Effects of Trehalose and Sucrose on Gene Expression Related to Senescence of Cut Astilbe (Astilbe × arendsii Arends) Flowers

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Cut astilbe (Astilbe × arendsii) inflorescences have many small florets with low ethylene sensitivity, and a short vase life. The senescence of flowers is complex and probably regulated by programmed cell death, but specific senescence-related genes have not been widely studied, apart from ethylene-sensitive species. In this study, we investigated the effects of continuous treatments with 4% sucrose (Suc), 2% trehalose (Tre), and a combination (Suc+Tre) on postharvest quality and senescence-related gene expression in cut flowers of the astilbe ‘Gloria Purpurea’. Vase life was extended from 3.7 days to 10.3, 9.4, and 12.4 days by treatment with Suc, Tre and Suc +Tre, respectively. Florets were sampled at 0 days (0 d) and 2 days after harvest (2 d). De novo RNA-sequencing (RNA-seq) of floret tissues produced 89,705 unique sequences, and 2,517, 979, 609, and 1,846 differentially expressed genes were identified from the 0d_Control (Cont) vs. 2d_Cont, 2d_Cont vs. 2d_Suc, 2d_Cont vs. 2d_Tre, and 2d_Cont vs. 2d_Suc+Tre libraries, respectively. Gene Ontology (GO) analysis indicated that expression of many photosynthesis-related genes was upregulated in 2d_Cont florets, suggesting the photosynthesis system is activated in senescing florets. Kyoto Encyclopedia of Genes and Genomes analysis showed that WRKY22, related to leaf senescence, was more highly upregulated in 2d_Cont compared with 0d_Cont, 2d_Suc, and 2d_Tre in the Mitogen-activated Protein Kinase (MAPK) signaling pathway. Using Fragments Per Kilobase per Million reads (FPKM) values from RNA-seq, candidate genes associated with senescence including the no apical meristem, ATAF1/2 and cup-shaped cotyledon transcription factor 29 (NAC029), senescence-associated gene 12 (SAG12), and peroxidase 21 (PER21) were selected. Suc+Tre in particular, the most effective treatment for prolonging vase life, downregulated NAC029, SAG12, and PER21 expression at 2 d. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) validated the RNA-seq results, i.e., similar expression trends were found in the three genes, although relatively weak expression levels were detected compared with FPKM values from RNA-seq. The effect of Tre alone on the three genes’ expression levels was relatively small compared with treatments containing Suc, suggesting that Tre may delay floret senescence by altering genes involved in water transport such as Aquaporin tonoplast intrinsic protein 1-3. This study revealed several candidate genes and GO terms involved in the senescence of cut florets, but further study is needed, especially on key genes including NAC029 and WRKY22.

Key Words: gene ontology (GO) enrichment, Kyoto encyclopedia of genes and genomes (KEGG), reverse transcription-quantitative polymerase chain reaction (RT-qPCR), RNA-sequencing (RNA-seq), senescence-related genes.

Introduction

Astilbe (Astilbe × arendsii) is a perennial plant native to eastern Asia, which blooms in early summer. Commercial cultivars are bred in Europe, especially by Georg Arends, and it is used as an ornamental garden plant. The vase life of cut inflorescences is relatively short, partly due to vascular occlusion as a physiological response to the stress of cutting or by microbial
colonization in the vascular system (Kalkman, 1985; Loubaud and van Doorn, 2004; van Doorn et al., 1993; Villanueva et al., 2019).

The plant hormone ethylene is known to promote petal senescence in several plant species, but some are less affected (Shibuya, 2012; van Doorn, 2001). Astilbe was not evaluated for ethylene sensitivity due to the occurrence of vascular blockage (Woltering and van Doorn, 1988), but its ethylene sensitivity was suggested to be relatively low (Villanueva et al., 2019).

Sucrose is one of the sugars used to extend the life of cut flowers (Cho et al., 2001; Ichimura and Hisamatsu, 1999; Ranwala and Miller, 2009). Sucrose treatment of cut rose flowers increases glucose and fructose concentrations in the vacuole, reduces the cellular osmotic potential and promotes water absorption into the cells, thereby promoting flowering (Norikoshi et al., 2016). In cut peony flowers, sucrose treatment was reported to extend vase life and affect expression of sucrose transporter and invertase genes, which are thought to be involved in maintaining the quality of peony petals (Xue et al., 2018).

Trehalose is a disaccharide with the structure α-D-glucopyranosyl-α-D-glucopyranosyl (Süssich et al., 1998). Trehalose is found in bacteria, yeasts, fungi, insects, and invertebrate animals, but is rarely found in higher plants (Müller et al., 1995). However, trehalose prolongs the vase life of cut flowers in several species such as gladiolus and tulip (Iwaya-Inoue and Takata, 2001; Otsubo and Iwaya-Inoue, 2000; Yamada et al., 2003; Yamane et al., 2005). Trehalose maintains turgor pressure in the petal tissues of cut flowers (Iwaya-Inoue and Takata, 2001; Otsubo and Iwaya-Inoue, 2000). Moreover, trehalose was reported as delaying the senescence process, i.e., programmed cell death (PCD), in gladiolus (Yamada et al., 2003).

In our previous paper (Villanueva et al., 2019), continuous treatment with trehalose and sucrose solutions was effective in maintaining development and delaying senescence of florets, consequently extending the vase life of cut astilbe inflorescences. Vase life was markedly prolonged by pulse-treatment with sugar, but was markedly prolonged by continuous treatment, suggesting that astilbe has high metabolic activity. In addition, combined treatment with trehalose and validamycin A, a potent inhibitor of trehalose metabolizing activity, induced severe wilting of florets and necrotic spots on leaves (Villanueva et al., 2019). However, to our knowledge, there are no reports on the effects of these sugar treatments on aging-related gene expression.

Senescence and PCD in petals are genetically programmed processes and are controlled by developmental stages (Rogers, 2006, 2013; van Doorn and Woltering, 2008). To identify regulators of the age-dependent PCD regulatory pathways in petal aging, regardless of ethylene signaling, ethylene-insensitive flowers including Hemerocallis hybrid (Panavas et al., 1999), Iris hollandica (van Doorn et al., 2003), and Alstroemeria (Breeze et al., 2004) were used. However, PCD-specific genes for petal senescence were not identified in these species to date. Conversely, recent studies suggested that a NAC transcription factor EPHEMERAL1 (EPH1) is an important regulator of age-dependent PCD in petal senescence in the morning glory Ipomoea nil (Shibuya et al., 2014, 2018).

NAC proteins are named after the initials of no apical meristem in petunia and ATAF1-2, and cup-shaped cotyledon 2 in Arabidopsis, the consensus sequences of which were used to characterize the NAC domain (Ooka et al., 2003). NAC family genes are plantspecific transcription factors and were reported to be involved in developmental processes and stress responses in various tissues (Ni et al., 2017; Olsen et al., 2005; Ooka et al., 2003; Puranik et al., 2012; Yeung et al., 2018). In particular, AtNAP (ANAC029), ORESARA1 (ORE1/ANAC029/AtNAC2), and VND-INTERACTING2 (VNI2/ANAC083) were reported to control leaf senescence (Balazadeh et al., 2010; Guo and Gan, 2006; Kim et al., 2009; Yang et al., 2011; Yeung et al., 2018). NAC genes were also implicated in petal aging as mentioned above (Shibuya et al., 2014, 2018; Trupkin et al., 2019).

WRKY transcription factor family genes are related to various biotic and abiotic stress responses (Woo et al., 2013). Particularly, WRKY22 and WRKY6 were suggested to control leaf senescence (Robatzek and Somssich, 2001; Woo et al., 2013; Zhou et al., 2011).

Expression of senescence-related gene 12 (SAG12) and peroxidases are upregulated in petal senescence (Noh and Asamino, 1999; Oh et al., 1997; Quirino et al., 2000). In addition, SAG12 is known as a marker gene for senescence (Quirino et al., 2000).

In this study, we investigated the effects of sucrose and trehalose treatments on gene expression using RNA-sequencing (RNA-seq) to search for senescence-related genes in cut astilbe flowers. Differentially expressed genes (DEGs) were analyzed using the Gene ontology (GO) (Harris et al., 2004) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000) databases to uncover gene expression profiles in senescing flowers. In addition, we focused on candidate genes and investigated expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Materials and Methods

Plant materials

Astilbe (Astilbe ×arendsii) ‘Gloria Purpurea’ plants were grown in an experimental field at Utsunomiya University (36°32'55" N, 139°54'42" E). Cut inflorescences without leaves were harvested when 50% of florets were open. The cut flowers were stored and evaluated at 20–21°C and 60% relative humidity under cool white fluorescent light of 15 μmol·m⁻²·s⁻¹ photo-
synthetic photon flux density in the following experiments. Cut inflorescences without leaves were continuously treated with distilled water as a Control (Cont), 4% sucrose solution (Suc), 2% trehalose solution (Tre), and 4% sucrose plus 2% trehalose (Suc+Tre). To prevent bacterial contamination, all solutions were treated with 200 ppm (v/v) Kathon CG (The Dow Chemical Company, Midland, MI, USA). Three to six cut inflorescences were used for each treatment. Vase life was evaluated daily until 50% of florets on the inflorescence were wilted or faded. We used flowers grown in 2017 for observation of florets under a stereo-microscope and RNA-seq, and those grown in 2018 for measurement of vase life, soluble sugar content, chlorophyll content, and RT-qPCR.

Measurement of soluble sugar content

Soluble sugar contents were measured in the florets of cut inflorescences from each treatment at 0, 2, 4, and 6 days after harvest (d). Florets were sampled from the lower part of the inflorescences and dried with a freeze dryer (FDU-1200; TOKYO RIKAKIKAI Co., Ltd., Tokyo, Japan). Five replicates of dried samples were weighed out to 0.05 g and 5% rhamnose was added as an internal standard. Soluble sugars in the samples were extracted with 80% ethanol at 80°C for 1 h. The samples were centrifuged at 1,550 rpm for 10 min and the supernatant recovered. Ethanol and water were re‐moved by a centrifugal evaporator (CVE-200D; TOKYO RIKAKIKAI) for 2–3 h. The resultant pellets were dissolved in 300 μL of ultra-pure water and filtered through a 0.22 μm filter. The samples were analyzed by high-performance liquid chromatography (SCL-6B; Shimadzu Corporation, Kyoto, Japan) using a refractive index detector (RI-8022; Tosoh Corporation, Tokyo, Japan). The separation column (TSK-gel Amide 80; Tosoh) was eluted with 75% acetonitrile at a flow rate of 1 mL·min⁻¹ and an oven temperature of 80°C. Glucose, fructose, sucrose, and trehalose were identified and quantified by comparison with authentic standards.

Total RNA isolation

Total RNA was isolated from florets of cut inflorescences from each treatment at 0, 2, 4, and 6 d. 100 mg samples of frozen plant tissue were ground in liquid nitrogen to a fine powder. Total RNA was isolated in two steps. First, total RNA was extracted twice using cetyltrimethylammonium bromide (CTAB) buffer and chloroform:isoamyl alcohol (24:1) according to the methodology of Chang et al. (1993). Then, total RNA was isolated using a Maxwell® RSC Instrument (Promega, Madison, WI, USA) with a Maxwell® RSC Plant RNA Kit (Promega) according to the protocol. The isolated total RNA was quantified and examined for protein contamination (A260/A280) and reagent contamination (A260/A230) using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). After that, RNA integrity was evaluated using a 1.0% agarose gel stained with ethidium bromide.

Library preparation and Illumina sequencing

The RNA integrity number (RIN) was determined for RNA-seq using an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) with an Agilent RNA 6000 Nano kit (Agilent Technologies). Libraries for RNA-seq were constructed using a KAPA Stranded mRNA-Seq Kit (Roche Sequencing and Life Science, Indianapolis, IN, USA), according to the manufacturer’s instructions. Double‐stranded cDNA (ds cDNA) fragments were purified to remove free nucleotides, enzymes, buffer, and RNA with AMPure XP beads (Beckman Coulter, Brea, CA, USA). The purified ds cDNA fragments were ligated with adaptors (Set A and Set B from TruSeq DNA Sample Prep LT Kits; Illumina, San Diego, CA, USA). After library preparation, the libraries were quantified with a LightCycler® 480 Instrument (Roche, Basel, Switzerland) and a Kapa Library Quantification Kit (Roche Sequencing and Life Science), according to the manufacturer’s instructions. Then, each library was pooled to 4 nM and quantified again. Finally, the pooled libraries were sent to a sequencing facility for cluster generation on a MiSeq Sequencer (Illumina). Each paired-end library was prepared following the protocol of the Illumina MiSeq Reagent Kit v3 (150 cycles; Illumina). Six biological replicates of the 0 d samples and three biological replicates of the 2d_Cont, 2d_Suc, 2d_Tre, and 2d_Suc+Tre samples were sequenced.

Bioinformatics analysis

The quality of the sequence reads was checked with FastQC v0.11.3. Raw reads were first processed using Trimmomatic-0.36 (Bolger et al., 2014) to remove adapter sequences, low quality ends (quality scores < 30) and the last 76th base, while reads shorter than 50-bp were discarded. The high-quality short reads were assembled into unigenes using the Trinity program version 2.4.0 (Haas et al., 2013). rRNA sequences were excluded from the unigenes by removing sequences matched to the SILVA rRNA database by the megablast program (Zhang et al., 2000). The high-quality short reads were mapped to the rRNA-removed unigenes using Bowtie (Langmead et al., 2009).

To estimate expression levels of the transcripts, the number of uniquely mapped reads for each unigene was determined and normalized to FPKM values (Fragments Per Kilobase per Million reads) using the RSEM method (Li and Dewey, 2011). The FPKM values were then compared pairwise as follows: 0d/2d_Cont, 2d_Cont/2d_Suc, 2d_Cont/2d_Tre, and 2d_Cont/2d_Suc+Tre. To investigate DEGs, the edgeR package (Robinson et al., 2010) was used to compute P values and fold change (FC) values. The P value was
used to identify genes expressed differentially between paired treatments. Significant DEGs were identified using a false discovery rate (FDR) threshold of ≤ 0.05 and |logFC| ≥ 1.

To predict the biological functions of the unigenes, the sequences were annotated against the Universal Protein Resource KnowledgeBase (UniProtKB)/Swiss-Prot database (NCBI swissprot) and NCBI nr/nt database (NCBI nucleotide collection) using the blastx and blastn programs. Additionally, putative coding regions were extracted from the Trinity transcripts using TransDecoder. Then, the coding regions were annotated by GO annotation against the Pfam database, and those data used for GO enrichment analysis. Results of GO enrichment analysis were shown by z-score bar plot of GO terms. A higher z-score indicated the GO term as increasing while a lower z-score indicated it as a decreasing GO term. Heatmaps of genes classified into five GO terms related to senescence were drawn using shinyheatmap (<http://shinyheatmap.com/>). The color intensity represented the up- (red) or down- (green) regulated FPKM values. Subsequently, KEGG pathway mapping of the unigenes was done using BlastKOALA (KEGG Orthology And Links Annotation) (Kanehisa et al., 2016).

Quantitative gene expression analysis

RT-qPCR was performed to validate the mRNA abundance of three genes that were significantly affected by sugar treatment in the RNA-seq analysis and previous reports. For each sample, 1 μg of total RNA was used with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. The cDNA was stored at −20°C until use in the RT-qPCR analysis. The RT-qPCR assay was performed for five biological replicates with three technical replicates using the QuantiTect SYBR Green PCR Kit (Qiagen) on a LightCycler® 480 Instrument (Roche) in a total volume of 10 μL. The PCR cycle comprised one 900 s cycle at 95°C, followed by 45 cycles at 94°C for 15 s, 62°C for 30 s and 72°C for 30 s. All amplified products were subjected to melt curve analysis. A negative control without a cDNA template was run with all analyses to evaluate the overall specificity. The reference gene Actin97 was used to normalize the total amount of cDNA in each reaction. The Actin gene is stably expressed during petal senescence (Yamada et al., 2007). Amplification efficiency and relative gene expression levels were calculated according to the Pfaffl method (Pfaffl, 2001). Gene-specific primers were designed using Primer3Plus (<https://primer3plus.com/cgi-bin/dev/primer3plus.cgi>; Table S1).

Measurement of chlorophyll content and observation of florets under a stereomicroscope

Chlorophyll content was measured with reference to Yamane et al. (2008). The astilbe florets used for RNA isolation were also used for measurement of chlorophyll content. A 30 mg sample was placed in a 1.5 mL tube. Then, 900 μL of 100% methanol was added to the sample tube. After 24 h, 100 μL of ultra-pure water was added to the tube with the sample and 100% methanol to obtain 90% methanol. OD$_{663}$ and OD$_{645}$ were measured and calculated using the following equation: chlorophyll $a$ (mg) = 0.00162 (45.6 OD$_{663}$ − 9.27 OD$_{645}$); chlorophyll $b$ (mg) = 0.00149 (82.04 OD$_{645}$ − 16.75 OD$_{663}$). The results were presented as the sum of chlorophyll $a$ and $b$.

Cont florets were observed under a stereomicroscope (MZ16F; Leica Microsystems, Germany) at 0 d, 3 d, 5 d, and 9 d.

Results and Discussion

Effects of sucrose and trehalose on ornamental quality and soluble sugar contents

Vase life was significantly extended from 3.7 days to 10.3, 9.4, and 12.4 days by Suc, Tre, and Suc+Tre, respectively (Fig. S1), re-confirming that the continuous application of sucrose and trehalose is effective for extending the vase life of cut ‘Gloria Purpurea’ inflorescences.

Suc+Tre increased the total soluble sugar content from 22.2 in Cont to 60.2 mg·g$^{-1}$FW (Fig. 1A). Suc and Tre significantly increased the total soluble sugar content compared with Cont at 4 d, while there was no significant difference between Tre and Cont at 6 d.

![Fig. 1.](image-url) Effects of continuous treatments with 4% sucrose (Suc), 2% trehalose (Tre), and Suc plus Tre (Suc+Tre) on total soluble sugar (A) and trehalose (B) concentrations in the florets of cut inflorescences of the astilbe ‘Gloria Purpurea’. Vertical bars indicate the SE (n = 5). Means with the same letter on the same day are not significantly different according to the Tukey–Kramer HSD test (P < 0.05).
The cut astilbe flowers absorbed trehalose in Tre and Suc+Tre, whereas trehalose was not detected in Cont or Suc (Fig. 1B). Pulse treatments were inadequate to maintain the sugar contents and respiration activity in our previous paper (Villanueva et al., 2019), while continuous treatments of Suc and Suc+Tre significantly raised sugar contents until 6 d (Fig. 1B). Contents of fructose, glucose and sucrose showed a similar tendency to total soluble sugar content (Fig. S2).

**De novo transcriptome assembly and DEG analysis**

The high-quality short reads from the transcriptome sequencing reads were assembled into unigenes using the Trinity program. rRNA sequences were excluded from the unigenes by removing those matched to the SILVA rRNA database. We obtained 89,705 unique sequences with an average sequence length of approximately 948 bp and a total sequence length of 85,029,113 bases (Table 1). N50 was 1,484 bp.

We compared gene expression among florets of 0d, 2d_Cont, 2d_Suc, 2d_Tre, and 2d_Suc+Tre and identified DEGs. The numbers of significant DEGs with FDR ≤ 0.05 and |logFC| ≥ 1 obtained by comparing 0d vs. 2d_Cont, 2d_Cont vs. 2d_Suc, 2d_Cont vs. 2d_Tre, and 2d_Cont vs. 2d_Suc+Tre libraries were 2,517, 979, 609, and 1,846, respectively. Details in Table S2.

**GO enrichment analysis of DEGs**

The significant DEGs in each treatment were subjected to GO enrichment analysis and most were categorized into ‘biological process’ (BP), ‘molecular function’ (MF), and ‘cellular component’ (CC).

Among the top hit MF categories, motor activity, structural constituent of cuticle, exodeoxyribonuclease VII activity and lipid binding were significantly enriched when comparing 0d_Cont vs. 2d_Cont (Fig. 2A). Structural constituent of ribosome, structural constituent of cuticle, protein binding, and sulfotransferase activity were the most enriched MF categories in 2d_Suc vs. 2d_Cont (Fig. 2B). Structural constituent of cuticle, protein binding, and sulfotransferase activity were the most enriched MF categories in 2d_Tre vs. 2d_Cont (Fig. 2C). Structural constituent of ribosome, structural constituent of cuticle, protein binding, and sulfotransferase activity were the most enriched MF categories in 2d_Suc+Tre vs. 2d_Cont (Fig. 2D).

**Table 1. Summary of assembly.**

| Total sequences | 89,705 |
|-----------------|--------|
| Total bases     | 85,029,113 |
| Min sequence length (bp) | 201 |
| Max sequence length (bp) | 11,535 |
| Average sequence length (bp) | 948 |
| N50 length (bp) | 1,484 |
| (G+C)s          | 43.01% |

![Fig. 2. GO enrichment analysis. Enrichment of gene ontology terms in DEGs in 0d_Cont vs. 2d_Cont (A), 2d_Suc vs. 2d_Cont (B), 2d_Tre vs. 2d_Cont (C), and 2d_Suc+Tre vs. 2d_Cont (D). The color intensity represented the value of z-score. A higher z-score indicated the GO term as increasing, while a lower z-score indicated it as a decreasing GO term.](image-url)
activity were the most highly enriched terms in 2d_Cont vs. 2d_Suc (Fig. 2B). In 2d_Cont vs. 2d_Tre, structural constituent of cuticle, heme binding, peroxidase activity, terpene synthase activity, protein kinase activity, and structural constituent of ribosome were in the top hit GO terms (Fig. 2C). Cysteine-type peptidase activity, sulfotransferase activity, oxidoreductase activity, acting on NAD(P)H, quinone or a similar compound as acceptor and chitin binding were among the most highly enriched terms of 2d_Cont vs. 2d_Suc+Tre (Fig. 2D).

In the BP category, protein polymerization, lipoprotein metabolic process and chitin metabolic process were significantly enriched between 0d and 2d_Cont (Fig. 2A), while translation was the most highly enriched term in 2d_Cont vs. 2d_Suc (Fig. 2B). Oxidation-reduction process, viral capsid assembly, pathogenesis and protein phosphorylation were enriched in 2d_Cont vs. 2d_Tre (Fig. 2C). Photosynthesis and transmembrane transport were among the most highly enriched terms in 2d_Cont vs. 2d_Suc+Tre (Fig. 2D).

In the CC category, myosin complex, photosystem, and protein complex were significantly enriched between 0d and 2d_Cont (Fig. 2A). No CC terms were significant in 2d_Cont vs. 2d_Tre (Fig. 2C).

Heatmaps of genes relevant to senescence or specifically changed GO terms, including cysteine-type peptidase activity (Fig. 3A; Table S3A), peroxidase activity (Fig. 3B; Table S3B), photosystem I (Fig. S3A; Table S3C), photosystem II (Fig. S3B; Table S3D), and oxidoreductase activity (Fig. S3C; Table S3E) were also constructed.

Interestingly, photosystem II oxygen evolving complex, photosystem II and photosystem I were the most highly enriched terms in 2d_Cont vs. 2d_Suc (Fig. 2B). Moreover, photosystem I, photosystem II and photosystem II oxygen evolving complex were among the most highly enriched terms of 2d_Cont vs. 2d_Suc+Tre (Fig. 2D). Photosynthesis-related gene expression showed a tendency to be upregulated by senescence of florets from GO enrichment analysis. According to the heatmap (Fig. S3A, B), the expression of major photosystem-related genes was upregulated in 2d_Cont. On the other hand, treatments containing sucrose significantly suppressed expression of these genes, probably due to sufficient supply of the final product of photo-

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Fig. 3. Heatmaps of cysteine protease (A) and peroxidase activity (B) by GO enrichment analysis.
synthesis. Sugar treatments also suppressed expression of oxidoreductase activity, acting on NAD(P)H, quinone or a similar compound as acceptor group genes (Figs. 2D and S3C) and most of them were predicted to express in chloroplast (Table S3E). Arabidopsis and petunia contain chloroplasts in their petals, and chloroplasts increase or decrease by petal development stage (Pyke and Page, 1998; Trivellini et al., 2015; Weiss et al., 1988). Under stereomicroscopic observation, Suc+Tre florets maintained more vivid pink petals, styles, filaments and receptacles, while the receptacles turned greenish in Cont florets (Villanueva et al., 2019). The tepals were greenest around 5 d and then turned to yellow (Fig. S4). These observations were consistent with chlorophyll contents being significantly higher in Cont florets at 4 and 6 d (Fig. S5). Thus, chloroplasts may play some role in the senescence process of astilbe florets. In appearance, ovaries of florets did not develop during senescence. It is thought that photosynthesis activity can increase to support embryo development in intact plants, but it is not sufficient to support development in cut inflorescences without leaves under indoor conditions. Additional studies are required to determine whether chloroplasts have functions during flower senescence.

 Peroxidase, antioxidant-related and pathogenesis-related genes were upregulated in 2d_Tre (Fig. 2C). Peroxidases are upregulated in senescing plants (Oh et al., 1997). Moreover, the expression of peroxidases is upregulated in response to wounding in plants (Cheong et al., 2002). PATHOGENESIS-RELATED 10.1, which is induced by ethylene, may be important in preventing premature organ senescence in rose petals (Khashkheili et al., 2018; Wu et al., 2017). It was suggested that trehalose may function to suppress floret senescence regardless of gene expression in the early stage.

Sucrose transporter and invertase genes

Sucrose transporter and invertase genes were reported to be involved in translocation of sucrose from a vase solution and maintaining the quality of peony petals (Xue et al., 2018). This study found two sucrose transporter genes, i.e., sucrose transport protein 2 (SUC2) and sucrose transport protein 4 (SUC4), and three invertase genes, i.e., acid invertase (vacuole), invertase (soluble isoenzyme I, vacuole) and invertase 1 (cell wall). Sucrose transporter genes were upregulated in 2d_Cnt (Fig. S6A, B). On the other hand, sugar treatments suppressed upregulation of sucrose transporter genes. In particular, expression of SUC2 was inversely proportional to vase life (Figs. S1 and S6A). These results were contrary to those of Xue et al. (2018). Re-translocation of sucrose from senescing florets into blooming buds was reported in gladiolus (Yamane et al., 1993) and daylily (Bielski, 1995). Even though the respiration rates of cut inflorescences were constant (Villanueva et al., 2019), contents of total soluble sugars, especially fructose, sharply decreased in florets between 2 d and 4 d (Figs. 1 and S2). Taken together, this suggests sugars are exported through sucrose transporters such as SUC2 and SUC4 in the senescing florets of cut astilbe inflorescences, but further study is required to confirm this.

Expression of invertase (soluble isoenzyme I, vacuole) was downregulated in 2d_Cnt and 2d_Tre, but Suc and Suc+Tre upregulated expression of invertase (soluble isoenzyme I, vacuole) (Fig. S7A), suggesting that sucrose concentration promoted gene expression and metabolism of sucrose. In contrast, expression of acid invertase (vacuole) (Fig. S7B) and Invertase 1 (cell wall) (Fig. S7C) was downregulated in 2d_Cont and 2d_Tre, but tended to be downregulated by Suc+Tre. From these results, the relationship between invertase genes and senescence of astilbe florets was not clarified. Further study is recommended to explore invertase genes.

KEGG pathway analysis of DEGs

Among the DEGs with KO annotations, those with \(|\logFC| \geq 1\) were mapped (red points). The numbers of DEGs with KO annotations and \(|\logFC| \geq 1\) obtained by comparing 0d vs. 2d_Cnt, 2d_Cnt vs. 2d_Suc and 2d_Cnt vs. 2d_Tre libraries were 7,036, 1,959, and 2,811 respectively. Details are shown in Table S4.

Among KEGG pathways, the mitogen-activated protein kinase (MAPK) signaling pathway (Figs. S8, S9, S12, S13, S16, and S17) and starch and sucrose metabolism (Figs. S10, S11, S14, S15, S18, and S19) were focused on because MAPK signal transduction cascades are known regulators of various aspects of plant biology (Andreasonn and Ellis, 2010). In the MAPK signaling pathway, WRKY22 was upregulated in 2d_Cnt compared with 0 d (Fig. S9), 2d_Suc (Fig. S12), and 2d_Tre (Fig. S16). It was reported that WRKY22 is related to leaf senescence in Arabidopsis thaliana (Woo et al., 2013; Zhou et al., 2011). Dark-treated AtWRKY22 over-expression and knockout lines showed accelerated and delayed senescence phenotypes, respectively (Zhou et al., 2011). Moreover, WRKY22 is regulated by WRKY6 and WRKY6, which were suggested to control senescence (Robatzek and Somssich, 2001; Zhou et al., 2011). Therefore, it is necessary to examine the relationship between the WRKY transcription factor family and the senescence of astilbe florets.

In our previous paper (Villanueva et al., 2019), combined treatment of trehalose and validamycin A induced severe disorders. Validamycin A inhibits trehalose metabolic activity and excessive amounts of trehalose accumulated in floret and leaf tissues. Accordingly, we assumed that trehalose metabolic pathways would change in florets under trehalose treatment, especially trehalase (EC: 3.2.1.28). Trehalase was not mapped in any comparisons, contrary to expectation. Analysis of trehalose metabolic pathways is thus needed to under-
stand its role in the senescence of astilbe florets.

**Senescence-related genes**

We selected three DEGs as senescence-related genes from the top 500 logFC values in 0d vs. 2d_Cond, **Senescence-associated gene 12 (SAG12)**, ** Peroxidase 21 (PER21)**, and ** NAC transcription factor 29 (NAC029)**, based on our FPKM data (Table S5) and previous reports. To validate the results from the transcriptome analysis, these DEGs were analyzed by RT-qPCR.

SAG12 encodes cysteine protease and is a marker gene for senescence (Noh and Amasino, 1999; Quirino et al., 2000). The FPKM of SAG12 was upregulated at 2 d in Cond, Suc and Tre, but downregulated in 2d_Suc+Tre (Fig. 4A). Expression of SAG12 in florets by RT-qPCR was also downregulated until 6 d by Suc+Tre (Fig. 5A), suggesting that it may contribute to prolonged vase life by Suc+Tre. GO enrichment analysis showed that DEGs associated with cysteine-type peptidase activity were highly enriched in 2d_Cond vs. 2d_Suc+Tre (Fig. 2D). The expression of 31 of 56 cysteine-type peptidase group genes was actually suppressed in 2d_Suc+Tre compared to 2d_Cond (Fig. 3A). These results also suggest that Suc+Tre suppressed expression of cysteine-type peptidase group genes including SAG12 and thereby delayed senescence by affecting protein degradation. However, there was no significant difference between 0d_Cond and 2d_Cond (Fig. 5A), which suggested that the senescence of the lower part of the inflorescences may have already begun at the time of harvest when 50% of the florets were open. The expression level of 0d_Cond for the 23rd to 36th genes was higher than other treatments, but these expression levels were very low i.e., maximum FPKM was 10 (Fig. 3A; Table S3A). Therefore, it is necessary to investigate the expression level of SAG12 at an earlier stage, such as in unopened buds.

In previous reports, peroxidase activity increased with the progression of senescence (Abarca et al., 2001; Passardi et al., 2004). In the RNA-seq data, the FPKM of PER21 was increased in 2d_Cond (Fig. 4B). However, in RT-qPCR, the expression level of PER21 showed a tendency of upregulation in 2d_Cond and Tre (Fig. 5B). A positive correlation was observed between FPKM and relative expression at 0 d and 2 d. Suc+Tre suppressed PER21 expression in both the FPKM (Fig. 4B) and RT-qPCR (Fig. 5B) data, and prolonged vase life (Fig S1), implying that PER21 expression is associated with senescence of florets. Also, expression
levels of peroxidase activity genes were significantly suppressed in 2d_Suc+Tre (Fig. 3B). Furthermore, expression patterns of 2d_Suc and 2d_Tre differed from 2d_Cont. Thus, various peroxidase activity related genes are thought to be involved in promoting and delaying senescence. Additional studies are required to determine the role of each peroxidase gene.

Recent research on leaf and petal senescence found that NAC transcription factors regulate PCD induced by age and stress during the senescence of leaves and petals (Balazadeh et al., 2010; Guo and Gan, 2006; Kim et al., 2009; Shibuya et al., 2014; Trupkin et al., 2019; Yang et al., 2011; Yeung et al., 2018). The FPKM of NAC029 showed a high expression level in 2d_Cont similarly to PER21 (Fig. 4B, C). The FPKM of NAC029 was highest in Cont, followed by Suc or Tre alone treatment, and was the lowest in Suc+Tre treatment (Fig. 4C), suggesting that the expression of NAC029 corresponded to the vase life (Figs. 4C and S1). In the relative expression data, NAC029 showed a tendency to upregulation in 2d_Cont (Fig. 5C). Conversely, Suc+Tre significantly decreased the FPKM at 2 d (Fig. 4C) and maintained relative expression until 6 d (Fig. 5C). Suc and Tre showed a similar tendency to Suc+Tre. These results and previous studies suggest that NAC029 is related to senescence of astilbe florets. In morning glory, it was proposed that the NAC transcription factor EPHEMERAL1 primarily controls petal senescence by experiments using RNAi (Shibuya et al., 2014) and genome editing (Shibuya et al., 2018). Experiments including overexpression and/or suppression of NAC029 are needed to understand its role in the senescence of astilbe florets. Moreover, functional analysis of NAC029 and the NAC transcription family could potentially clarify the mechanism of ethylene-independent senescence.

Trehalose only treatment increased the expression levels of SAG12 (Figs. 4A and 5A) and PER21 (Figs. 4B and 5B). However, trehalose treatment maintained ornamental value (Fig. S1), and alleviated the increase in expression level of NAC029 (Figs. 4C and 5C). Interestingly, the expression level of Aquaporin tonoplast intrinsic protein 1-3 (Aquaporin TIP 1-3) showed a downregulation tendency only in 2d_Tre (Fig. 6), suggesting that trehalose is involved in water retention as reported previously (Iwaya-Inoue and Takata, 2001; Otsubo and Iwaya-Inoue, 2000) and may have quite a different function from sucrose. However, the mechanism by which trehalose delays the senescence of florets is not known.

WRKY22 was excluded from the RT-qPCR analysis because its logFC and FPKM values were smaller than those of the other three genes (Fig. 4D). However, its |logFC| was greater than 1 and we confirmed that its expression changed in the KEGG pathway analysis. Therefore, WRKY22 needs to be investigated in the future.

Conclusions

In this study, we investigated the effects of sugar treatments on gene expression in astilbe florets by next-generation sequencing and RT-qPCR. Suc, Tre, and Suc+Tre extended the vase life of cut inflorescences. GO enrichment analysis showed photosynthesis activity was increased in senescing florets. GO analysis also showed DEGs related to cysteine-type peptidase activity, sulfotransferase activity and oxidoreductase activity were downregulated in 2dSuc+Tre compared with 2d_Cont. KEGG analysis suggested the WRKY transcription factor family in the MAPK signaling pathway is involved in the senescence of florets.

The expression level of NAC029 was upregulated in 2d_Cont. These results and previous studies suggest that NAC029 is related to the senescence of astilbe florets. Experiments using astilbe with overexpressed or inhibited NAC029 are required to understand the role of NAC029 in the senescence of astilbe florets. A limitation of this study is that plants cultivated outdoors in 2017 and 2018 were used for RNA-seq and RT-qPCR, respectively; therefore, results may have been affected by weather conditions. Moreover, the mechanism by which trehalose delays the senescence of florets is not understood. In conclusion, this study revealed several candidate genes and GO terms such as cysteine-type peptidase activity and peroxidase activity, involved in the senescence of cut florets, but further study is required especially for key genes including NAC029 and WRKY22.

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