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A Protein Disulfid Isomerase from *Rosa hybrida* Influences Flowering Time and Flower Durability in Transgenic Tobacco Plants

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Abstract

Extending the vase-life of cut flowers is a major global challenge. Among various genetic factors that influence the lifespan of cut flowers, protein disulfide isomerases (PDIs) are the most important. The PDI family comprises several proteins that play a key role in disulfide bond formation, which is often overexpressed in response to stresses such as drought, salinity, and senescence in flowers. In this study, *Rosa hybrida* protein disulfide isomerase A6 (*RhPDIA6*) was synthesized and transferred to tobacco (*Nicotiana tabacum*) plants via Agrobacterium. In transgenic lines, plant height, flower longevity, time to flowering, CAT activity, and SOD activity decreased, while the levels of proline and hydrogen peroxide in comparison to wild-type and control plants increased in the T0 and T1 generations. The results demonstrated a complex relationship between *RhPDIA6* and the characters studied, so understanding their interaction could potentially lead to advances in agricultural practices, such as breeding flower varieties with longer shelf-life.

Keywords: Senescence, flower longevity, protein disulfide isomerase A6, overexpression

Key Findings

A protein disulfide isomerase gene from *Rosa Hybrida (RhPDIA6)* was a candidate to introduce into a model plant, tobacco, for functional analysis during flower senescence. Transgenic tobacco lines expressing *RhPDIA6* exhibited altered plant height, flower longevity, and flowering time. The study suggests a complex and effective relationship between *RhPDIA6*, the studied traits, and the floral senescence.

Introduction

The genus Rosa includes several cultivars and is a prominent ornamental flower traded as a cut flower in global markets [1]. Therefore, understanding the unknown genetic aspects of this flower is essential. The quality of cut flowers is typically assessed based on their longevity, color, and fragrance. However, in recent times, the lifespan of flowers has received increasing attention [2].

Senescence is a genetically regulated process that occurs during the final phase of a flower's life [3]. The breakdown of deoxynucleotides, ribonucleotides, and proteins is crucial for determining shelf life, which is facilitated by the upregulation of several nucleases [4,5]. During this process, an increase in the levels of amino acids and reactive oxygen species (ROS) commonly takes place. One of the main free amino acids in plants is proline, and its levels rise under stressful conditions such as drought, salinity, and high light intensity [6,7].

In the early stages of growth, plant buds are well protected from the harmful effects of superoxide. However, as the flowers begin to bloom, the plant's capacity for antioxidant defense becomes limited. This makes the reproductive organs, particularly rose petals, more sensitive to reactive oxygen species (ROS) [8,9].

On other hand, ethylene plays a crucial role in the senescence of ethylene-sensitive flowers [10-12]. Petal senescence driven by ethylene is marked by several changes, including petal wilting, increased oxidative stress, degradation of proteins and nucleic acids, and autophagy [13,14]. Ethylene also interacts with other growth regulators. In cut roses, flower blooming is primarily regulated by ethylene through the expression of two ethylene receptor genes and two CTR genes in the petals [15,16]. As a result of these processes, morphological changes often occur, such as the bending of the neck and the wilting and dropping of petals.

The visual signs of senescence in plants include withering, color changes, and the falling off of various parts, such as flowers, petals, and buds [5,17].

In general, delays in plant senescence can be achieved through genetic engineering before harvesting flowers or by manipulating environmental factors during the post-harvest period. Key environmental factors that can affect the vase life of flowers include light, weather, humidity, and drought stress, as these can enhance catabolic processes [19,20]. High humidity levels in greenhouses or storage areas where cut flowers are produced or kept can lead to the growth of pathogenic fungi, which in turn accelerates senescence [21,22]. While most environmental factors that influence the post-harvest stage have immediate effects and cannot be passed on to the next generation, the production of genetically modified plants appears necessary.

A common method for transferring genes into plants is through Agrobacterium, a genus of bacteria known for its ability to deliver DNA to plants. Genetic analyses have shown that many genes can either upregulate or downregulate during the stages of senescence, leading them to be classified as senescence-associated genes (SAGs) [23,24]. Several genes related to senescence have been studied in roses, specifically in *Rosa hybrida*. Among them, a number of ethylene receptor genes have been identified, including *RhETR1, RhETR2, RhETR3, RhETR4,* and *RhETR5* [26,27], which *RhETR1, RhETR3*, and *RhETR5* are responsible for differences in longevity and appearance quality among various rose cultivars.

The *RhAC* fragment was previously studied by analyzing the expression of genes related to bud and open flower development in *Rosa hybrida* cultivars, Black Magic and Marroussia, during two stages: bud and flower. This was achieved using the cDNA amplified fragment length polymorphism (cDNA-AFLP) method [28]. The BLASTN and BLASTP search and alignment of the expressed sequence tags (ESTs) for *RhAC* in the National Center for Biotechnology Information (NCBI) database revealed the highest similarity with At3g54960 from *Arabidopsis thaliana* and a notable similarity (67%) with the probable protein, protein disulfide isomerase (PDI) from *Rosa chinensis*, respectively. We successfully obtained the full-length gene of *RhPDI* (1397 bp) through a homology-based approach and confirmed its identity through sequence analysis and structural features [28,34].

The Protein Disulfide Isomerase (PDI) family is one of the largest joint protein families found in humans, animals, and plants. Different organisms possess a variety of PDIs and their associated domains, but similar homologs typically perform equivalent functions across species [29]. The PDI family includes several PDI and PDI-like proteins that contain a thioredoxin domain, which is essential for organizing disulfide bond formation [29-31]. These multi-modular redox catalysts play a role in the formation, reduction, or isomerization of disulfide bonds, especially in the endoplasmic reticulum [32]. Post-translational modifications, such as glycosylation and protein folding, are essential for the export of proteins from the endoplasmic reticulum to other organelles. These processes depend on the activity of chaperones as well as the catalytic function of disulfide isomerase proteins, which act as sulfide carriers to form disulfide bonds [31,33]. The protein disulfide isomerase family in *Rosa chinensis* consists of 41 members, but their features and functions have not yet been well characterized [34].

Given the competitive nature of the ornamental plant industry, it is crucial to research and develop new products while also enhancing the quality of existing ones. In the present study, after synthesizing the *RhPDIA6* gene which was isolated from *Rosa hybrid*, previously [34], we transferred it to the tobacco model plant using Agrobacterium. The goal of the project was to analyze the expression of the PDI gene to investigate its function in tobacco plants and to provide information about its role in the senescence with respect to plant morphology and physiology.

Material and Methods

Gene synthesis

The *PDI* gene (1130 bp) was synthesized in the pUC57 vector between *Nde*I (which cuts at CA^TATG sites) and *Sal*I (which cuts at G^TCGAC sites), based on NEB Cutter design (https://nc3.neb.com/NEBcutter/ Shanghai Shine Gene Molecular Biotech Co., China). The resulting pUC plasmid with the insert measured 3538 bp (SnapGene 6.2). The pRI vector (TakaRa, Japan), containing a kanamycin resistance marker, was used for expression. pUC57-RhPDI and pRI were extracted using a Genet Bio kit (South Korea), then digested with *Nde*I and *Sal*I (Biolabs, UK, and BioRad, USA). The DNA fragment was purified using a PrimePrep Gel Purification Kit (AddBio, South Korea). The *RhPDIA6* gene was ligated into pRI with T4 DNA ligase (Y-TA, Iran). The resulting pRI-RhPDI was transformed into *E. coli* DH5α and grown on Murashige and Skoog medium [35] supplemented with 50 µg/L of kanamycin. Gene assembly in pRI was confirmed by PCR colony screening using M13 and NPT primers (Table 1). The *RhPDIA6* gene was transferred to *Nicotiana tabacum* Xanthi via *Agrobacterium tumefaciens* GV3101 (pRI-PDI) using the leaf disc method [36]. Controls included leaf discs infected with Agrobacterium carrying the empty vector and uninfected wild-type leaf discs.

Transformation of tobacco by Agrobacterium tumefaciens

Agrobacterium tumefaciens strain GV3101 was cultured in LB medium supplemented with 30 mg/L ampicillin and 50 mg/L kanamycin until it reached an OD600 of 0.5 and was used to infect tobacco leaf discs for 10 minutes. The leaf discs were then placed on a hormone-free and antibiotic-free co-culture medium. Transgenic calli were selected using a medium containing hormones and antibiotics, with a pH of 5.8. For the selection of transgenic calli, a tobacco selective medium was used, which included the hormones naphthalene acetic acid and benzyl aminopurine, along with the antibiotics meropenem (250 mg/L) and kanamycin (80-150 mg/L). After two months, rooted seedlings (T0) were transferred to soil. PCR using specific primers for the *RhPDIA6* gene and *virG* (Table 1) confirmed the presence of *RhPDIA6* and absence of bacterial contamination, respectively. T0 plants were moved to the greenhouse, and T1 seeds were harvested after about 100 days. T1 seeds were planted in kanamycin--

containing medium to select for transgenic seedlings, which were then transferred to the greenhouse for adaptation.

Table 1: Primers used in *RhPDIA6* cloning and qRT-PCR

Primer name	(5´-3´) Sequence	Tm (°C)	PCR product (bp)	Annotation
PDI2F2	Forward CAA GTT TGG AGT TTC TGG TTA CC	58.5	588	Gene cloning
PDI2R3	Reverse GAT GGC TTC TTC TCC TCA CTG C			
NPT	Forward GTCGCCTAAGGTCACTATCAGCTAG			
	Reverse ATGTTTGAACGATCGGGGATCATG			
virG	Forward GGTCGCTATGCG GCATC			
	Reverse CCTGAGATTAAGTGTCCAGTCAG			
RhPDI1F	GTGTTTCCGGTGTACTCTCGTT	60	158	qRT-PCR
RhPDI1R	ACAAGCTATTGCAGTCCTCAGTTC			
β-actinF	CCCAGAAGAGCACCCCATT	60	191	qRT-PCR
β-actinR	GGCTAACACCATCACCAGAGT			

Analysis of the physiological and morphological characteristics of the T0 and T1 generations

We analyzed the morphological traits (flowering time, flower durability, plant height at 30 and 60 days after adaptation) and physiological characteristics, including catalase (CAT) [37] and superoxide dismutase (SOD) [38] activity, proline content [39], and H_2O_2 concentration [40] in triplicate. Physiological traits were measured at 30 and 63 days after adaptation. The presence of the *RhPDIA6* gene was also assessed in seedlings of wild-type, control, and transgenic plants.

RNA extraction and cDNA synthesis

Total RNA was extracted from 50 mg of plant flowers using the RNeasy Plant Mini-Prep Kit (Zymo Research, USA), following the manufacturers protocol. To eliminate any residual DNA, 5 µl of *DNase* I (5000 U/µl) was added to each sample, as specified in the RNeasy Plant Mini-Prep Kit instructions. cDNA synthesis was then carried out using the cDNA synthesis kit from Fermentas (Canada).

Gene expression analysis

To investigate gene expression, primers for *RhPDIA6* were designed using Oligo 7 software. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using an RT-PCR system with 10 ng of cDNA and SYBR Green PCR Master Mix (YTA, Iran). The thermal cycling profile consisted of an initial step at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 5 seconds, 60 °C for 15 seconds, and 72 °C for 25 seconds. The specific gene expression levels were quantified by normalizing to the *β-actin* housekeeping gene [34].

Bioinformatics analysis

The PDI query using accession number XP_024189320.1, probable protein disulfide-isomerase A6 [*Rosa chinensis*], was conducted against senescence-related genes in the Leaf Senescence Database (LSD). This website employs the BLOSUM62 substitution matrix to score amino acid substitutions during protein sequence alignment. *Arabidopsis thaliana* LHY/CCA1-like 1 (L-CL1), as the reference gene, is known with protein_id="NP_568108.1" and TAIR: AT5G02840 (https://www.ncbi.nlm.nih.gov). The LCL1 promoter is active in all tested tissues and developmental stages, especially in drought, salty, and light conditions

[41].

Statistical analysis

The experiment used a completely randomized design with three replications. Data were analyzed using R software (4.5.1) with a two-way ANOVA for transgenic, wild-type, and control plants. Mean differences were analyzed using Student's t-test ($p <$ 0.05 and 0.01). Gene expression data were analyzed using REST software, based on [42]. Treatments included three biological and technical replications, and relative gene expression levels were evaluated using the $2^{-\Delta\Delta CT}$ method [43].

Results

RhPDIA6 Cloning

Synthesized gene (Figure 1) sequence analysis in the tobacco genome showed 77.47% similarity to probable protein disulfide-isomerase A6 (LOC107807082). *RhPDIA6* presence was confirmed by amplifying a 588 bp fragment in pUC, pRI vector (Figure 2a), and Agrobacterium (Figure 2b). Fragments of 14-day-old tobacco were inoculated with Agrobacterium containing the *RhPDIA6* gene. A 588 bp band, used for gene tracing, was observed in regenerated calli (Figure 2a-c). Kanamycin-resistant, *virG*-negative plants were selected (Figure 2a), rooted, and transferred to soil (Figure 3a-h). Seeds (T1) were collected. Three *RhPDIA6*-positive lines (1, 2, and 3) were selected for examination, but line 3 was removed due to environmental issues. Adaptation steps were repeated for T1 seedlings (Figure 4a-d).

Morphological characteristics of T0 and T1 off-springs

The results did not demonstrate any significant differences between the two generations, T0 and T1, and between each of lines in a generation in height, time to flowering, and durability of flowers (Figure 5a-c). Moreover, the height of the transgenic lines was approximately 20 cm shorter than that of the control and wild-type plants, and it did not change significantly over time. Interestingly, the time to flowering in transgenic lines, across both generations, was approximately 10 days shorter than in wild and control plants (Figure 5b). On the other hand, in wild-type and control plants, the maximum duration of flowering lasted about 13 days, while in the transgenic lines (T0 and T1), it decreased to approximately 7 days (Figure 5c).

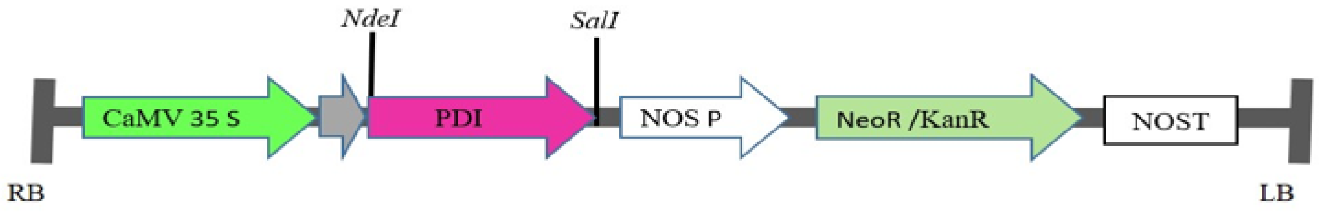


Figure 1: Schematic diagram of the T-DNA region in the plasmid pRI. RB, right border; LB, left border; CaMV 35S, cauliflower mosaic virus 35S promoter; PDI, Protein Disulfide Isomerase; *Nde*1= restriction site within T-DNA; *Sal*I= restriction site within T-DNA; *NOS-Pro*, promoter sequences of the *nopaline synthase* gene; *KanR*, *kanamycin resistance* gene. *NeoR*- is the protein name of KanR Aminoglycoside 3'-phosphotransferase; NOS-T: nopaline synthase terminator.

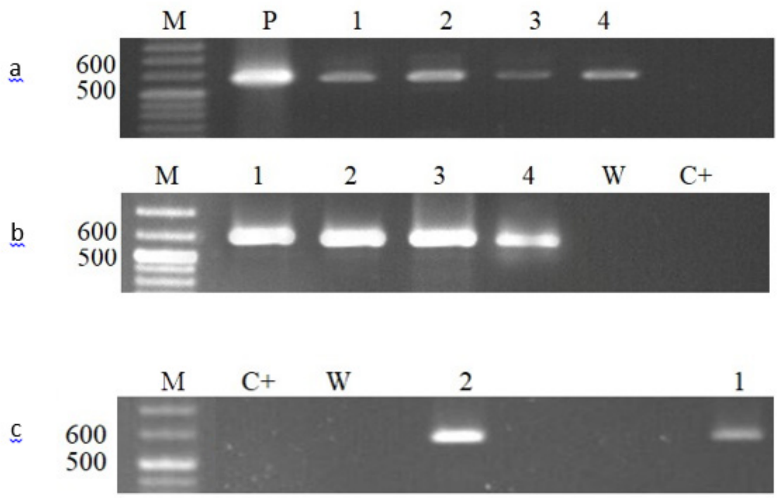


Figure 2: Polymerase chain reaction (PCR) analysis of the transgenic Agrobacteria and transgenic plants. (a) The PCR clones analysis of *Rosa hybrid* Protein Disulfide Isomerase (*RhPDIA6*) gene (588 bp) isolated from transformed Agrobacteria. P, plasmid (positive control), 1–4 are transgenic lines. (b) PCR amplification of the *RhPDIA6* gene (588 bp) from the T0 lines, 1–4 are transgenic lines, W, wild-type, and C^+, control. (c) The PCR analysis of the *RhPDIA6* gene (588 bp), 1–2 are T1 transgenic lines, W, wild-type, and C+, control. M, 1500 bp DNA marker.

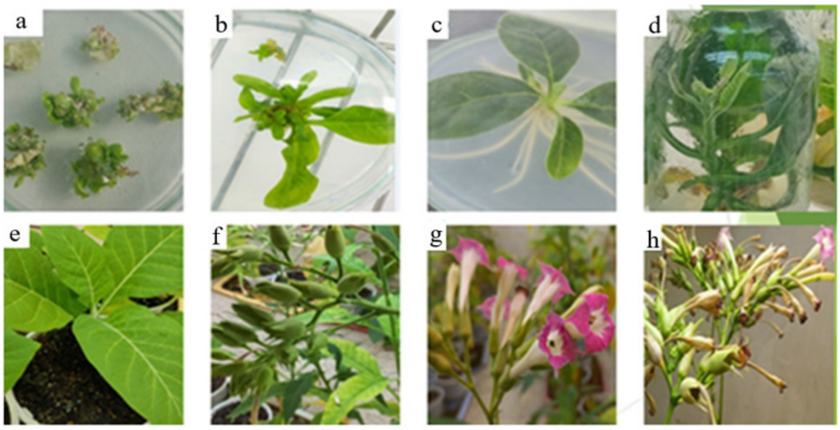


Figure 3: Adaptation of *RhPDIA6* transgenic plants in the greenhouse included the following stages: a) callus formation from leaves inoculated with Agrobacterium after 16 days, b) regeneration phase after 30 days, c) rooting of seedlings in glass containers after 45 days, d) mature plant development in glass bottles after 60 days, e) adaptation of the transgenic plant in the pot (16 days after moving to the pot), f) budding stage ~55 days after moving to the pot, g) flowering stage ~75 days after moving to the pot, and h) flower shedding and seed setting ~85 days after moving to the pot.

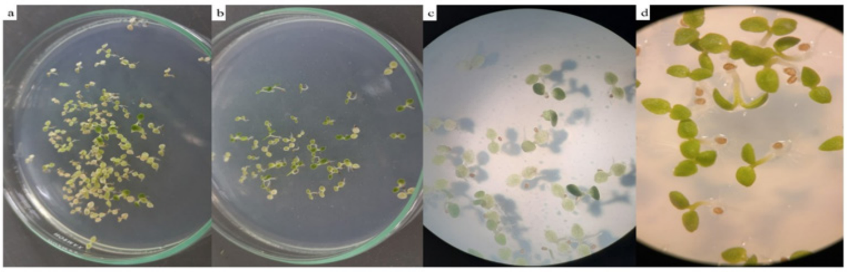


Figure 4: Growth of the T1 transgenic seedlings of tobacco on the medium containing the Kanamycin antibiotic: a) Transgenic seedlings line 1, b) Transgenic seedlings line 2, c) Control, with green seedlings indicating Kanamycin resistance, and d) Wild--type cultivated on medium free of antibiotics.

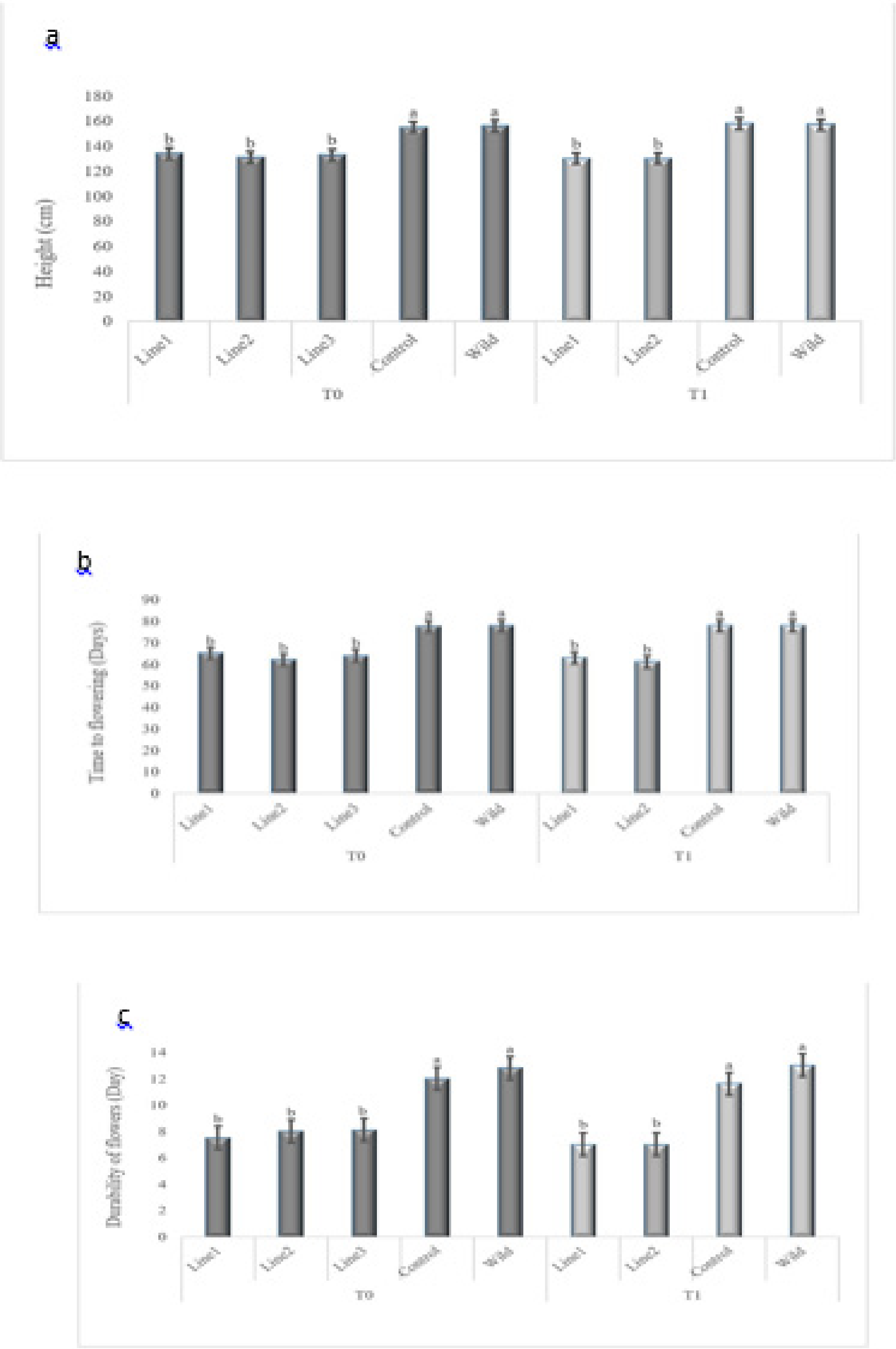


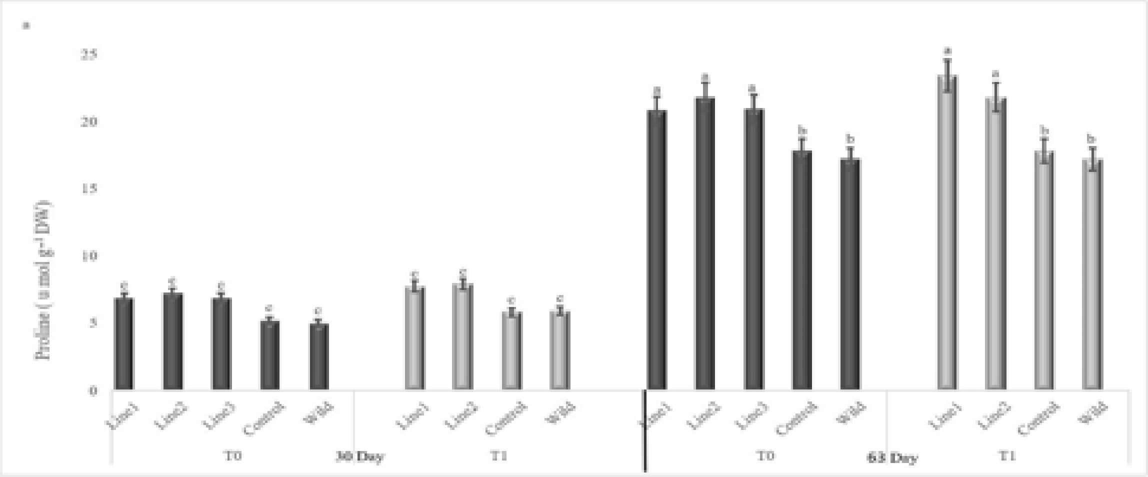
Figure 5: Morphological features of 70-day-old wild-type, control, and transgenic plants: a) durability of flower, b) plant aerial part height, and c) time to flowering. Data were analyzed using R (v4.5.1) with the 'agricolae' package for ANOVA. Different lowercase letters indicate significant differences with significance levels set at 0.05 and 0.01 among wild-type, control, and transgenic plants. The differences between the mean standard deviation (n = 3) were analyzed using the Student's t-test; different letters indicate significant differences.

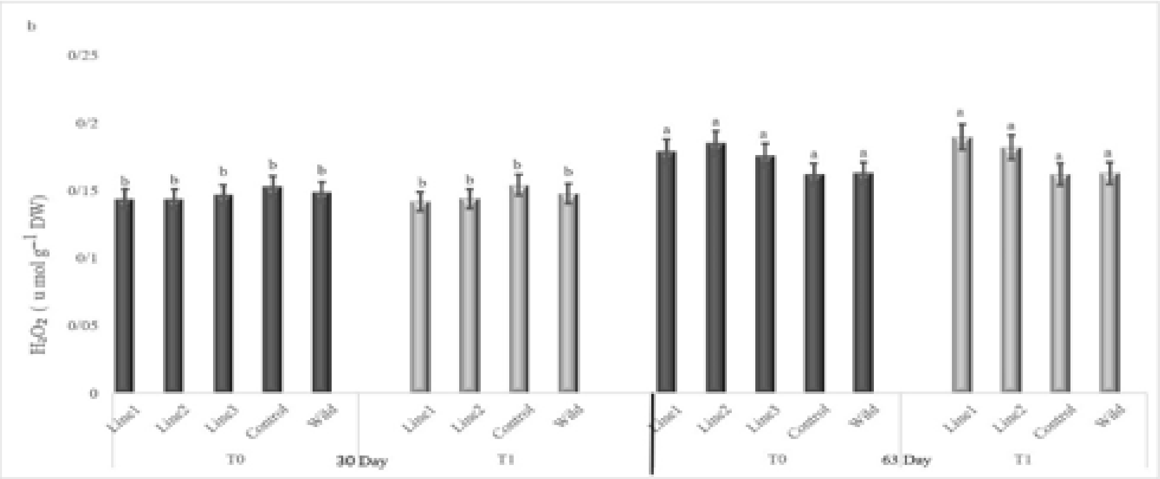
Physiological characteristics of transgenic plants in the T0 and T1 generations

According to the results, the proline content was statistically similar across all transgenic lines and was higher than control and wild-type plants after 30 days; however, as the plants developed (63-day-old plants) and reached the flowering stage, the proline content increased in all samples. Most samples demonstrated a significant increase, reaching up to three times, whereas the wild and control plants displayed the lowest content than transgenic lines (Figure 6a).

In this regard, the content of hydrogen peroxide (H_2O_2) was roughly similar in all samples after 30 days (Figure 6b), although its amount increased significantly in all samples after 63 days, its level in wild-type and control plants was lower, but not meaningfully, than that in transgenic plants (Figure 6b).

Incidentally, the activity of the superoxide dismutase enzyme was somewhat similar across all transgenic lines in both generations, but it was roughly lower than that of wild-type and control plants after 30 days (Figure 6c). Over time, SOD activity decreased in all plants; however, it remained higher in wild-type and control plants (Figure 6c). This study found that in 30--day-old plants, the activity of CAT was slightly higher in wild-type and control plants than in transgenic plants across both generations. However, as the plants developed, enzyme activity decreased by up to one and a half times in most plants, although it remained higher in wild-type and control plants (Figure 6d).





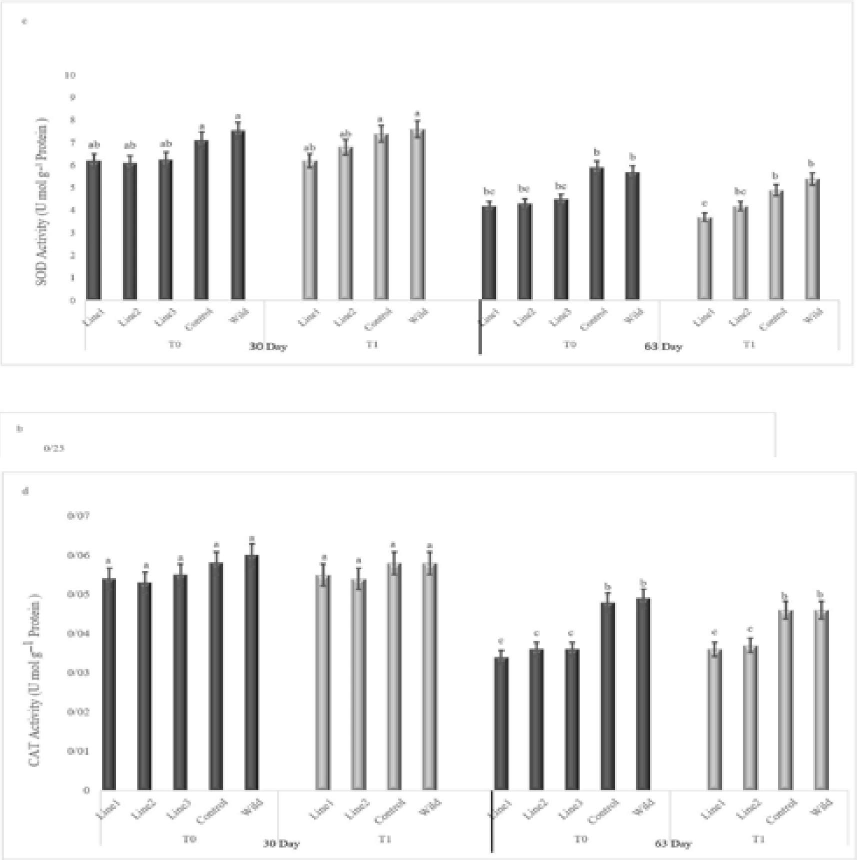


Figure 6: The impact of time (30 and 63 days after plant adaptation) on the physiological traits of transgenic lines (Line 1, 2, and 3 in T0 generation and Line 1 and 2 in T1 generation), as well as control (Cnt) and wild-type (WT) plants of tobacco, was examined. The traits assessed included: a) proline content, b) hydrogen peroxide (H_2O_2) content, c) superoxide dismutase (SOD) activity, and d) catalase (CAT) activity. Data were analysed using R (v4.5.1) with the 'agricolae' package for ANOVA. Different lowercase letters indicate significant differences with significance levels set at 0.05 and 0.01 among wild-type, control, and transgenic plants. The differences between the mean standard deviation (n = 3) were analyzed using the Student's t-test; different letters indicate significant differences.

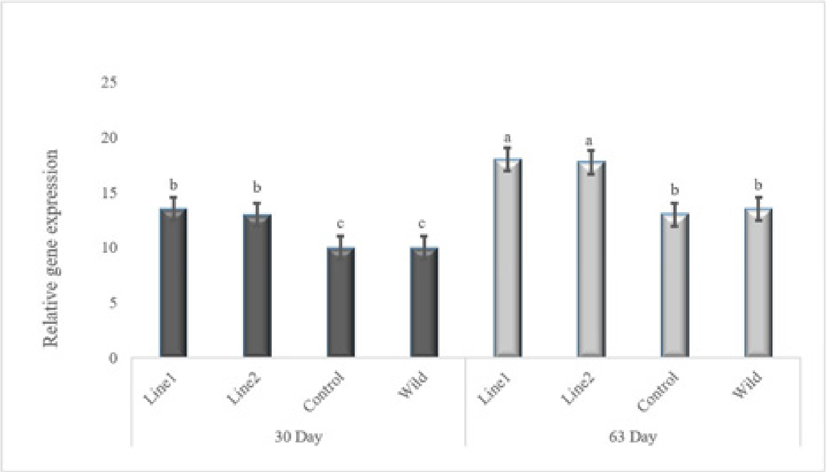


Figure 7: The expression patterns of *RhPDIA6* at two transgenic lines of tobacco, Line1 and Line2, control plants, and wild--types (wild) at two developmental stages of flowers, which were analyzed using qRT-PCR. Data analysis were carried out by REST software and the vertical bars represent standard deviations (n = 3).

Monitoring the expression pattern of RhPDIA6 through qRT-PCR

The cDNAs were successfully synthesized using total RNAs extracted from transgenic lines, wild-type, and control plants. Initially, in 30-day-old plants, the expression levels of the gene in wild-type and control plants were lower than in transgenic lines. After reaching the flowering stage, the expression levels increased significantly for all plants (Figure 7).

RhPDIA6 BLAST P

The protein sequence of probable *RhPDIA6* shows high homology with senescence-related proteins of leaves found in the Leaf Senescence Database https://ngdc.cncb.ac.cn/lsd/ (Figure 8) with a higher (81) score alignment related to AT1G35620.1, which encodes a protein disulfide isomerase-like (PDIL) protein, PDI-like 5-2, in *Arabidopsis thaliana*, which is a member of a multigene family within the thioredoxin (TRX) superfamily (Figure 8 and Table 2).

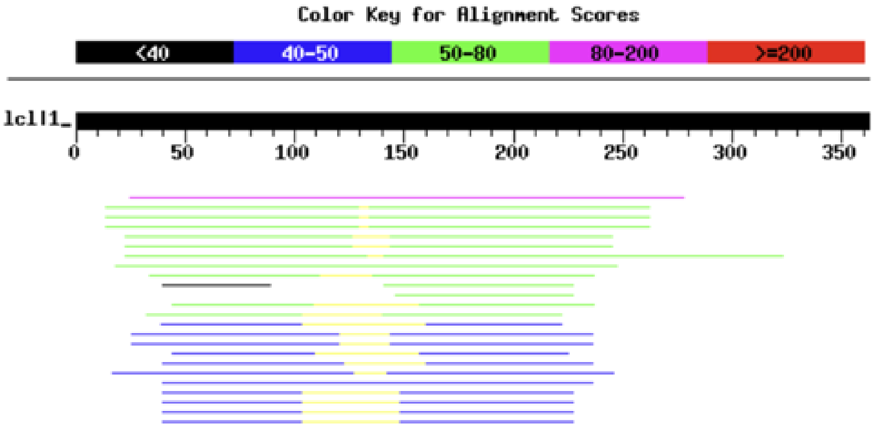


Figure 8: Analyses of alignment scores for various sequences against the reference sequence LHY/CCA1-like 1 (LCL1). The 1c1l1 color-coded key indicates the alignment score ranges for RhPDI, with colors corresponding to specific score thresholds: blue (<40), green (40-50), yellow (50-80), orange (80-200), and red (≥200). If the majority of the sequences display high alignment scores, indicating significant conservation among the tested variants with lcl11. Notably, sequences in the red category suggest strong homology, which may highlight evolutionary relationships.

Table 2: Sequences producing significant alignments for *RhPDIA6* in the Leaf Senescence Database

Sequences producing significant alignments:	Score(bits)	EValue
AT1G35620.1	81	$2E^{-16}$
AT1G07960.2	75	$2E^{-14}$
AT1G07960.1	75	$2E^{-14}$
AT1G07960.3	75	$2E-^{14}$
AT4G27080.1	70	$4E^{-13}$
AT4G27080.2	70	$4E^{-13}$
AT3G16110.1	68	$2E^{-12}$
AT1G50950.1	59	$7E^{-10}$
AT1G19730.1	53	$7E^{-08}$

AT2G15570.1	52	$9E^{-08}$
AT2G15570.2	52	$1E^{-07}$
AT1G45145.1	50	$3E^{-07}$
AT4G26160.1	50	$4E^{-07}$
GSMUA_Achr10T18740_001	49	$1E^{-06}$
AT3G08710.2	48	$2E^{-06}$
AT3G08710.1	48	$2E^{-06}$
AT5G42980.1	47	$5E^{-06}$
AT5G16400.1	46	$8E^{-06}$
AT4G21990.1	45	$1E^{-05}$
AT3G02730.1	45	$1E^{-05}$
AT1G08570.2	41	$2E^{-04}$
AT1G08570.1	41	$2E^{-04}$
AT1G08570.3	41	$2E^{-04}$
AT1G08570.4	41	$2E^{-04}$
GSMUA_Achr11T15150_001	38	0.002

Discussion

Senescence involves cell death and degradation, controlled by genetic programs and environmental factors. Flowering, a critical stage affecting yield and stress resistance, is influenced by plant age and environmental cues like photoperiod and nutrient availability. Stress accelerates flowering. The main flowering pathways are light-dependent, gibberellin, cold, and autonomous. Prior research [34] explored the role of *RhPDI* and *RhPDIA6* genes in rose aging and their response to ethylene. This study investigates *RhPDIA6* function in plant senescence, particularly flower aging.

RhPDIA6 cloning

Previously, a protein sequence alignment of At3g54960 revealed there are two probable proteins, *PDIA6* and *PDI*, with a high similarity (67%) in the *Rosa chinensis* genome. PDI proteins participate in the correct folding of misfolded or denatured proteins during plant growth, as well as in stress tolerance. Thus, understanding the relationship between PDI proteins and the phenomenon of senescence in roses has become interesting. After analyzing gene expression in various reproductive tissues of roses [34], we hypothesized that *RhPDIA6* may accelerate flowering and aging by influencing ascorbic acid levels, increasing reactive oxygen species (ROS) levels, and enhancing sensitivity to external ethylene. Additionally, ascorbate serves as a reducing agent, which may enhance the activity of protein disulfide isomerase (PDI).

After introducing the synthesised gene (Figure 1) into tobacco, initial investigations showed that *PDI* is present in tobacco as well, but its sequence had only slight similarity to the *RhPDIA6* sequence. Confirmation of gene presence in transgenic plants was achieved using *RhPDIA6*-specific, kanamycin, and *VirG* primers (Figure 2). Meanwhile, after monitoring all transgenic plants in the T0 generation, the presence of the *RhPDIA6* was confirmed in only three lines, L1, L2, and L3. To continue investigating the gene function in offspring, seeds were collected from the aforementioned lines and planted in pots. Given that seed production in line 3 was very low, only seeds from lines 1 and 2 were planted (T1) (Figure 4a-d).

Morphological characteristics

Results revealed that the height of T0 and T1 plants was shorter than that of the wild-type and control plants (Figure 5a). Furthermore, the presence of *RhPDIA6* in the T1 progeny contributed to their reduced height compared to the T0 progeny. Evidence suggests that protein disulfide isomerases (PDIs) may play a role in gibberellin signaling pathways, influencing how plants respond to gibberellins during development and in response to stress. PDIs may help stabilize gibberellin-related proteins or signaling molecules, thereby enhancing their effectiveness during critical growth phases [44]. Moreover, studies have indicated that gibberellic acid is crucial for stem growth. The gene responsible for stem elongation is *LUE1*, which is directly activated by gibberellic acid (GA). The *LUE1* gene is essential for maintaining proper alignment and functionality of microtubules, making it a key component in orchestrating plant cell growth and development through hormonal regulation. Given the role of PDIs in maintaining protein homeostasis during stress, their interaction with LUE1 and modulation by GAs suggest that these factors are interconnected in influencing the overall health and lifespan of plants. The coordination between hormone signaling (GAs), stress response (LUE1), and protein folding (PDIs) represents a complex network through which plants adapt to their environment [45]. Regulations that reduce the effects of GA can lead to shorter plant stature [46]. On the other hand, ethylene is often considered as an inhibitor of stem elongation in many dicots. Studies indicate that it can cause reduced stem height by affecting cell growth characteristics [25]. This is evidenced by the transfer of the *RhPDIA6* gene to tobacco plants, which resulted in decreased height in both the T0 and T1 transgenic generations (Figure 5a). However, more studies need to be conducted on the relationship between RhPDIA6 expression, Gibberellin signaling pathways, and ethylene levels.

Physiological characteristics

It has been shown in numerous studies that the production of proline is increased in drought, salinity, light conditions, and developmental stages [47]. Proline is a natural stimulant, which is produced for regulating osmotic pressure and tolerating stress. It is the source of carbon and nitrogen, which, by degradation of reactive oxygen species, regulates the reduction potential and gives stability to cell organelles such as membranes [48]. Here, results indicated that proline and hydrogen peroxide concentrations increased (Figure 6a and b), and CAT enzyme activity and SOD enzyme activity decreased (Figure 6c and d); therefore, it can be concluded that *RhPDIA6* gene probably plays an important role in flower senescence by increasing ROSs content and causes the shorter flower lifespan.

The interplay between PDIs and proline accumulation is crucial for plant resilience to environmental stresses. Proline not only protects against oxidative stress but also facilitates the optimal functioning of PDIs in refolding and stabilizing proteins. Understanding this interaction can potentially lead to advances in agricultural practices, such as breeding stress-tolerant varieties through the manipulation of proline metabolism and PDI expression [6,49]. Also, the studies indicated that the activity of various PDI gene families is often upregulated in conditions where plants are exposed to prolonged stress. This upregulation suggests a synergistic relationship where proline enhances the effectiveness of PDIs, allowing better management of protein misfolding that occurs under stress [50].

Many studies have examined the transfer of senescence-associated genes (SAGs) to tobacco leaves [51], indicating a rise in ROS levels in chloroplasts and involvement in the regulation of the senescence process [52]. Among the ROSs it can be mentioned to peroxide hydrogen, which is a signaling molecule that produces a toxic product (·OH) in cell metabolism, and it extremely regulates its cellular level under normal conditions [53]. A high concentration of endogenous H_2O_2 has also been reported in iris flowers due to reduced ascorbate peroxidase (APX) and CAT activities, along with lipoxygenase (LOX) [54]. According to the previous reports, the activities of CAT, peroxidase (POD), and ascorbate peroxidase increased until stage 5 of rosebud growth (fully opened petals) and then decreased. The results indicated a reduced activity of antioxidant enzymes like CAT and SOD, which were negatively correlated with elevated H_2O_2 levels (Figure 6a-d). Therefore, the likely reason for premature flower senescence in transgenic lines is the addition of internal H_2O_2 levels and the reduction in antioxidant enzyme activity, which are partly responsible for the onset of senescence in flowers, as has been formerly reported in roses [9].

In this study, the activity of SOD in transgenic plants decreased over time and exhibited a negative correlation with H_2O_2 levels throughout the experiment (Figure 6c). The relationship between SOD activity and H_2O_2 levels in aging flowers is a crucial aspect of understanding oxidative stress and plant physiology, particularly in the context of floral senescence and longevity. A negative correlation between SOD activity and H_2O_2 levels suggests that as flowers age, their ability to dismutate superoxide radicals ($.O^{2-}$) into less harmful species, including H_2O_2, diminishes while H_2O_2 levels increase.

Protein disulfide isomerase is a crucial enzyme located in the endoplasmic reticulum (ER) that facilitates the oxidative folding of proteins by catalyzing the formation of disulfide bonds. This process is essential for the proper folding of many secretory and membrane proteins [59]. During oxidative protein folding, H_2O_2 is generated as a byproduct; it is estimated that about 25% of the cellular reactive oxygen species (ROS) produced during protein synthesis comes from this pathway [55]. Recent studies highlight that PDI accumulation leads to heightened oxidative protein folding, resulting in elevated levels of H_2O_2 released into the nucleus. This H_2O_2 can then induce the expression of various stress-related proteins, such as PDIs, which are known to further promote cellular senescence [34,55]. Previous qRT-PCR studies showed that *RhPDI6* gene expression was higher in Cool Water (with a shorter shelf-life), compared to Marussia (with a higher shelf-life), in response to ethylene across various reproductive tissues [34]. This aligns with [28], who reported elevated *RhAC (RhPDI6)* expression in the short-lived Black Magic rose cultivar during natural senescence [28]. Accordingly, in transgenic lines, the expression of *RhPDIA6* along with H_2O_2 was higher than in wild-type and control plants upon reaching the flowering stage (Figure 7).

Gene similarity to other genes associated with flower senescence

Numerous studies have shown that thousands of genes are regulated in specific tissues at any given time, with hundreds exhibiting expression changes during PCD or senescence [18], and PDIs are among them. A study was conducted to investigate the potential similarity between the *RhPDIA6* sequence and known functional sequences in databases during senescence, utilizing the Leaf Senescence Database. Then, the resulting visualization helps researchers quickly identify which sequences have significant alignments based on the color-coded scores, aiding in comparative analysis and interpretation of biological data. *Arabidopsis thaliana* LHY/CCA1-like 1 (LCL1), as the reference gene, regulates the Circadian Clock of plants, which regulates the timing of leaf senescence, syncing it with environmental cues like light and day length [56]. Also, LCL1 negatively regulates genes involved in senescence, delaying the aging process. Also, LCL1 modulates ABA, ethylene, and jasmonic acid pathways, which are key senescence signals. Its activity balances growth and aging by suppressing early activation of these hormonal signals. As a light-responsive gene, LCL1 helps delay senescence under favorable light conditions. Under stress or darkness, its expression may drop, allowing senescence to proceed [57].

As a result, *RhPDI6* showed high homology with senescence-associated genes (SAGs) (Figure 8) and exhibited an identity of 81 bits, homologous to *PDIL5-2 PDI-like 5-2* from *Arabidopsis thaliana* (Table 2). It encodes a protein disulfide isomerase-like (PDIL) protein, a member of a multigene family within the thioredoxin (TRX) superfamily [58], which confirms the findings regarding the gene's role in accelerating aging within flower organs. However, investigating *RhPDIA6* function in roses via gene transfer, gene deletion, or gene editing techniques can be highly effective in elucidating its role in metabolic processes, particularly flower aging.

Conclusion

Understanding the genetics of roses, particularly concerning their senescence and flower quality, is paramount for the advancement of cut flower longevity. The role of proteins such as protein disulfide isomerases (PDIs) and the involvement of hormones like ethylene highlight the complexities of flower development and aging. The research on the *RhPDIA6* gene from *Rosa hybrida*, which was successfully transferred to tobacco plants, provides valuable insights into the genetic manipulation of flower traits. The observed changes in physiological parameters, including proline levels and antioxidant enzyme activities, suggest that targeted genetic engineering can significantly influence flowering time and durability. Overall, these findings have implications for enhancing agricultural practices and breeding programs aimed at producing roses with extended shelf-life, thus addressing the significant challenges faced in the floral industry. Previously, bioinformatics studies indicated the presence of ethylene-responsive elements in the promoter regions of the *RhPDIA6* gene [34]. The intricate interplay between ethylene and protein disulfide isomerases elucidates important pathways in plant growth, stress response, and ultimately, lifespan. In point of fact, studying the interaction of *RhPDIA6* and plant hormones such as ethylene is essential to understanding gene function during the aging process. The evidances suggests that manipulating PDI expression or activity can serve as a strategy to enhance the post-harvest longevity of crops and ornamental plants. Continued exploration of the intricate relationships between genes and flower characteristics will be essential for future developments in horticulture and floriculture.

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Author Contributions

Conceptualization, K.R.; methodology, T.L., M.A., and K.R.; software, T.L. and M.A.; validation, K.R., T.L., and A.M.; investigation, M.A.; writing—original draft preparation, M.A.; writing—review and editing, K.R, T.L., and A.M.; visualization, M.A.; resources, M.A; data curation, K.R. and M.A.; supervision, K.R. and T.L.; project administration, K.R.

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Data Availability Statement

The authors confirm that all the data about the manuscript are already presented in this manuscript or available from the authors on reasonable request.

References

1. Pourghorban M, Azadi P, Khaghani S, Mirzakhani A, Changizi M, Edrisi B (2020) Propagation of three cultivars of Rosa hybrida L. through stenting method. Int J Hort Sci & Technology, 7: 27-36.

2. Salunkhe DK, Bhat NR, Desai BB (2012) Postharvest biotechnology of flowers and ornamental plants. Springer Berlin Heidelberg.

3. Rogers HJ (2012) Is there an important role for reactive oxygen species and redox regulation during floral senescence? Plant Cell Environ, 35: 217-33.

4. Rani P, Singh N (2014) Senescence and postharvest studies of cut flowers: A critical review. Pertanika J Trop Agric Sci, 37: 159-201.

5. Ahmad SS, Tahir I (2016) Increased oxidative stress, lipid peroxidation and protein degradation trigger senescence in Iris versicolor L. flowers. Physiol Mol Biol Plants, 22: 507-14.

6. Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A (2012) Role of proline under changing environments: A review. Plant Signal Behav, 7: 1456-66.

7. Per TS, Khan NA, Reddy PS, Masood A, Hasanuzzaman M, Khan MIR, Anjum NA (2017) Approaches in modulating proline metabolism in plants for salt and drought stress tolerance: Phytohormones, mineral nutrients and transgenics. Plant Physiol Biochem, 115: 126-40.

8. Kumar N, Pal M, Srivastava GC (2009) Proline metabolism in senescing rose petals (Rosa hybrida L. ‘First Red’). J Hort Sci Biotechnol, 84: 536-40.

9. Kumar N, Srivastava G, Dixit K (2008) Senescence in rose (Rosa hybrida L.): Role of the endogenous antioxidant system. J Hort Sci Biotechnol, 83: 125-31.

10. Dar RA, Nisar S, Tahir I (2021) Ethylene: A key player in ethylene sensitive flower senescence: A review. Sci Hortic, 290: 110491.

11. Ul Haq A, Farooq S, Lone M, Parveen S, Altaf, Tahir I (2023) Flower senescence coordinated by ethylene: An update and future scope on postharvest biology in the “Buttercup” family. J Plant Growth Regul, 43.

12. Wang Y, Zhao H, Liu C, Cui G, Qu L, Bao M, Wang J, Chan Z, Wang Y (2020) Integrating physiological and metabolites analysis to identify ethylene involvement in petal senescence in Tulipa gesneriana. Plant Physiol Biochem, 149: 121-31.

13. Parveen S, Altaf F, Farooq S, Lone ML, Haq AU, Tahir I (2023) The swansong of petal cell death: Insights into the mechanism and regulation of ethylene-mediated flower senescence. J Exp Bot.

14. Shibuya K (2018) Molecular aspects of flower senescence and strategies to improve flower longevity. Breed Sci, 68: 99-108.

15. Singh P, Singh AP, Sane AP (2019) Differential and reciprocal regulation of ethylene pathway genes regulates petal abscission in fragrant and non-fragrant roses. Plant Sci, 280: 330-9.

16. Xia Y, Kuan C, Chiu CH, Chen XJ, Do YY, Huang PL (2016) Global transcriptomic analysis of targeted silencing of two par-

alogous ACC oxidase genes in banana. Int J Mol Sci, 17.

17. Shahri W, Tahir I (2011) Flower senescence-strategies and some associated events. Trop Bot Rev, 77: 152-84.

18. Tripathi SK, Tuteja N (2007) Integrated signaling in flower senescence: An overview. Plant Signal Behav, 2: 437-45.

19. Teixeira da Silva J (2006) Ornamental cut flowers: Physiology in practice.

20. Oloo-Abucheli G, Aguyoh JN, Liu G (2017) Effect of light and temperature through poly film covers on anthocyanin content in rose cut flowers. Int J Plant Soil Sci, 20: 1-10.

21. Khan MR, Haque Z (2024) Diseases of ornamental, aromatic and medicinal plants. Bentham Science Publishers Pte. Limited.

22. Verma J, Singh P (2021) Post-harvest handling and senescence in flower crops: An overview. Agric Rev.

23. Fridman AL, Tainsky MA (2008) Critical pathways in cellular senescence and immortalization revealed by gene expression profiling. Oncogene, 27: 5975-87.

24. Panavas T, Pikula A, Reid PD, Rubinstein B, Walker EL (1999) Identification of senescence-associated genes from daylily petals. Plant Mol Biol, 40: 237-48.

25. Iqbal N, Khan NA, Ferrante A, Trivellini A, Francini A, Khan MIR (2017) Ethylene role in plant growth, development and senescence: Interaction with other phytohormones. Front Plant Sci, 8: 475.

26. Ha STT, In B-C (2021) Ethylene biosynthesis, signaling, and regulation in roses. Flower Res J, 29: 1-10.

27. Khatami F, Najafi F, Yari F, Khavari-Nejad RA (2020) Expression of etr1-1 gene in transgenic Rosa hybrida L. increased postharvest longevity through reduced ethylene biosynthesis and perception. Sci Hortic, 263: 109103.

28. Hajizadeh H, Mostofi Y, Razavi K, Zamani Z, Mousavi A (2013) Gene expression in opening and senescing petals of rose (Rosa hybrida L.). Acta Physiol Plant, 36: 199-206.

29. Wilkinson B, Gilbert HF (2004) Protein disulfide isomerase. Biochim Biophys Acta, 1699: 35-44.

30. Laurindo FR, Pescatore LA, Fernandes Dde C (2012) Protein disulfide isomerase in redox cell signaling and homeostasis. Free Radic Biol Med, 52: 1954-69.

31. Soares Moretti AI, Martins Laurindo FR (2017) Protein disulfide isomerases: Redox connections in and out of the endoplasmic reticulum. Arch Biochem Biophys, 617: 106-19.

32. Xu X, Chiu J, Chen S, Fang C (2021) Pathophysiological roles of cell surface and extracellular protein disulfide isomerase and their molecular mechanisms. Br J Pharmacol, 178: 2911-30.

33. Victor P, Sarada D, Ramkumar KM (2021) Crosstalk between endoplasmic reticulum stress and oxidative stress: Focus on protein disulfide isomerase and endoplasmic reticulum oxidase 1. Eur J Pharmacol, 892: 173749.

34. Anbaz M, Bayanati M, Lohrasebi T, Mousavi A, Razavi K (2023) Are protein disulfide isomerases effectively involved in the

rose senescence phenomenon? Sci Hortic, 311: 111783.

35. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant, 15: 473-97.

36. Gallois P, Marinho P (1995) Leaf disk transformation using Agrobacterium tumefaciens-expression of heterologous genes in tobacco. Methods Mol Biol, 49: 39-48.

37. Maehly AC (2006) The assay of catalases and peroxidases.

38. Giannopolitis CN, Ries SK (1977) Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol, 59: 309-14.

39. Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil, 39: 205-7.

40. Junglee S, Urban L, Huguette S, Lopez F (2014) Optimized assay for hydrogen peroxide determination in plant tissue using potassium iodide. Am J Anal Chem, 5: 730-6.

41. Bélanger JG (2019) The expansion and diversification of the CCA1-LHY-RVE gene family in monocots and dicots. McGill University (Canada).

42. Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res, 30: e36.

43. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods, 25: 402-8.

44. Castro-Camba R, Sánchez C, Vidal N, Vielba JM (2022) Plant development and crop yield: The role of gibberellins. Plants, 11: 2650.

45. Carrillo R, Iwai K, Albertson A, Dang G, Christopher DA (2024) Protein disulfide isomerase-9 interacts with the lumenal region of the transmembrane endoplasmic reticulum stress sensor kinase, IRE1, to modulate the unfolded protein response in Arabidopsis. Front Plant Sci, 15.

46. Steffens B, Wang J, Sauter M (2006) Interactions between ethylene, gibberellin and abscisic acid regulate emergence and growth rate of adventitious roots in deepwater rice. Planta, 223: 604-12.

47. Heuer B (2010) Role of proline in plant response to drought and salinity.

48. Valentovič P, Luxová M, Kolarovič L, Gašparíková O (2006) Effect of osmotic stress on compatible solutes content, membrane stability and water relations in two maize cultivars. Plant Soil Environ, 52: 186-91.

49. Zhang H, Zhang Y, Cui K, Liu C, Chen M, Fu Y, Li Z, Ma H, Zhang H, Qi B, Xu J (2024) A global identification of protein disulfide isomerases from 'Duli' pear (Pyrus betulaefolia) and their expression profiles under salt stress. Genes (Basel), 15.

50. Selles B, Jacquot J-P, Rouhier N (2011) Comparative genomic study of protein disulfide isomerases from photosynthetic organisms. Genomics, 97: 37-50.

51. Pak Dek MS, Padmanabhan P, Sherif S, Subramanian J, Paliyath G (2017) Upregulation of phosphatidylinositol 3-kinase

(PI3K) enhances ethylene biosynthesis and accelerates flower senescence in transgenic Nicotiana tabacum L. Int J Mol Sci, 18: 1533.

52. Mayta ML, Lodeyro AF, Guiamet JJ, Tognetti VB, Melzer M, Hajirezaei MR, Carrillo N (2018) Expression of a plastid-targeted flavodoxin decreases chloroplast reactive oxygen species accumulation and delays senescence in aging tobacco leaves. Front Plant Sci, 9: 1039.

53. Gechev TS, Hille J (2005) Hydrogen peroxide as a signal controlling plant programmed cell death. J Cell Biol, 168: 17-20.

54. Zeng F, Pan K, Xu S, Hu C, Geng X (2025) Differential effects of hydrogen peroxide on the senescence and antioxidant defence mechanisms in ethylene-sensitive and ethylene-insensitive cut flowers. Hortic Environ Biotechnol.

55. Cheng F, Ji Q, Wang L, Wang CC, Liu GH, Wang L (2023) Reducing oxidative protein folding alleviates senescence by minimizing ER-to-nucleus H2O2 release. EMBO Rep, 24: e56439.

56. Ding Z, Doyle MR, Amasino RM, Davis SJ (2007) A complex genetic interaction between Arabidopsis thaliana TOC1 and CCA1/LHY in driving the circadian clock and in output regulation. Genetics, 176: 1501-10.

57. Marcolino-Gomes J, Rodrigues FA, Fuganti-Pagliarini R, Bendix C, Nakayama TJ, Celaya B, Molinari HBC, De Oliveira MCN, Harmon FG, Nepomuceno A (2014) Diurnal oscillations of soybean circadian clock and drought responsive genes. PLoS One, 9: e86402.

58. Hori K, Okunishi T, Nakamura K, Iijima K, Hagimoto M, Hayakawa K, Shu K, Ikka T, Yamashita H, Yamasaki M (2022) Genetic background negates improvements in rice flour characteristics and food processing properties caused by a mutant allele of the PDIL1-1 seed storage protein gene. Rice, 15: 13.

59. Zito E (2015) ERO1: A protein disulfide oxidase and H2O2 producer. Free Radic Biol Med, 83: 299-304.