *et al*., 2018). In the present study, TYLCV-infected cultivars subjected to salt stress for 21 days exhibited a down-regulation of the *Csl-H1* gene when compared with the 1st day, which was consistent with the results of Seo *et al*. (2018). It has been suggested that the down-regulation of these genes may play trigger reduced growth and leaf curling in TYLCV-infected plants (Li *et al*., 2019). However, the down-regulation fold changes were similar and of low magnitude between the MK and YG tomato cultivars experiencing salt stress, and infected by TYLCV and ToCV, for the *Csl-H1* gene. Also, various transcription factors and some hormones have been shown to affect cellulose biosynthesis in salt stressed plants (Dabravolski and Isayenkov, 2023). *Csl* genes related to cellulose biosynthesis were also down-regulated, suggesting that these genes can be negative modulators of salt tolerance (Zhang *et al*., 2019). From this perspective, the present study results related to this gene are also consistent with previous studies. However, the other *Csl* gene *Csl-D3,1* exhibited up-regulation in both TYLCV- and ToCV-infected tomato cultivars (except for YG-ToCV, 1st day) as duration of salt stress increased (21st day), compared with day 1.

The present study has demonstrated significant up-regulation in the other three *CesA/Csl* genes (with the exception of the *Csl-H1*) after 21 days of salt stress. This up-regulation was particularly prominent in the MK ToCV-infected tomato cultivar. In the YG cultivar, the *Csl-D3,1* gene was 2-fold increased on the 1st day, and was 64-fold up-regulated on the 21st day. Similarly, to these results, the *Csl-D3,1* and *Csl-D3,2* genes were up-regulated in TYLCV-infected tomato cultivars (Choe *et al*., 2021). In the MK TYLCV-infected cultivar, no significant up-regulation was observed between the 1st and 21st days of salt stress. Nonetheless, considering the up-regulated profiles of the four genes which assessed, it can be inferred that these genes contribute to tomato response to TYLCV, irrespective of time (1st day or 21st day). Particularly noteworthy was the extreme down-regulation of the *Csl protein G2* gene (*CslG2; Solyc07g043390*) observed in TYLCV-infected plants. Chantreau *et al*. (2015) have suggested that the constitutive overexpression of the *Csl* gene can mitigate the severity of TYLCV symptoms, enhance disease tolerance, and increase productivity in TYLCV-infected tomato plants.

RNA-Seq analyses of uninfected and TYLCV-infected tomato cultivars were conducted by Seo *et al*. (2018) to investigate 38 *CesA/Csl* genes. The *Ces* family gene (*Csl-H1g043390.2.1*), a homolog of the *AtCESA8* gene, was found to be highly down-regulated among the genes implicated in TYLCV infection outcomes. This result underscores the critical role of cellulose in provoking stunted growth and leaf curls in response to TYLCV infections. Symptoms of TYLCV infections may involve substantial down-regulation of the *Ces* family gene to reduce cellulose levels. In the present study experiments, and in alignment with previous results, both tomato cultivars exhibited down-regulation of the *Ces-A2* and *Csl-D3,2* genes at the 1st day compared to the 21st day of salt stress. On the 21st day, an up-regulation profile was observed only for the *Csl-D3,2* gene in both tomato cultivars. It can therefore be postulated that the *Csl-D3,2* gene is linked to salt stress, directly or indirectly. Up-regulation was especially observed for the *Csl-D3,2* gene in the MK ToCV-infected (21st day) plants, with a 64-fold change. In the MK cultivar, no significant up-regulation differences were detected between days 1 and 21 of salt stress. Up-regulation of *CesA* (*Solyc03g005450.2.1*) in response to salt stress in tomato plants has also been previously documented (Renau-Morata *et al*., 2017).

Tomato plants infected with TYLCV and ToCV have been subjected to drought stress for 25 days, resulting in increased expression of *Ces-A2, Csl-D3,2* and *Csl-H1* genes, especially in the MK cultivar (Mirzayeva *et al*., 2023). Similarly, in the present study, both TYLCV- and ToCV-infected MK plants had increaseed *Csl-D3,2* and *Ces-A2* gene expressions (21st day of salt stress). However, unlike in drought stress (Mirzayeva *et al*., 2023), the *Csl-H1* gene was down-regulated in all the virus infected MK cultivar. This suggests that this gene may

be expressed differently under drought and salt stress.

Plant cell walls predominantly consist of polyphenolic compounds, including lignin and polysaccharides such as cellulose, pectin, and hemicellulose, which form primary plant biomass (Hu *et al*., 2018). Cell walls also have key roles in plant growth and maturation, as they determine cell shape and size, providing essential structural support. Additionally, cell walls act as a defense mechanism against environmental stressors (Malinovsky *et al*., 2014; Le Gall *et al*., 2015).

For the MK and YG tomato cultivars infected with TYLCV/ToCV without salt stress, high coefficients were detected in these plants during physiological measurements. Statistically significant reductions in mean dry weights were observed in both experimental groups (virus-grafted and virus plus salt-treated plants) of the MK and YG cultivars compared to non-inoculated controls. Leaf tissues had reduced biomass in response to TYLCV and ToCV inoculation, indicating that virus infections enhanced plant endurance under salt stress conditions, with leaflet biomass allocation in TYLCV- and ToCV-grafted plants allowing host adaptation to saline environments. Assessments of physiological water balance parameters in healthy and virus TYLCV- and ToCV-grafted tomato plants previously revealed substantial differences in plant their responses to salt stress (Gharsallah *et al*., 2020).

When TYLCV- and ToCV- infected samples with salt stress applied were examined as another experimental group, significant reductions were detected in leaf area in the MK and YG cultivars for virus-treated plants. Similarly, LA decreased by approx. 6 $cm^2$ in these plants compared to experimental controls. The experiments demonstrated LA increases in salt-treated plants in both cultivars, with the plants responding positively to salinity. However, the LA was reduced in salt-treated plants in both MK and YG cultivars. LA measurements were taken for both control plants and those exposed to salt stress, showing this parameter gradually increased. However, the LA of control plants was greater under favorable conditions than in salt-stressed plants (Bacha *et al*., 2017).

Sandy *et al*. (2014) measured 20 to 25% reductions plant dry mass in drought-treated tomato plants. Transgenic overexpressing genes *SlSOS2* and *LeNHX2* exhibited increased growth and biomass production when cultivated in an NaCl-rich condition compared to their wild-type counterparts (Maach *et al*., 2021; Roșca *et al*., 2023). Although tomato is categorized as moderately salt-tolerant, salt accumulation in the soil can reduce plant production. This was evident for plants grown in compost conditions and those treated with mineral fertilizers, which had increased nutrient content, indicating adaptation to salt stress (Savy *et al*., 2022). These results indicate that tomato defence systems adapt to unfavourable growth conditions by activation from viruses and from high soil salt contents.

Wang *et al*. (2021) demonstrated that moderate salt stress inhibited tomato leaf growth, degree of inhibition increasing with time. Also, tomato plants subjected to salinity stress had reduced height and decreased leaf area. *Taraxacum officinale* and *Ambrosia artemisiifolia* had weak correlations with other parameters. Conversely, LA in *Tilia tomentosa* and *Aesculus hippocastanum* were positively correlated with specific leaf weights (SLW) but negatively correlated with specific leaf area (SLA). Furthermore, fractal dimensions (FD) in *T. tomentosa* and *A.s hippocastanum* were negatively correlated with SLA values, while FDs in *T. officinale* and *A. artemisiifolia* were negatively correlated with SLWs (Terada *et al*., 2021).

Relative water content (RWC) is an indicator of cellular water status, and is associated with abiotic stress tolerance. Reductions in RWC were observed in virus-inoculated tomato plants, and RWC was reduced in plants exposed to salt stress compared to the controls. Patane *et al*. (2022) showed that RWC values exceeding 75% were normal even under drought stress conditions. Hosseini *et al*. (2018) found that experimental plants exhibited reduced water content when exposed to a combination of drought stress and cucumber mosaic virus (CMV) compared to non-inoculated cultivars. Silencing of *SlCBL3-1* also reduced shoot and root growth, as well as RWC (Hosseini *et al*., 2018). In the present study, reductions in RWC were observed in TYLCV/ToCV-infected tomato cultivars MK and YG. The combined stresses reduced shoot fresh and dry weights, leaf area, and RWC in all cultivars assessed by Zhou *et al*. (2017). Increased RWC contributes to reductions in osmotic stress during the recovery periods (Tiwari *et al*., 2016), with leaf RWC being greatest in control plants and least in salt-exposed plants (Tanveer *et al*., 2019). Salt stress also severely inhibits seedling growth and biomass accumulation (Parvin *et al*., 2019, 2020). Restrictions in plant growth caused by salt have been attributed to salt-mediated reductions in cell growth. In addition to ion toxicity, salinity induces osmotic stress by altering water potential in growth media and within plants (Raziq *et al*., 2022).

Osmotic stress in tomato plants is evident through reduced leaf RWC, as high NaCl concentrations can damage root systems, leading to reduced water absorption (Zeng *et al*., 2011). Nahar *et al*. (2016) reported that salinity enhances proline levels, potentially increasing stress resistance by maintaining osmotic potential, promoting leaf expansion, enhanc-

ing stomatal conductance, and facilitating photosynthesis. Zhao *et al*. (2021) showed that root water and osmotic potentials improved in tomato plants exposed to salinity stress following biochar applications, and leaf RWC increased after biochar application under salinity stress. Water deficit is detrimental to plant growth, primarily due to reductions in RWC and water potential (Diouf *et al*., 2018). In the present study increased water deficits were detected in SV and V tomato plants. TYLCV- and ToCV-infected plants of the MK and YG cultivars had increased RWC in response to salt stress, although these approached levels similar to those of non-inoculated samples. Previously research revealed that tomato seeds exhibited maximum RWC when exposed to *Trichoderma viride* in a non-saline MS medium, but displayed minimum RWC when subjected to 100 mM NaCl (Metwally and Shereen S., 2023). TYLCV infection resulted in a 58% reduction in plant height when the plants were adequately watered, although infection did not affect numbers of leaves (Botto *et al*., 2023). Decreased shoot fresh and dry weights, leaf area, and RWC were also recorded in several cultivars under combined stresses (Zhou *et al*., 2017). These results show that plants resist salt stress do so by producing particular proteins (Maach *et al*., 2020).

## CONCLUSIONS

While previous studies have explored the physiological and biochemical processes of tomato plants under various biotic and abiotic stress factors, there has been absence of research reports on the intricate interplay between biotic stress, such as that caused by virus infections, and abiotic stress, particularly salinity.

The present study has addressed this knowledge gap by utilizing virus susceptible tomato cultivars which were subjected to TYLCV and ToCV inoculations through single-leaflet grafting. The experimental design involved exposure to virus infections and salt stress to investigate the potential role of *CesA/Csl* genes biosynthesis in modulating host plant defense mechanisms. The study also aimed to assess the physiological effects of combined biotic and abiotic stress in tomato leaf tissues. The results highlight key physiological responses and gene expression patterns under the combined stress conditions. Alignment between gene expression data and physiological measurements indicates that the YG tomato cultivar was as sensitive to salt and virus stresses as the MK cultivar. The results also emphasize the importance of up-regulating specific genes, including *Csl-D3,1*, *Csl-D3,2*, and *Ces-A2*, which contribute to resistance against the combined effects of salt and virus stresses during dual stress exposure. Almost similar results were obtained during physiological measurements in MK and YG cultivars. As a result of TYLCV/ToCV infections and the combined effects of virus and salt stress, key physiological parameters, including relative water content, dry biomass, water deficit (WD), and leaf area, exhibited high variation coefficients in experimental plants. An exception was observed in relative water content measurements, where plants subjected to combined ToCV and salt stress exhibited values close to those of non-inoculated controls. This stability indicates a distinct physiological adaptation in response to the combined ToCV and salt stress conditions. However, when the broader physiological and gene expression data indicate that the YG tomato cultivar was similarly sensitive to stress conditions as the MK cultivar, suggesting that both tomato cultivars are similarly affected by virus and salt stresses.

This research provides insights into the molecular and physiological responses of tomato plants to the concurrent challenges of virus infection and salt stress. The observed regulation of specific *CesA/Csl* genes, along with distinct physiological alterations, highlight the complex interplay between biotic and abiotic stress signalling pathways. This study advances the understanding of stress adaptation mechanisms, and has potential to inform development of resilient tomato cultivars through targeted breeding or biotechnological approaches aimed at enhancing tolerance to multiple stress factors.

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## AUTHORS CONTRIBUTIONS

S.M., conceptualization, investigation and visualization, data analyses, drafting of original draft manuscript; İ.H., author of the idea project topic; İ.Ö.E., provided infected plant collections; C.Y.Ö., methodology, statistical analyses, revised original manuscript; A.E., methodology, conducted experiments, provided laboratory and the necessary chemical reagents. All authors read and approved the final version of the manuscript.

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