Postharvest Biology and Technology 152 (2019) 60–64

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Isolation and expression patterns of two novel senescence-associated genes *RhAA* and *RhCG* in rose (*Rosa hybrida*)

M. Bayanati[a,b], K. Razavi[b,*], A. Tehranifar[a], T. Lohrasebi[b], S.H. Neamati[a], N. Ahmadi[c]

[a] Department of Horticultural Science, Ferdowsi University of Mashhad, P.O. Box 9177948974, Mashhad, Iran
[b] Department of Agriculture Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), P.O. Box 14965-161, Tehran, Iran
[c] Department of Horticultural Sciences, Tarbiat Modares University, P.O. Box 14115-336, Tehran, Iran

ARTICLE INFO

Keywords:
Cut Rose
Ethylene
Senescence-associated genes
qRT-PCR
RhAA
RhCG

ABSTRACT

The functional life and marketability of flowers is restricted by senescence and /or abscission. Many senescence-associated genes have been identified in the Arabidopsis plant. In this research, we isolated two full length clones of *RhAA* and *RhCG* in rose (*Rosa hybrida*) and compared the expression patterns of these genes in cultivars with short ('Cool Water') and long ('Marroussia') shelf lives. The *RhAA* gene is more similar to unknown proteins *Cucurbita maxima, Gossypium arboretum* and senescence-associated protein *Medicago truncatula,* while the *RhCG* is closer to the unknown proteins *Pisum sativum* and *Dendrobium catenattum.* The results of quantitative real-time polymerase chain reaction (qRT-PCR) analysis showed that the *RhAA was* expressed at higher levels in at the bud and open stages in most tissues of 'Cool water' compared with 'Marroussia' in response to the ethylene treatment. In gynoecia, the *RhCG* gene showed the highest level of expression in both cultivars at the second and seventh stages. It seems that the *RhAA and RhCG* were encoding a plant-specific protein of unknown function, which may relate to in the degradative and remobilization processes of senescence.

1. Introduction

Molecular biological approaches are being used to recognize genes that may be involved in the initiation and regulation of the senescence program. The recognition and characterization of these senescence-related (SR) genes have begun to provide us with a clear and better understanding of the process of senescence (Jones, 2004). *Rose* is the most economically important cut flower and it is critical to control the senescence of rose flowers during postharvest period (Fukuchi-Mizutani et al., 2000)

Ethylene in the environment (exogenous) or produced by senescing plant organs and in response to various stressors has negative effects on postharvest quality (Hegelund et al., 2017). During the transport and handling of cut roses, sensitivity to ethylene has serious impacts in markets and other areas where the air is commonly contaminated with ethylene (Müller et al., 2000). Most cultivars exhibit ethylene sensitivity (Xue et al., 2009). In attempts to clarify the molecular causes for the differences in flower longevity among different rose cultivars, the expression of transcripts for ethylene biosynthetic genes, ethylene receptor genes and other ethylene signal transduction genes have been investigated (Al-Salem and Serek, 2017). Microarray experiments have revealed changes in the expression of more than 1400 genes during leaf development and senescence in Arabidopsis as a model plant (Buchanan-wollaston et al., 2003). The expression of many genes is up-regulated during the senescence processes (Gepstein et al., 2003).These genes are determined as senescence-associated genes (SAGs) by Lohman et al. (1994), and often encode proteins that are capable of implementing cell death (Thompson et al., 2007; Nam, 2019, 1997). A combination of physiological, biochemical, genetic and molecular assets is another important aspect of SAG that will be required to fully illuminate the regulation of flower senescence (Tripathi and Tuteja, 2007). Seyed Hajizadeh et al. (2014) identified two fragments in rose petals: *RhAA* (*Rosa hybrid*a AA) and *RhCG* (*Rosa hybrida* CG) by cDNA-AFLP, the fragments being similar to putative senescence- associated proteins of *Picea abies* and *Pisum sativum*, respectively. The objective of our study was investigate the role of these genes in flower senescence. We isolated full-length cDNAs of the *RhAA and RhCG* genes from rose and identified that they were expressed in various flora tissues and in response to ethylene. We then used these genes to compare the cultivar rose 'Cool Water' which a group with average vase life 6. 3 d, and 'Marroussia', which is widely grown in the cool climates typical of northern Europe and has an average vase life of 14.3 d (Nabigol et al., 2009).

* Corresponding author.
E-mail address: razavi@nigeb.ac.ir (K. Razavi).

https://doi.org/10.1016/j.postharvbio.2019.02.011
Received 8 August 2018; Received in revised form 12 February 2019; Accepted 12 February 2019

2. Material and methods

2.1. Plant material and ethylene treatments

R. hybrida cvs. 'Cool Water' (short vase life) and 'Marroussia' (long vase life) were harvested at two stages of flower opening (stage 2: partially opened bud and stage 7: fully opened flower) as demonstrated by Wang et al. (2004). The flowers were immediately brought to the laboratory of the Tarbiat Modares University, Tehran, Iran and the stems were adjusted to 45 cm of length and placed in deionized water (DW) for further processing. For the exogenous ethylene treatment, cut rose stems of each cultivar were placed in 200 L glass chambers and were exposed to 8 μL L^{-1} ethylene for 24 and 48 h based on our previous work (Ahmadi et al., 2008; Daneshi and Ahmadi, 2014). Control cut flowers were placed in an identical glass chamber, which received regular air. 100 mL solution of 1 M NaOH was placed into the chambers to maintain low CO_2 concentrations from respiration during treatment. The experiment was conducted at 22 ± 2 °C, 60–65% RH, and a 12/12 h light/dark photoperiod at an illumination of 15 μmol $m^{-2}s^{-1}$. After the ethylene treatment the petals, sepals, stamens, gynoecia and receptacles of the flowers were separately taken and were individually and immediately collected, frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA isolation and cDNA synthesis

The total RNA was extracted by the RNeasy Plant Mini Prep Kit (Zymo Research, USA) from 100 mg of petals and 60 mg stamen, gynoecia, and receptacle tissues, according to the manufacturer's protocol. To eliminate residual DNA, 5 μl *DNase* I was added to each sample according to protocol the RNeasy Plant Mini Prep Kit (Zymo Research, USA).The cDNA synthesis was performed according to the cDNA synthesis kit (Fermentas, USA). To obtain full length cDNAs, several pairs of degenerate primers were designed on the basis of alignment of fragments of *RhAA* and *RhCG*.

2.3. Cloning, sequencing, and sequence analysis

cDNA fragments of 472bp and 576bp were ligated to the TA plasmid vector to the protocol provided by the pTG19-T cloning vector Vivantis kit (Malaysia). By PCR cloning and specific primers, positive transformed bacteria were selected, then the clones were incubated in 5 ml of liquid LB medium containing ampicillin at 37 °C. The plasmid DNA was extracted by the protocol provided by the prime prep plasmid DNA extraction kit and sequenced by the Gen Fanavaran Co. (Iran). The sequences were analyzed with the CLUSTAL W program, European Bioinformatics Institute (EMBL). The homology search was done using the BLUSTN program National Center for Biotechnology Information (NCBI).

2.4. qRT-PCR analysis

To determine gene expression, primers were designed using the Oligo 7 software with Tm (60 °C), CG content (51%), and amplicon length (150bp). The qRT-PCR was carried out in a RT-PCR system using 10 ng of cDNA and SYBR Green PCR Master Mix. The thermal profile was initially heated for 10 min at 95 °C, followed by 40 cycles at 95 °C of 5 s, 60 °C of 15 s and 25 s at 72 °C. The specific gene expression was quantified by normalization to the ß-actin (for petal and stamen), ubiquitin (*RhUB 15* and *RhUB12* for gynoecia and receptacle, respectively) housekeeping genes (Meng et al., 2013).

2.5. Statistical analysis

This experiment was designed in a randomized completely design with three replications in a controlled environment postharvest room. The analysis of data was performed using the relative expression software tool (REST) as described by Pfaffi and et al (2002). Three biological and technical replications were used for each treatment and the $2^{-\Delta\Delta CT}$ method was used for evaluation of the relative levels of gene expression.

2.6. Measurements of ethylene production

After the efflux of ethylene, three flowers from each treatment were sealed in a 1 L glass bottle, and kept at 20 ± 2 °C for 48 h. The gas samples were taken after 24 and 48 h through headspaces via a gas-tight syringe and injected into a gas chromatograph (GC Agilent 6890 N) equipped with a capillary column and a flame ionization (FID) detector. The carrier gas was helium at 6.5 mL min^{-1}, and the injection and column temperatures were 180 °C and 60 °C, respectively.

3. Results

3.1. Sequences analysis of the full-lengths RhAA and RhCG cDNA

We isolated full length cDNAs of *RhAA* and *RhCG* genes from rose petals. The full length cDNAs of the *RhAA* and *RhCG* genes are 472 bp and 576 bp, respectively, and the putative protein encoded by *RhAA* has 157 amino acids. The alignment of the deduced amino acid sequence of this gene indicated 95% similarity to *Gossypium barbadense* (accession No. PPR88595.1) and 94% similarity to *Cucurbita maxima* (accession No. XP_022975737.1), respectively. The phylogenetic analysis predicted that the *RhAA* protein (Fig.1A) is closer to the senescence-associated protein *Medicago truncatula* (accession No. XP_013443005.1), uncharacterized proteins from *Cucurbita maxima* (accession No. XP_022975737.1), and *Gossypium arboretum* (accession No. XP_017621534.1). The *RhCG* gene encodes a protein that has 197 amino acids and the phylogenetic tree shows (Fig. 1B) that *RhCG* has less genetic distance to the *Pisum sativum* (BAB33421.1) and *Dendrobium catenattum* (accession No. XP-020704835) proteins.

3.2. Expression pattern analysis of RhAA and RhCG

We investigated the expression of *RhAA* and *RhCG* in different floral tissues including sepals, petals, stamens, gynoecia, and receptacles at stages 2 and 7 with and without ethylene treatment. In the petal and stamen tissues, *RhAA* was up-regulated at 24 and 48 h after the ethylene treatment. In stamens, the *RhCG* showed the highest level of expression at 48 h after ethylene treatment at both stages (Fig. 2)

In gynoecia, the *RhAA* was down-regulated 24 h after treatment at the bud stage in both cultivars but increased after 48 h (Fig. 3), whereas in the 'Cool water' gynoecia the relative expression was 8 fold, compared to 'Marroussia', which was observed as 6 fold in the open stage at 48 h (Fig. 3). Also, the *RhCG* expression was more than the *RhAA* gene in 'Marroussia' at both stages at 24 and 48 h after the ethylene treatment in gynoecia while in stamen, the *RhCG* showed the highest level of expression at 48 h after the ethylene treatment at both stages (Fig. 2).

In the receptacle and sepal tissues, the *RhAA and RhCG* showed a higher level of expression in 'Cool water' compared with 'Marroussia' during ethylene treatment at the open stage. Also, the relative expression of *RhAA* increased slowly from 1.95-fold at bud stage to 3.31-fold at an open stage after 24 h and then to 4.01-fold at an open stage after 48 h in 'Marroussia' (Fig. 3) (Fig. 4)

RhAA and RhCG expression in 'Cool water' dramatically increased in most tissues at 24 and 48 h after the ethylene treatment (Fig. 4).

3.3. Ethylene production

Ethylene production during first 24 h was higher than second 24 h (48 h) for all treatments (Fig. 5). The lowest amount of ethylene was produced in the controls in both cultivars at bud stage at 24 h. At 48 h

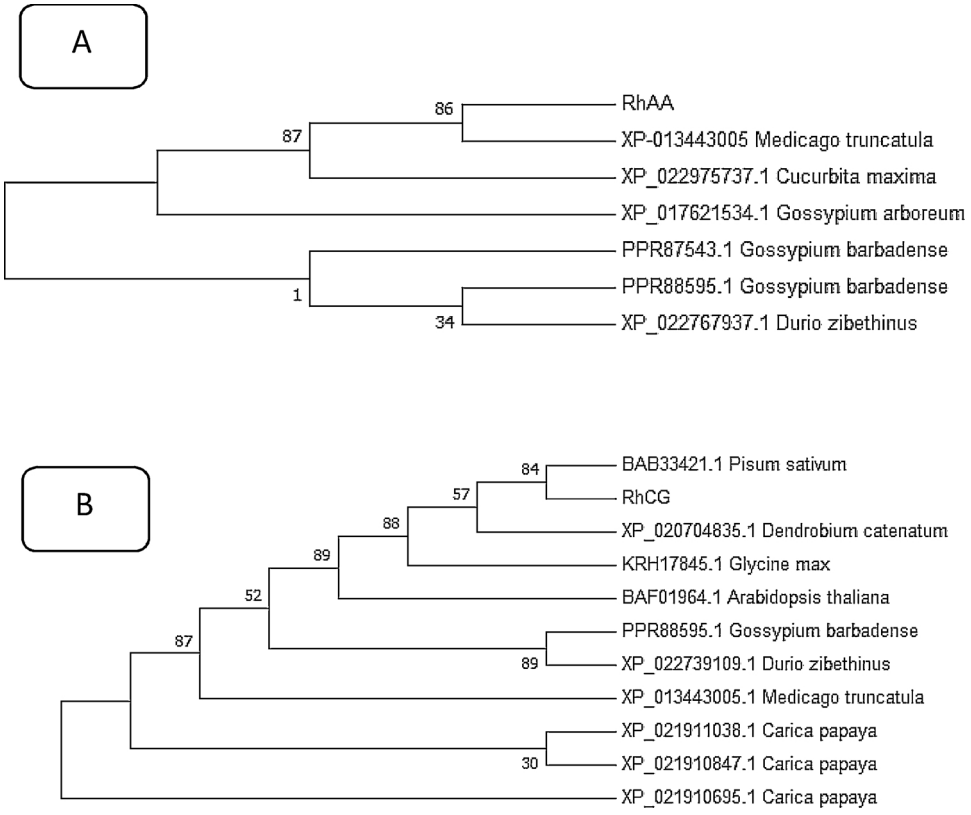

Fig. 1. The phylogenetic tree of the *RhAA* (A), and *RhCG* (B) proteins in *Rosa hybrida*. Sequence alignment analysis was performed using a CLUSTAL W method and the tree constructed with MEGA 7.0.

after exposure to ethylene, the highest ethylene production was found at both stages in 'Cool water', while in 'Marroussia' at the open stage, the controls and treated flowers had high ethylene production (Fig. 5).

4. Discussion

Thousands of genes are regulated in a particular tissue at any time, and hundreds of them may change expression during PCD/senescence (Tripathi and Tuteja, 2007). Most of the senescence-related genes are expressed at basal levels in non-senescing tissues (green leaves, fruits, and young flowers) and increase in abundance during senescence, while a smaller number of SR (as senescence-related genes) are only detected in senescing tissues and represent senescence or ripening-specific genes (Jones, 2004). According to the alignment of the sequence of the *RhAA* gene, at the amino acid level, *RhAA* shows the highest similarity of 95% to *Gossypium barbadense* (accession No. PPR88595.1), 94% to *Cucurbita maxima* (accession No. XP_022975737.1), and 88% to *Medicago truncatula* (accession No. XP-013443005) that encode proteins with unknown functions. Also, the *RhCG* gene is highly homologous (81% similarity) to the unknown protein *Carica papaya* (accession No. XP-0211911038.1), and is 73% and 72% similar to a putative senescence-associated protein *Pisum sativum* (accession No. BAB 33421.1) and senescence-associated protein *Medicago truncatula* (accession No. XP 013442997.1), respectively respectively (Fig. 1 A and B).

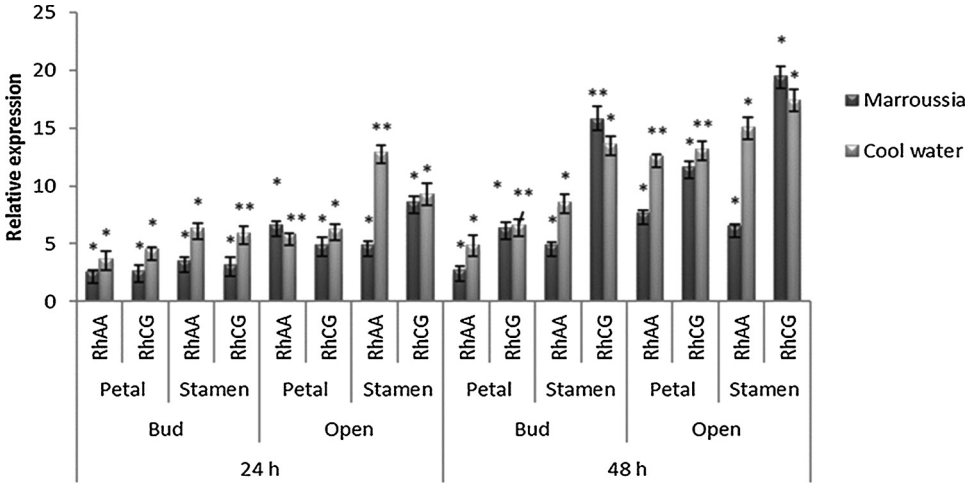

Fig. 2. Expression patterns for the *RhAA* and *RhCG* at two developmental stages in petals and stamens of 'Cool Water' and 'Marroussia' after 24 and 48 h of ethylene treatment as determined by qRT-PCR. Vertical bars represent standard deviations (n = 3) and significant differences are indicated by **, $P < 0.01$; and *, $P < 0.05$.

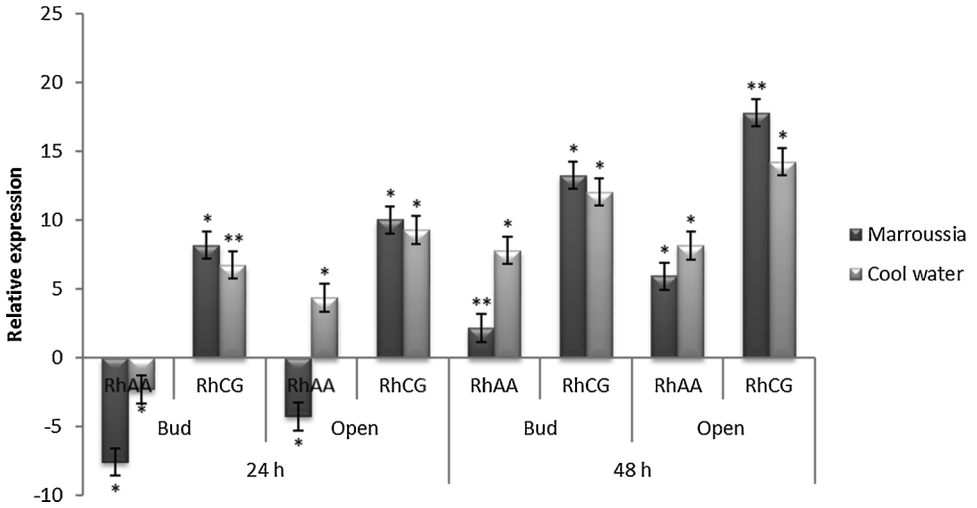

Fig. 3. Expression patterns for the *RhAA* and *RhCG* at two developmental stages in gynoecia of 'Cool Water' and 'Marroussia' after 24 and 48 h of ethylene treatment as determined by qRT-PCR. Vertical bars represent standard deviation (n = 3) and significant differences are indicated by **, $P < 0.01$; and *, $P < 0.05$.

SAGs genes such as *RhAA* and *RhCG* have no sequence similarity to genes in the database with any known function in senescence that is obvious. We found that the expression of *RhAA* was also at a high level in 'Cool water' with short longevity compared with the response 'Marroussia' to the ethylene treatment. The results show that the expression had a different pattern during flower development in the gynoecia and receptacle compared with other tissues (Figs. 3 and 4). Also, this result suggests that the expression of the *RhAA and RhCG* genes in the floral tissues are different in the variation in flower longevity in these cultivars. Our results are in agreement with the results of Seyed Hajizadeh et al. (2014) where a high expression level of TDF *RhAA* showed in short lasting cultivar, 'Black Magic' during natural senescence. In Norway spruce (*Picea abies* L. Karst), an EST encoding senescence-associated protein with the highest homology with TDF *RhAA*, was relatively more abundant in the early-flushing families of *P. abies* (Yakovlev et al., 2005)

On the other hand, according to the Arabidopsis Information Resource database (TAIR), SAG13 and 12 are up-regulated in the flower in response to ethylene. The Rlos1, a Senescence-related gene in rose, showed high expression in ethylene treatment, especially in aging petals. The gynoecia have the most sensitivity to ethylene in the very early stage of ethylene induction, different observations reported that the *Rh-ACS2* in Rose (Xue et al., 2008) and the *DC-ASC2* in carnation, two senescence-associated genes, respond immediately to ethylene treatment in the gynoecia compared to the petals. However, in our results, at first, the *RhAA* gene was induced in the petals and then in the gynoecia at bud stage after 24 h of ethylene treatment. Our results confirm that exogenous ethylene increases ethylene production, which is similar to reports on the cut rose 'Sparkle' (Daneshi Nergi and Ahmadi, 2014) or 'Samantha' (Ma et al., 2006). Also, plant sensitivity to exogenous ethylene is effective on the level of *RhAA* and *RhCG* expression (Ahmadi et al., 2008), whereas ethylene production in 'Cool water', was equal at bud and open stage after 48 h ethylene treatment and was more than in 'Marroussia', generally. On the other hand, the level of *RhAA* expression was higher in 'Cool water' in most tissues.

We assume that the up-regulation of the *RhAA* gene in gynoecia modulates with time after the ethylene treatment. Also, these patterns suggest that age-related factors may regulate the up-regulation of the *RhAA* and *RhCG* genes during flower aging.

It seems that *RhAA and RhCG* were encoding a plant-specific protein of unknown function, which may relate to in the degradative and remobilization processes of senescence. Further investigations are required to clarify the details of mechanism and function of the *RhAA* and *RhCG* genes. Also, using transgenics/mutants of the *RhAA* and *RhCG,* the analysis of physiological, biochemical and molecular behaviour will be helpful to elucidate the regulation of flower senescence.

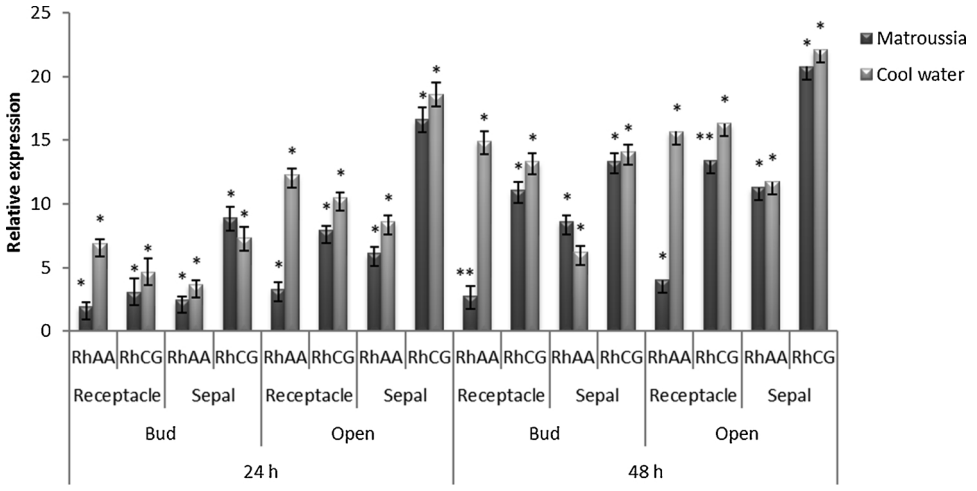

Fig. 4. Expression patterns for the *RhAA* and *RhCG* at two developmental stages sepals and receptacles of 'Cool Water' and 'Marroussia' after 24 and 48 h of ethylene treatment as determined by qRT-PCR. Vertical bars represent standard deviations (n = 3) and significant differences are indicated by **, $P < 0.01$; and *, $P < 0.05$.

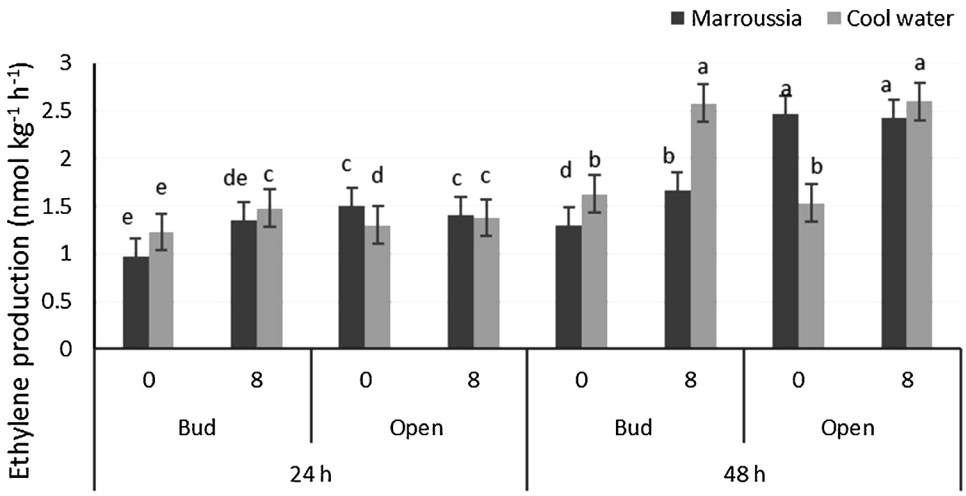

Fig. 5. Ethylene production in *Rosa hybrida* 'Cool water' and 'Marroussia' after 24 h and 48 h ethylene treatment (0 and 8 μL). Vertical bars represent standard deviations (n = 3). Differences between means are indicated by different letters ($P < 0.5$).

5. Conclusion

Full length *RhAA* and *RhCG* genes were isolated using the homology approach. We found that the expressions of *RhAA* and *RhCG* in roses were increased by ethylene. We presume that the *RhAA* and *RhCG* are involved in floral senescence. Further research on the *RhAA* and *RhCG* functions and their use to regular the senescence process will enable a better understanding of the roles that these genes might have in plant response to stress conditions in general and to ethylene in particular.

Acknowledgments

The present study was supported by grants from the National Institute of Genetic Engineering and Biotechnology (NIGEB), Iran, No. 314 and ICRP-NO. 2321. We would also like to thank the Tarbiat Modares University, Iran, for allowing us to use its postharvest physiology lab space.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2019.02.011.

References

Ahmadi, N., Mibus, H., Serek, M., 2008. Isolation of an ethylene-induced putative nucleotide laccase in miniature roses (*Rosa hybrida* L.). J. Plant Growth Regul. 27, 320–330. https://doi.org/10.1007/s00344-008-9059-2.

Al-Salem, M., Serek, M., 2017. Expression analysis by RT-PCR of genes involved in ethylene synthesis and signal transduction in miniature roses. Sci. Hortic. 216, 22–28. https://doi.org/10.1016/j.scienta.2016.12.029.

Buchanan-wollaston, V., Earl, S., Harrison, E., Mathas, E., Navabpour, S., Page, T., 2003. The molecular analysis of leaf senescence – a genomics approach. Plant Biotechnol. J. 3–22.

Daneshi Nergi, M.A., Ahmadi, N., 2014. Effects of 1-MCP and ethylene on postharvest quality and expression of senescence-associated genes in cut rose cv. Sparkle. Sci. Hortic. 166, 78–83. https://doi.org/10.1016/j.scienta.2013.12.015.

Fukuchi-Mizutani, M., Ishiguro, K., Nakayama, T., Utsunomiya, Y., Tanaka, Y., Kusumi, T., Ueda, T., 2000. Molecular and functional characterization of a rose lipoxygenase cDNA related to flower senescence. Plant Sci. 160, 129–137. https://doi.org/10.1016/S0168-9452(00)00373-3.

Gepstein, S., Sabehi, G., Carp, M.J., Hajouj, T., Nesher, M.F., Yariv, I., Dor, C., Bassani, M., 2003. Large-scale identification of leaf senescence-associated genes. Plant J . 36, 629–642. https://doi.org/10.1046/j.1365-313X.2003.01908.x.

Hegelund, J.N., Lütken, H., Müller, R., 2017. Postharvest Physiology: Ethylene in Roses BT - Reference Module in Life Sciences. https://doi.org/10.1016/B978-0-12-809633-8.05082-2.

Jones, M.L., 2004. Changes in Gene senescence. In: Nooden L, D. (Ed.), Plant Cell Death Processes. E-Publishing Inc., Academic Press, pp. 51–71.

Lohman, K.N., Gan, S., John, M.C., Amasino, R.M., 1994. Molecular analysis of natural leaf senescence in Arabidopsis thaliana. Physiol. Plant. 322–328.

Ma, N., Tan, H., Liu, X., Xue, J., Li, Y., Gao, J., 2006. Transcriptional regulation of ethylene receptor and CTR genes involved in ethylene-induced flower opening in cut rose (*Rosa hybrida*) cv. Samantha. J. Exp. Bot. 57, 2763–2773. https://doi.org/10.1093/jxb/erl033.

Meng, Y., Li, N., Tian, J., Gao, J., Zhang, C., 2013. Identification and validation of reference genes for gene expression studies in postharvest rose flower (*Rosa hybrida*). Sci. Hortic. 158, 16–21. https://doi.org/10.1016/j.scienta.2013.04.019.

Müller, R., Stummann, B.M., Serek, M., 2000. Characterization of an ethylene receptor family with differential expression in rose (*Rosa hybrida* L.) flowers. Plant Cell Rep. 19, 1232–1239. https://doi.org/10.1007/s002990000251.

Nabigol, A., Naderi, R., Mostofi, Y., Khalighi, A., Bujar, M., 2009. Soluble carbohydrates content and ethylene production in cut rose cultivars. Hortic. Environ. Biotechnol. 50, 122–126.

Nam, H.G., n.d. The molecular genetic analysis of leaf senescence 200–207.

Seyed Hajizadeh, H., Mostofi, Y., Razavi, K., Zamani, Z., Mousavi, A., 2014. Gene expression in opening and senescing petals of rose (*Rosa hybrida* L.). Acta Physiol. Plant. 36, 199–206. https://doi.org/10.1007/s11738-013-1400-0.

Thompson, J.E., Hopkins, M., Taylor, C., Liu, Z., Ma, F., Mcnamara, L., Wang, T., Thompson, J.E., 2007. Regulation and execution of molecular disassembly and catabolism during senescence 201–214.

Tripathi, S.K., Tuteja, N., 2007. Integrated signaling in flower senescence: an overview. Plant Signal. Behav. 2, 437–445. https://doi.org/10.4161/psb.2.6.4991.

Wang, D., Fan, J., Ranu, R.S., 2004. Cloning and expression of 1-aminocyclopropane-1-carboxylate synthase cDNA from rosa (*Rosa x hybrida*). Plant Cell Rep. 22, 422–429. https://doi.org/10.1007/s00299-003-0721-7.

Xue, J., Li, Y., Tan, H., Yang, F., Ma, N., Gao, J., 2008. Expression of ethylene biosynthetic and receptor genes in rose floral tissues during ethylene-enhanced flower opening. J. Exp. Bot. 59, 2161–2169. https://doi.org/10.1093/jxb/ern078.

Xue, J., Yang, F., Gao, J., 2009. Isolation of Rh-TIP1, an aquaporin gene and its expression in rose flowers in response to ethylene and water deficit. Postharv. Biol. Technol. 51, 407–413. https://doi.org/10.1016/j.postharvbio.2008.08.011.

Yakovlev, I.A., Fossdal, C.C., Skrøppa, T., 2005. Analysis of gene expression during bud burst initiation in Norway spruce via ESTs from subtracted cDNA libraries. Tree Genetics & Genomes. https://doi.org/10.1007/s11295-005-0031-z.