RESEARCH PAPER

Effects of abscisic acid on ethylene biosynthesis and perception in *Hibiscus rosa-sinensis* L. flower development

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Abstract

The effect of the complex relationship between ethylene and abscisic acid (ABA) on flower development and senescence in *Hibiscus rosa-sinensis* L. was investigated. Ethylene biosynthetic (*HrsACS* and *HrsACO*) and receptor (*HrsETR* and *HrsERS*) genes were isolated and their expression evaluated in three different floral tissues (petals, style–stigma plus stamens, and ovaries) of detached buds and open flowers. This was achieved through treatment with 0.1 mM 1-aminocyclopropane-1-carboxylic acid (ACC) solution, 500 nl l^{-1} methylcyclopropene (1-MCP), and 0.1 mM ABA solution. Treatment with ACC and 1-MCP confirmed that flower senescence in hibiscus is ethylene dependent, and treatment with exogenous ABA suggested that ABA may play a role in this process. The 1-MCP impeded petal in-rolling and decreased ABA content in detached open flowers after 9 h. This was preceded by an earlier and sequential increase in ABA content in 1-MCP-treated petals and style–stigma plus stamens between 1 h and 6 h. ACC treatment markedly accelerated flower senescence and increased ethylene production after 6 h and 9 h, particularly in style–stigma plus stamens. Ethylene evolution was positively correlated in these floral tissues with the induction of the gene expression of ethylene biosynthetic and receptor genes. Finally, ABA negatively affected the ethylene biosynthetic pathway and tissue sensitivity in all flower tissues. Transcript abundance of *HrsACS*, *HrsACO*, *HrsETR*, and *HrsERS* was reduced by exogenous ABA treatment. This research underlines the regulatory effect of ABA on the ethylene biosynthetic and perception machinery at a physiological and molecular level when inhibitors or promoters of senescence are exogenously applied.

Key words: Abscisic acid content, ACO, ACS, ERS, ethylene production, ETR, floral tissue, flower senescence.

Introduction

The senescence of many flowers is controlled by the plant hormone ethylene. The induction and production of ethylene vary during the development of the flower, but its effect, as a trigger of flower senescence, occurs during full anthesis (Manning, 1985; Peiser, 1986; Woodson *et al.*, 1992). Studies of ethylene biosynthetic pathways have led to the identification of the main enzymes involved, such as 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and oxidase (ACO). In addition, the availability of inhibitors acting at different levels in the ethylene signalling pathway could lead to a better understanding of its regulation (Wang *et al.*, 2002).

A flower organ is made up of several different tissues, which have a differential rate of ageing. In carnation flowers it has been observed that an increase in ethylene production occurs first in the ovaries, followed by the styles, the receptacle, and then the petals (Jones and Woodson, 1997, 1999). Therefore, the coordination of the senescence process in flowers is due to intraorgan signals. The subsequent production of ethylene by different tissues is thought to be the signal that triggers the corolla senescence in response to pollination (Nichols, 1977).

With the isolation of genes coding for enzymes involved in the biosynthetic pathway of ethylene, a differential

expression of these genes in various floral tissues was observed (O'Neill *et al.*, 1993; ten Have and Woltering, 1997; Jones, 2003). Unlike ethylene production, the gene expression is initially high in the styles and then in other tissues in the presence of exogenous ethylene (Jones, 2003). However, in carnation flower, it has been demonstrated that the driving force for the activation of senescence, and the genes involved in it, is determined by the ovary (Shybuya *et al.*, 2000; Nukui *et al.*, 2004). An over-regulation has been reported for ACS and ACO genes, which are the direct precursors for the synthesis of ethylene. The involvement of ethylene in flower senescence and the role of different floral tissues have been clearly demonstrated.

Although ethylene plays an important role, which is necessary for senescence, the presence of ethylene is not enough to cause it. In fact, the presence of ethylene receptors and how these are expressed in different floral tissues are also essential to carry on the ageing process. Ethylene receptors have been described for ornamental plants such as carnation (Shibuya *et al.*, 2002), rose (Müller *et al.*, 2000*a*), and geranium (Dervinis *et al.*, 2000). To date, five ethylene receptors have been identified in *Arabidopsis*: ETR1 (Chang *et al.* 1993), ERS1 (Hua *et al.*, 1995), ETR2 (Sakai *et al.*, 1998), EIN4, and ERS2 (Hua and Meyerowitz, 1998). Hua and Meyerowitz (1998) classified the receptors into two subfamilies based on sequence similarities and the structural features of proteins.

The regulation of the expression of receptor genes may has the purpose of increasing tissue sensitivity to ethylene by reducing the number of receptors, or decreasing the sensitivity by increasing expression of the receptor (Bleecker, 1999). However, some studies have also shown an induction of receptor gene expression in response to stress during senescence or in response to exogenous ethylene (Lashbrook *et al.*, 1998; Tieman and Klee, 1999; Müller *et al.*, 2000*a*, *b*). Indeed, the results obtained by studying the genes involved in ethylene perception can sometimes be in conflict, which is why this issue needs to be clarified.

In addition to ethylene, abscisic acid (ABA) accumulation also accelerates the senescence of cut flowers, as has been seen in carnation, rose, narcissus, and daylily, and thus is generally considered as a promoter of flower senescence (Mayak and Halevy, 1972; Mayak and Dilley, 1976; Eze *et al.*, 1986; Le Page-Degivry *et al.*, 1991; Panavas *et al.*, 1998; Hunter *et al.*, 2004). Flowering potted plants treated with exogenous ABA quickly lose their ornamental value. Potted roses treated with ABA shed their leaves and the senescence of flowers is accelerated. After ABA treatment some cultivars have shown an uncommonly early bud abscission, premature flowers, and leaf senescence (Müller *et al.*, 1999). The ABA appears to increase the sensitivity of flowers to ethylene, as the gene expression of some ethylene receptors increased after exogenous ABA treatment (Müller *et al.*, 2000*a*). Therefore, ABA may accelerate senescence by increasing the sensitivity of the flower, acting at the level of ethylene receptors.

Hibiscus rosa-sinensis provides a good model system for studying the developmental process of senescence, as

previously reported by Woodson *et al.* (1985). In the ephemeral flowers of *H. rosa-sinensis* L., the flower life is limited to 24 h from corolla opening to corolla senescence; thus, the process that leads the flower to senesce is fast and dramatic.

In this work, the hormonal changes of ethylene and ABA, and the isolation and characterization of two putative ethylene receptor genes (*HrsETR1* and *HrsERS1*) and two putative ethylene biosynthetic genes (*HrsACS* and *HrsACO*) were carried out in three different floral tissues during two distinct developmental stages (buds and fully opened flowers) of *H. rosa-sinensis* cv LaFrance, upon ACC, 1-MCP, and ABA treatment. The aim of this research was to elucidate the complex interplay among different effectors involved in the flower senescence process.

Materials and methods

Plant materials and growth conditions

Hibiscus rosa-sinensis L. 'La France' plants were used in all experiments. The plants were grown in a greenhouse under natural environmental conditions. Detached flowers were used as experimental material. All experiments were performed between 15 May and 30 September. Flowers were harvested between 07:30 h and 08:00 h either on the morning of flower opening (fully opened flowers) or on the morning of the day before opening (bud flowers). The lengths between the receptacles and the top of the curly corolla were recorded daily at (08.00 h) and were used to define the stages of bud development. The development and opening of buds to open flower is reported in Fig. 1. The bud stage (stage –1) used in these experiments referred to buds that were ready to open, meaning 24 h before opening. The open flowers (stage 0) were harvested when the anthers had not yet opened. The cut flowers were immediately placed in a controlled growth chamber under the following environmental conditions: cool-white fluorescent light (100 µmol m^{-2} s^{-1}) at 22±2 °C and ~60% relative humidity.

Treatment of flowers with chemicals

Whole detached flowers (bud and opened flowers), derived from the developmental stages described above and trimmed to 5 cm in stem length, were placed in 20 ml of distilled water for the control, in 20 ml of 0.1 mM ACC (Sigma, Italy) solution, or in a 0.1 mM ABA (Sigma, Italy) solution. For the 1-MCP treatment, the flowers were placed in 20 ml of distilled water and treated with 500 nl l^{-1} 1-MCP (AgroFresh, Italy), by sealing them in a glass chamber and releasing the gas from the calculated weight of bound 1-MCP. Petals, style–stigma plus stamens, and ovaries were then carefully isolated from each treated flower and used in the subsequent molecular and physiological analysis.

Determination of endogenous ABA and ethylene production

ABA was determined by an indirect enzyme linked immunosorbent assay (ELISA) based on the use of DBPA1 monoclonal antibody, raised against S(+)-ABA (Vernieri *et al.*, 1989). The ELISA was performed according to the method described by Walker-Simmons (1987), with minor modifications. Petals, style–stigma plus stamens, and ovary samples [100 mg fresh weight (FW)] were collected, weighed, frozen in liquid nitrogen, and then stored at –80 °C until analysis. ABA was measured after extraction in distilled water (water:tissue ratio=10:1 v/w) overnight at 4 °C. Plates were coated with 200 µl per well of ABA–4'-bovine serum albumin (BSA) conjugate and incubated overnight at 4 °C, then

washed three times with 75 mM phosphate-buffered saline (PBS) buffer, pH 7.0, containing 1 g l^{-1} BSA and 1 ml l^{-1} Tween-20, keeping the third washing solution for 30 min at 37 °C. Next a 100 µl ABA standard solution or sample and, subsequently, 100 µl of DBPA1 solution (lyophilized cell culture medium diluted in PBS buffer containing 10 g l^{-1} BSA and 0.5 ml l^{-1} Tween-20, at a final concentration of 50 µg ml^{-1}) were added to each well, and competition was allowed to occur at 37 °C for 30 min.

Plates were then washed again as described above and 200 µl per well of secondary antibody (alkaline phosphatase-conjugated rabbit anti-mouse; Sigma, Italy) in PBS buffer containing 10 g l^{-1} BSA and 0.5 ml l^{-1} Tween-20, at a final dilution of 1:2000) was added and incubated for 30 min at 37 °C. Plates were washed again and 200 µl per well of *p*-nitrophenyl phosphate were added and incubated for 30 min at 37 °C. Absorbance readings at 415 nm were obtained using an MDL 680 Perkin-Elmer microplate reader.

For each treatment, three independent samples were assayed in triplicate. Ethylene production was measured by enclosing petals or style–stigma plus stamens in 30 ml air-tight containers, whereas the ovaries, given their small dimension, were enclosed in 3 ml air-tight containers. Gas samples (2 ml) were taken from the headspace of the containers after 1 h incubation at 22±2 °C. The ethylene concentration in the sample was measured by a gas chromatograph (HP5890, Hewlett-Packard, Menlo Park, CA, USA) using a flame ionization detector (FID), a stainless steel column (150×0.4 cm i.d. packed with Hysep T), column and detector temperatures of 70 °C and 350 °C, respectively, and a nitrogen carrier gas at a flow rate of 30 ml min^{-1}. Quantification was performed against an external standard and results were expressed on a fresh weight basis (pl h^{-1} g^{-1} FW).

Cloning of putative ethylene receptor and biosynthetic genes

Hibiscus rosa-sinensis genomic DNA was used as a template for PCR. Total DNA was isolated from leaves with a DNeasy plant mini kit (Qiagen, Italy), using 100 mg of ground plant material. A sequence comparison was made between the known ETR1, ERS1, ACS, and ACO sequences of different plant species from the NCBI gene bank (http://www.ncbi.nlm.nih.gov/). Degenerate primers for ETR/ERS were designed on highly conserved regions across plant species using multialignment software. The degenerate primers for ETR/ERS were designed on the amino acid conserved region ECALWMP/QFGAFIVLCGATH for forward primers and VVADQVAVAL/SHAAILE for reverse primers, while ACS primers were designed on IQMGLAENQL for forward and on MSSFGLVS for reverse primers. Finally the ACO degenerate primers were designed on the conserved regions DWESTF for forward and NLGDQLEVITNG for reverse primers (Table 1). Degenerate primer sequences and the consensus region corresponding to the relative amino acid residues of already known *Arabidopsis* ethylene receptors and biosynthetic genes are listed in Table 1. PCR was performed using a GoTaq Green Master Mix (Promega, Italy). PCR conditions for *HrsETR*, *HrsERS*, *HrsACS*, and *HrsACO* were: 2 min at 95 °C (initial denaturation), then 40 cycles consisting of 40 s at 95 °C (denaturation), 40 s at 40 °C (10 cycles), 45 °C (10 cycles), 50 °C (10 cycles), or 55°C (10 cycles) (annealing), and 50 s at 72 °C (extension), 5 min at 72 °C (final

Fig. 1. Stages of *H. rosa-sinensis* flower development. Stage –4, sepals cover petals in length with an initial separation of sepals showing dark red coloured petals. Stage –3 and stage –2, petals are dark red, tightly curled within sepals. Stage –1, petals reach their full length with an initial petal unrolling. Stage 0, flower fully open. Petals are bright red. Stigmas are dark pink and soft in appearance. Multiple stamens are visible and the anthers are all undehisced, positioned below the stigma. Stage +1, corolla is in-rolled, petals are wilted, and the flower appearance has deteriorated. The stigma is not as soft as previously. All the anthers are dehisced and are discoloured with dark pink areas.

Table 1. Primer pair used for the isolation of HrsETR, HrsERS, HrsACS, and HrsACO, and real-time PCR

All primers were designed using primer select software (Lasergene) or Primer3 online software (http://fokker.wi.mit.edu/primer3/input.htm).

	Sequences 5'→3'	bp
Degenerate primers		19
ETR for	ARTGTGCHYTDTGGATGCC	
ETR rev	CAGCATGWGAVAGWGCWACDGC	22
ERS for	CAGTTTGGTGCTTTYATHGTTCTKTG	26
ERS rev	TCYARDATDGCAGCATGWGAVAG	23
ACS for	ATHCARATGGGTCTXGCXGARAATCA	26
ACS rev	GANACCAADCCRAARCTNGACAT	23
ACO for	TSGAYTTGGGAAAGYACHTTT	21
ACO rev	CTTGAGGTGATCACCAATGG	20
Real-time primers		
*Hrs*ETR for	TGCGGTCATGGTCCTTATTCTCC	23
*Hrs*ETR rev	TCGGCGAGCCAAGTCTAAGTCA	22
*Hrs*ERS for	CAGACCAGGTCGCCGTTGCT	20
*Hrs*ERS rev	GCCTCTCGACGAGCCAAGTCC	21
*Hrs*ACS for	GGCCTCCTTTGCACTCCTGA	21
*Hrs*ACS rev	TTCACCAGGATCAGCCAAGCAAAG	25
*Hrs*ACO for	CTGGACACGGTGGAAAGATTGAC	23
*Hrs*ACO rev	CTTGGCTCCTTGCACGTCTTGTAA	24
18S rRNA for	CATGGCCGTTCTTAGTTGGTG	21
18S rRNA rev	CAATGATCTCCCCATCACG	19

extension). The specific PCR product of 747 bp in size was amplified from a *Hibiscus* gene termed *HrsETR*, another PCR product of 724 bp was amplified from another gene termed *HrsERS*, a third PCR product of 920 bp was amplified from a *Hibiscus* gene called *HrsACS*, and a fourth of 179 bp was amplified from another gene called *HrsACO*. Fragments were purified using Wizard SV Gel and a PCR Clean-Up System (Promega) and cloned in a Topo-TA Cloning Kit (Invitrogen, Italy) for sequencing. Sequencing was performed using external facilities.

RNA extraction in floral tissues

Total RNA from petals, style–stigma plus stamens, and ovaries was extracted using a phenol–chloroform method. The extraction buffer contained 100 mM LiCl, 100 mM Tris–HCl, 10 mM EDTA, and 1% SDS (pH 8), and the RNA was extracted by phenol/chloroform (1:1 v/v) and then precipitated by 4 M LiCl overnight at –20 °C. The precipitate was washed twice with 70% and 100% ethanol, respectively. The pellet was then dissolved in 1.5 ml of water, and 1/10 vol. of 3 M Na-acetate and 2.5 vols of 100% ethanol were added and incubated overnight at –20 °C. The precipitate was washed twice with 70% and 100% ethanol, respectively, and then dissolved in RNase- and DNase-free water.

Quantitative reverse transcription-PCR (qRT-PCR) analysis

Genes expression was determined by qRT-PCR (ABI 7300, Applied Biosystem, Italy) using specific primers. Reverse transcription was performed with Superscript III (Invitrogen, Italy) using 1 μg of total RNA and a mix of random primers and oligo(dT). In order to avoid genomic DNA amplification, total RNA was treated with DNase I (Invitrogen, Italy) and the specific forward primer was designed across an intron-splicing zone. The SYBR green chemistry was used for gene expression analyses. Dissociation curves were performed to check for the absence of primer dimers and other amplification by-products. The amplification program was set to: one cycle at 50 °C for 2 min, then at 95 °C for 2 min; 40 cycles at 95 °C for 30 s; 55 °C for 1 min and 72 °C for 30 s (signal acquisition stage); 72°C, 10 min, and dissociation curve. The 18S rRNA was used as an internal control (Table 1). Specific primer pairs were designed across the splicing regions.

Results

Hibiscus rosa-sinensis flower development.

One *H. rosa-sinensis* flower takes 6 d to complete its development from bud sepal opening (showing colour) to full petal in-rolling (Fig. 1). In this work, two stages were considered, stage –1 and stage 0. Stage –1 was defined as a bud flower (on the morning of the day before opening) and stage 0 as a fully open flower (on the morning of flower opening). No differences in morphology or in the rate of development were noted for the flowers at different times during the flowering period. Moreover, in *H. rosa-sinensis*, detached flowers harvested at both stage –1 and stage 0 senesced over the same period as attached flowers, with full senescence symptoms at 24 h.

Cloning of two putative ethylene receptor gene fragments and two putative ethylene biosynthetic gene fragments

Isolation of putative ethylene receptors and enzymes involved in the biosynthesis of ethylene was performed using degenerate primers designed on highly conserved regions

(Table 1). All PCR amplifications were performed using genomic DNA extracted from young leaves. PCRs yielded different fragments, which were isolated and cloned. Sequencing and BLAST analyses showed that partial genomic amplified DNAs had a high similarity to receptor genes and ethylene biosynthesis enzymes of other species. Amino acid multiple alignments of putative *H. rosa-sinensis* ethylene receptors was very similar to those of both ETR and ERS genes of other plant species. Blast analyses of *HrsETR* (accession no. EF190514) cDNA showed an identity of 83, 80, and 84%, respectively with ETR1 of *Arabidopsis thaliana*, *Solanum lycopersicum* (formerly *Lycopersicon esculentum*), and *Prunus persica*. *HrsETR1* was 747 bp long with an internal intron of 217 bp detected with the splicing-specific sequence GT and AG.

The DNA fragment that had a high similarity to the ERS1 of other plant species, *HrsERS1* (accession no. EF190113), belonged to subfamily 1 of ethylene receptors. The sequenced fragment was 747 bp in length and contained one intron with a size of 190 bp. At the nucleotide level, the *HrsERS1* partial cDNA showed a 76, 73, 79, and 82% similarity to the ERS genes of *A. thaliana*, *S. lycopersicum*, *Glycine max*, and *Pisum sativum*, respectively.

PCR amplifications with ACS degenerate primers yielded a DNA fragment of 920 bp. Blast analyses showed a high similarity with ACS genes of other plant species. A multiple alignment of *HrsACS* (accession no. EU370930) showed that the amino acids were very similar to those of ACS genes of *A. thaliana*, *Gossypium hirsutum*, and *Nicotiana tabacum* with an identity of 69, 84, and 76%, respectively (Fig. 1B). The DNA fragment of 920 bp contained three introns with a total size of 428 bp. The DNA fragments amplified with ACO degenerate primers were 179 bp long. The blast analysis showed a similarity with the ACO gene family. A multiple alignment of *HrsACO* (accession no. GU233512) showed a similarity with that of other ACS genes of *A. thaliana*, *G. hirsutum*, and *Lactuca sativa* with an identity of 77, 83, and 78%, respectively.

Transcript accumulation of HrsACS, HrsACO, HrsETR, and HrsERS genes in different stages and flower tissues

The gene expression of enzymes involved in ethylene biosynthesis and perception was studied in different flower development stages and tissues. Spatial (flower tissues) and temporal (developmental stages) expression profiles are important in order to understand the initial signals of ethylene evolution and tissue sensitivity. The results should give a better overview of senescence initiation and the mechanisms of interorgan developmental regulation in *H. rosa-sinensis* flowers.

The transcript abundance of each gene was compared with the expression levels in different flowers at 1 h (see Supplementary Tables S1, S2, S7 available at *JXB* online). The gene expression of *HrsACS* in buds during the 9 h after detachment changed significantly in the three flower tissues (Fig. 2; Supplemetary Table S8).

An up-regulation of *HrsACS* was detected after 6 h and declined at 9 h in petals. In style–stigma plus stamen tissues the transcripts only increased after 3 h. Instead in the ovaries the *HrsACS* gene expression was statistically higher at 3 h and 9 h. *HrsACO*, the enzyme in the last ethylene biosynthesis step, showed the same trend in the petals, with an up-regulation at 6 h. In style–stigma plus stamens, on the other hand, the *HrsACO* expression level was higher only at 9 h, while in the ovaries it was only higher at 3 h.

The ethylene perception genes and, in particular, *HrsETR* showed no change in the petals at the bud stage. The transcript abundance only increased after 3 h in style–stigma plus stamen tissues. The *HrsETR* expression level was significantly ($P < 0.05$) lower in the ovaries after 6 h.
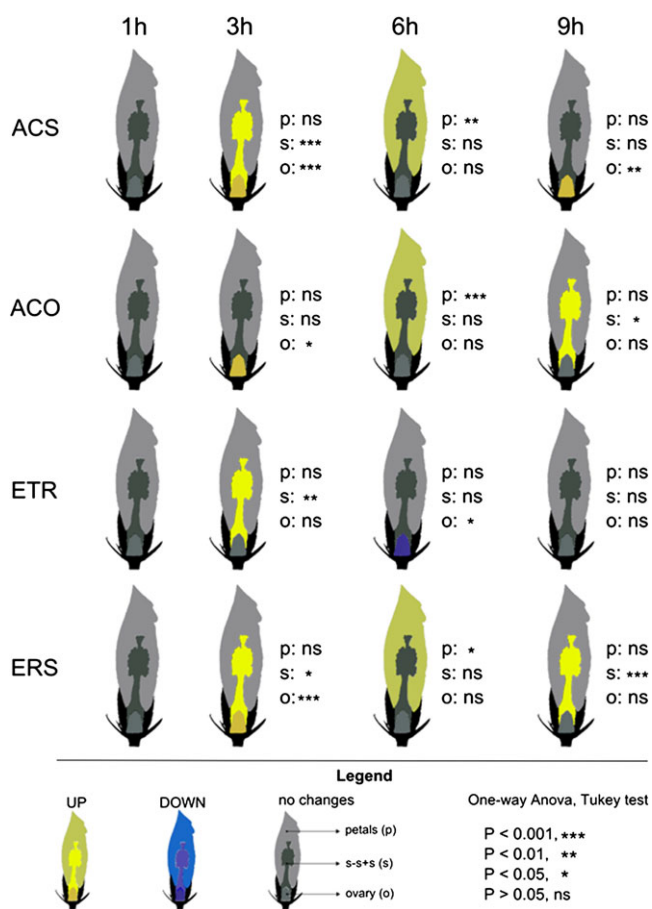

Fig. 2. Time-dependent expression of ethylene biosynthetic genes (*HrsACS* and *HrsACO*) and ethylene perception genes (*HrsETR1* and *HrsERS1*) in *Hibiscus rosa-sinensis* after flower bud removal from the plant at stage −1. The relative expression level for all genes in petal (p), style–stigma plus stamens (s), and ovary (o) is expressed as fold changes in the expression level of all genes at 1 h, which is defined as 1.0. Values are the means of three biological replicates, and data were subjected to a one-way analysis of variance. Different colours for each floral tissue examined indicate significant differences (Tukey test, *P* <0.05). Grey indicates no change in gene expression, yellow indicates significant up-regulation of gene expression, and blue indicates significant down-regulation of gene expression, relative to controls at 1 h.

HrsERS showed higher variations compared with *HrsETR*; in the petals the *HrsERS* expression level increased after 6 h and in style–stigma plus stamens it was significantly higher after 3 h and 9 h. In the ovaries *HrsERS* strongly increased after 3 h.

In the open flower stage *HrsACS* changed in expression only in the ovaries and was down-regulated after 3 h and 9 h compared with the flowers at 1 h (Fig. 3; Supplementary Table S9 at *JXB* online). No differences were found in petals and style–stigma plus stamen tissues. The *HrsACO* gene expression changed in all flower tissues. In petals, *HrsACO* transcripts were higher after 3 h and 9 h. The highest expression level was observed after 9 h in petals. In style–stigma plus stamen tissues the *HrsACO* expression level oscillated, decreasing after 3 h and 9 h with an increase at 6 h. On the other hand in the ovaries the expression of *HrsACO* increased only after 6 h.

HrsETR expression levels in open flowers were only lower in petals after 6 h. No differences were observed in style–stigma plus stamen and ovary tissues. The *HrsERS* expression profiles changed after 3 h and 9 h. In petals, *HrsERS* significantly decreased after 3 h and increased after 9 h. In style–stigma plus stamen tissues this ethylene receptor only increased after 3 h, while in the ovaries it was repressed at the same sampling time.

Temporal and spatial distribution of ethylene production and ABA content in bud and open flowers

Ethylene and ABA are the most important plant hormones involved in flower senescence. The relationship between them is not completely clear. Ethylene production and endogenous ABA content were determined during flower senescence (Table 2).

Ethylene production was higher in the ovaries and lower in petals, while intermediate ethylene production was shown in style–stigma plus stamen tissues. In the ovaries ethylene declined during the first 3 h and progressively increased after 6 h and 9 h, reaching on average the highest values of 15 nl g^{-1} h^{-1} (Table 2). Ethylene production in petals and style–stigma plus stamens increased during the time frame of the experiment. Compared with the values obtained at 9 h and 1 h, ethylene production increased by 2.1-fold in petals, 5.4-fold in style–stigma plus stamens, and 4.1-fold in ovaries.

In open flowers the ethylene evolution in the different organ tissues had the same trend as observed in buds. The highest values were observed in the ovaries, intermediate values were found in style–stigma plus stamens, and the lowest values were in petals. Ethylene production increased over time: from 1 h to 9 h by 6.3-fold in petals, 8.2-fold in style–stigma plus stamens, and 17.7 fold in ovaries (Table 2).

The endogenous ABA measured in the different flower organs of the bud stage was higher in petals and style–stigma plus stamens in the first 6 h. In these tissues, the ABA content increased over time. In the ovaries, ABA content oscillated but without significant differences, while
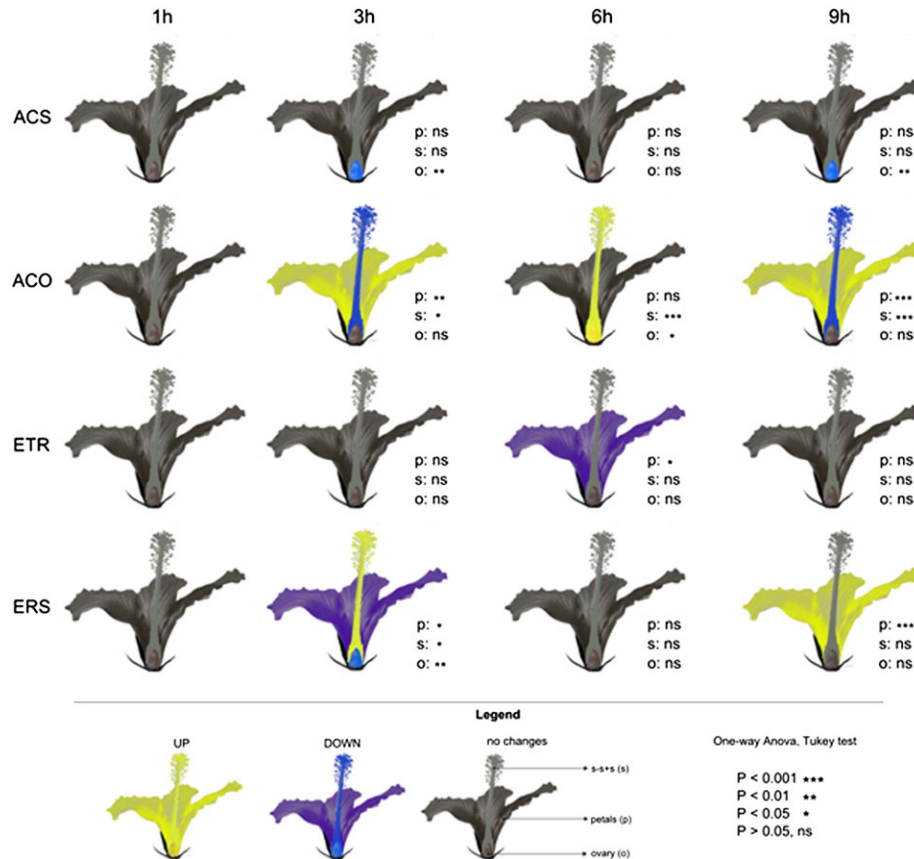
Fig. 3. Time-dependent expression of the ethylene biosynthetic genes (*HrsACS* and *HrsACO*) and ethylene perception genes (*HrsETR1* and *HrsERS1*) in *Hibiscus rosa-sinensis* opened flowers removed from the plant at stage 0. The relative expression level for all genes in petal (p), style–stigma plus stamens (s), and ovary (o) is expressed as fold changes to the expression level of all genes at 1 h, which is defined as 1.0. Values are the means of three biological replicates, and data were subjected to a one-way analysis of variance. Different colours for each floral tissue examined indicate significant differences (Tukey test, $P <0.05$). Grey indicates no change in gene expression, yellow indicates significant up-regulation of gene expression, and blue indicates significant down-regulation of gene expression, relative to controls at 1 h.

after 9 h it increased significantly, reaching the highest values among flower organs at the bud stage.

In the open flowers the ABA content was higher in petals compared with style–stigma plus stamens and ovaries. In petals, ABA decreased during the intermediate data points (3 h and 6 h) and the highest values were found after 1 h and 9 h. In style–stigma plus stamens the ABA content increased after 3 h and progressively declined to its lowest values of 51 ng g^{-1} FW. In the ovaries endogenous ABA oscillated, with an increase after 3 h and 9 h (Table 2).

Effects of exogenous ACC, 1-MCP, and ABA on H. rosa-sinensis *flowers*

To better understand the specific temporal/tissue ethylene production and endogenous ABA accumulation, the morphological changes of buds (stage –1) and flowers (stage 0) were first monitored after removal from the plant and following ACC, 1-MCP, and ABA treatments. The application of treatment for 9 h did not cause any visible morphological alteration in *Hibiscus* bud flowers (data not shown). In open flowers (Fig. 4), ACC treatment significantly promoted flower senescence; in fact, the characteristic petal in-rolling that accompanies *Hibiscus* flower senescence was accelerated under ACC treatment compared with the control. In addition, exogenously applied ABA accelerated flower senescence, and the effect was slightly less pronounced compared with the ACC application. In contrast to the ACC and ABA treatments, 1-MCP significantly impeded in-rolling of the petals.

Effects of exogenous ACC, 1-MCP, and ABA on the ethylene biosynthesis of H. rosa-sinensis *flowers*

The interaction between ethylene and ABA in *Hibiscus* flower development was investigated using an ethylene inhibitor (1-MCP) or promoter (ACC) and the hormone ABA.

In petals from ACC-treated bud flowers, ethylene production increased after 1 h and 9 h of treatment. The ethylene evolution increased after 9 h of 1-MCP treatment, showing the highest value (Fig. 5A). The ABA-treated flowers significantly decreased ethylene production after 6 h and 9 h. In style–stigma plus stamens from ACC-treated

Table 2. Changes in ethylene production and endogenous ABA content for each stage of *H. rosa-sinensis* flower development (B, bud; OF, open flower) and senescence

The flowers were held for 1, 3, 6, and 9 h in water solution. Data were subjected to a one-way analysis of variance and differences between developmental stages were analysed by a Bonferroni post-test. Different letters within columns denote significant differences at $P \leqslant 0.05$.

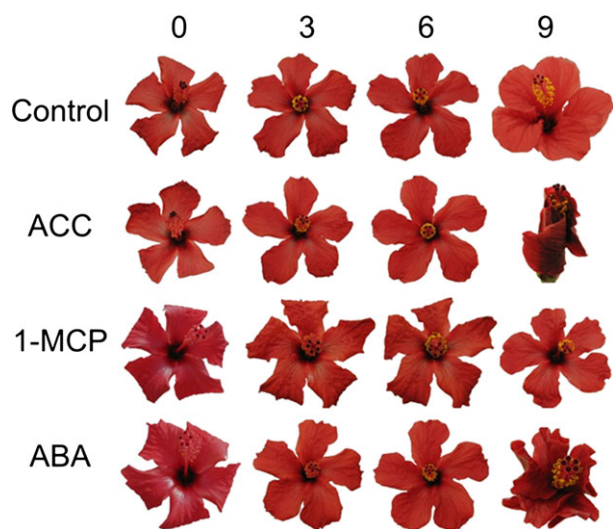| Stage	Timing (h)	Petals	S–S+S	Ovaries
Ethylene (pl g $^{-1}$ h $^{-1}$ FW)				
B	1	683 a	1698 a	3747 a
	3	723 a	1874 a	2630 a
	6	1231 b	6488 b	5625 b
	9	1438 b	9160 c	15 322 c
OF	1	598 a	819 a	950 a
	3	436 a	1049 a	4995 b
	6	2010 b	6644 b	9672 c
	9	3750 c	6683 b	16842 d
ABA content (ng g $^{-1}$ FW)				
B	1	83 a	173 a	48 a
	3	179 b	167 a	101 a
	6	239 c	198 a	87 a
	9	164 b	238 a	284 b
OF	1	347 a	104 a	85 a
	3	125 b	208 b	139 b
	6	196 c	163 c	73 a
	9	313 a	51 d	164 b

Fig. 4. Effects of ACC, 1-MCP, and ABA on opened flowers (stage 0). Flowers were treated with 0.1 mM ACC, 500 ppb 1-MCP, and 0.1 mM ABA for 9 h.

bud flowers, ethylene production increased after 3 h, but the highest values were found after 6 h and 9 h (Fig. 5B). Ethylene production in style–stigma plus stamens from 1-MCP-treated buds increased for 6 h then decreased to the initial values.

Ethylene production in ABA-treated buds was similar to that in the controls. Ethylene production in ovaries of bud flowers (Fig. 5C) responded early to ACC and 1-MCP treatments. After 1 h the production increased by almost 1-

and 2-fold, in ovaries from ACC- and 1-MCP-treated buds, respectively. Ethylene production in 1-MCP-treated ovaries peaked at 3 h and decreased at the termination point. ACC treatment increased the ethylene evolution, by ~2-fold at 6 h, and then remained at a constant level. The ovaries from ABA-treated bud flowers showed an increase in ethylene production after 6 h.

In petals of opened flowers ethylene production (Fig. 5D) was clearly enhanced, by ~3-fold, after 3 h of ACC treatment, but after 6 h and 9 h it did not increase. Petals from ABA-treated open flowers showed an appreciable reduction in ethylene evolution after 9 h of treatment. The style–stigma plus stamen tissues in ACC-treated open flowers (Fig. 5E), responded by considerably enhancing ethylene production, which peaked at 6 h, and was then maintained at a constant level. The style–stigma plus stamens from 1-MCP-treated open flowers showed a doubled ethylene production after 6 h, but ABA treatment, on the other hand, reduced the evolution after 6 h and 9 h. Ethylene production of ovaries from ACC-treated flowers (Fig. 5F) showed an increase after 3 h, reached its maximum value at 6 h, and was then constant. After 3 h, 1-MCP treatment significantly enhanced ethylene production in the ovary tissues, but after 9 h ethylene production was reduced. The ovary tissues of open flowers responded to ABA treatment by diminishing ethylene production after 6 h and 9 h.

A particularly interesting finding was that ovary tissues in the early stage of development initially responded to ACC and 1-MCP treatments. The results show that generally ABA suppressed ethylene evolution at the late time points; however, ABA treatment was found to be effective in accelerating senescence in *H. rosa-sinensis* flowers.

ABA content in different floral tissues treated with ACC and 1-MCP

The changes in ABA content, in bud and open flowers, after treatment with ACC and 1-MCP were monitored throughout the experiment. The ethylene receptor inhibitor increased the ABA content in petals of buds immediately after 1 h of treatment and remained constant throughout the experiment (Fig. 6A). The ACC treatment showed the same trend as the controls for 3 h then decreased and reached the lowest values after 9 h. The ABA in style–stigma plus stamen tissues rapidly increased in ACC-treated flowers. reaching the highest values, 340 ng g $^{-1}$ FW, after 6 h (Fig. 6B). The 1-MCP lowered the ABA content in style–stigma plus stamens throughout the whole experimental period although there were no significant differences. In the ovaries (Fig. 6C) the exogenously applied ACC on bud flowers prevented ABA accumulation, with values ranging from 180 ng g $^{-1}$ FW to 90 ng g $^{-1}$ FW. The ovaries from 1-MCP-treated flowers showed the same ABA trend as the controls but with higher values, which almost doubled for the first 6 h.

In the open flowers the effects of treatments were similar to those observed in the bud stage. The ABA content in
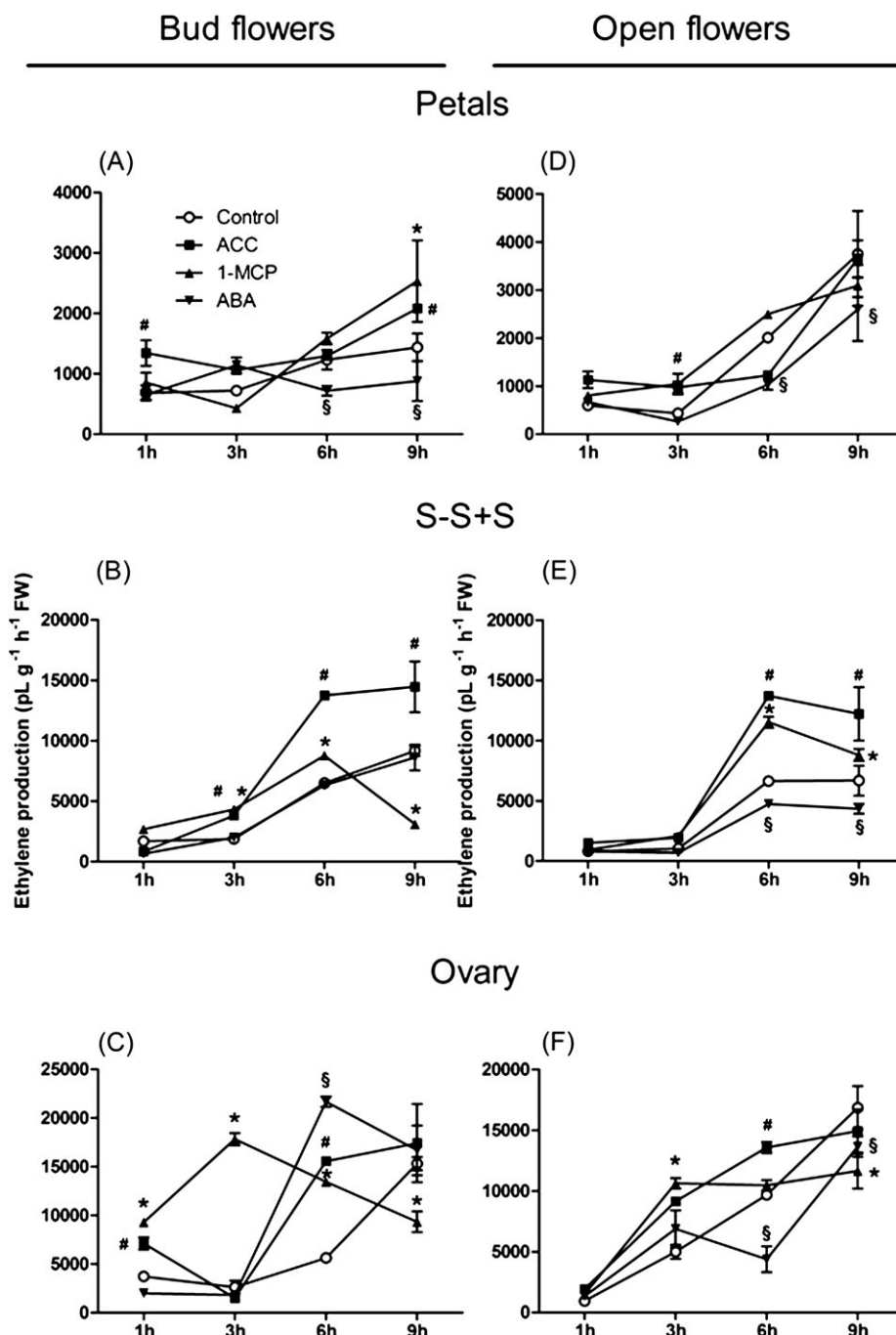
Fig. 5. Effects of exogenous ACC, 1-MCP, and ABA treatments on ethylene production in flower buds and open flowers of *H. rosa-sinensis* at four different time points. The flowers were treated with 0.1 mM ACC (filled squares), 500 ppb 1-MCP (filled upright triangles), 0.1 mM ABA (filled inverted triangles), or untreated (open circles). Data were subjected to a two-way analysis of variance. The *, #, and § symbols above each data point indicate a significant difference (Bonferroni post-test, $P < 0.05$) of ethylene production after ACC, 1-MCP, and ABA treatment (related to the control), respectively. S–S+S: style–stigma plus stamens. Error bars indicate the standard deviation.

petals (Fig. 6D) from flowers treated with ACC did not increase and showed the same trends as the controls for the first 6 h, but then significantly decreased. In 1-MCP-treated flowers the ABA content was similar for 3 h then sharply increased, reaching the highest value of 400 ng g^{-1} FW. After 9 h the ABA content was <200 ng g^{-1} FW. In the style–stigma plus stamens (Fig. 6E) in ACC-treated flowers, the ABA content did not change significantly for the first 6 h, while after 9 h it was higher compared with the controls. In contrast, 1-MCP stimulated ABA biosynthesis and doubled it for the first 3 h, and then declined, showing values <100 ng g^{-1} FW after 6 h and 9 h. In the ovaries the ABA content in ACC-treated flowers showed the same trends as the controls for 6 h, and then was significantly lower. In the ovaries from 1-MCP-treated flowers the ABA content increased after 1 h then declined until it reached the same concentrations as those found in the ACC treatments (Fig. 6F).
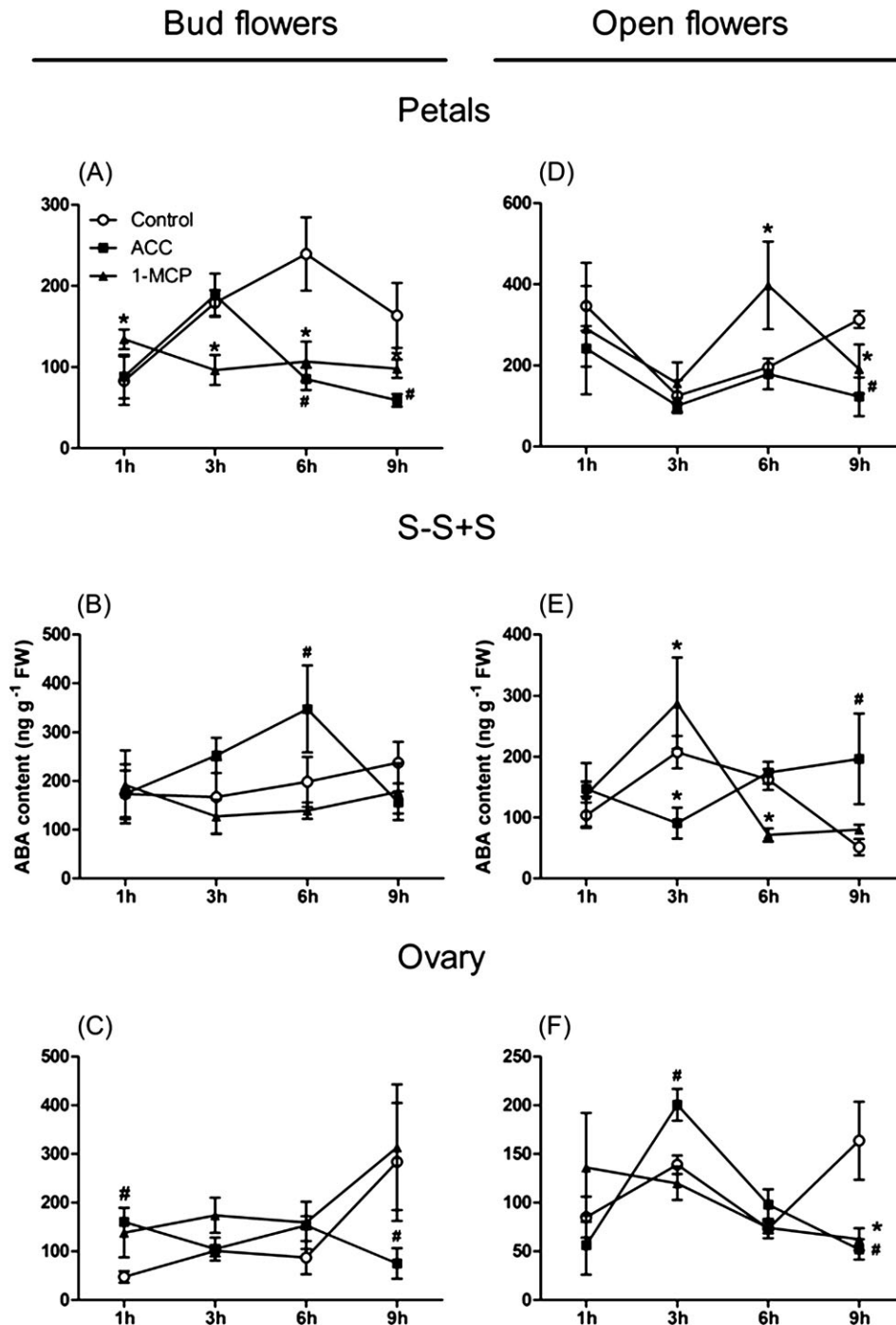
Fig. 6. Effects of exogenous ACC and 1-MCP treatments on endogenous ABA content in flower buds and open flowers of *H. rosa-sinensis* at four different time points. The flowers were treated with 0.1 mM ACC (filled squares) or 500 ppb 1-MCP (filled triangles), or untreated (open circles). Data were subjected to a two-way analysis of variance. The * and # symbols above each data point indicate a significant difference (Bonferroni post-test, $P < 0.05$) of ethylene production upon ACC or 1-MCP treatment (related to the control), respectively. S–S+S: style–stigma plus stamens. Error bars indicate the standard deviation.

Expression of HrsACS, HrsACO, HrsETR, *and* HrsERS *genes in bud flowers after ACC, 1-MCP, and ABA treatments*

The ethylene biosynthetic and perception pathway was also investigated at a molecular level in bud flowers (Fig. 7; Supplementary Tables S1, S3, S5 at *JXB* online). The *HrsACS* expression in petals from ACC-treated flowers was

up-regulated at the bud stage. During the experimental period the *HrsACS* expression declined and no differences were observed after 9 h. In petals from 1-MCP-treated buds, the *HrsACS* expression did not differ from the controls for 3 h, while it was strongly down-regulated after 6 h. In ABA-treated flowers, *HrsACS* was up-regulated after 1 h and down-regulated after 6 h, while no differences
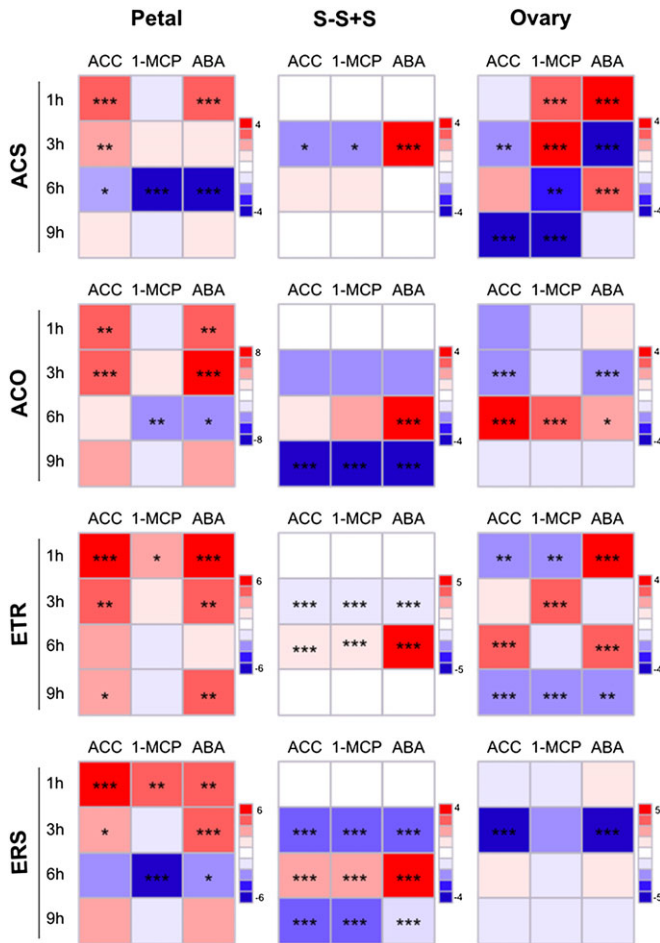
Fig. 7. Effects of ACC, 1-MCP, and ABA treatments on the expression of genes involved in ethylene biosynthesis and perception. Bud flowers were treated with 0.1 mM ACC, 500 ppb 1-MCP, and 0.1 mM ABA. Gene expression levels were determined by qRT-PCR, and are expressed as fold change relative to the transcript abundance in the control flowers. The flower tissues examined were: petals, style–stigma plus stamens (S–S+S), and ovaries. Values are the means of three biological replicates and are displayed as a row of coloured boxes. Each timing treatment is represented by a row of coloured boxes, and each exogenous treatment is represented by a single column. Effects of chemicals shown as red to brighter red and blue to brighter blue indicate significant up-regulation or down-regulation of gene expression, respectively. The maximum value for image contrast was determined for each gene assessed. The *, ** and *** symbols in each coloured box indicate significant differences in gene expression (related to the controls) at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, assessed by a Bonferroni post-test.

were observed after 3 h and 9 h. In the style–stigma plus stamen tissues of flower buds, the *HrsACS* transcripts were affected by treatments only after 3 h. They were down-regulated by ACC and 1-MCP, while they were up-regulated in the ABA treatment. In the ovaries of buds *HrsACS* was significantly down-regulated in the ACC treatment after 3 h and 9 h. After 1-MCP was exogenously applied, *HrsACS* expression was strongly induced within

1 h and 3 h, and was then down-regulated at 6 h and 9 h. In the ABA-treated flowers the *HrsACS* transcripts increased after 1 h and 6 h, while they were significantly lower after 3 h.

Regarding the expression of the last enzyme involved in ethylene biosynthesis, *HrsACO* was strongly up-regulated by ACC and ABA in petals after 1 h and 3 h. In contrast, after 6 h *HrsACO* was down-regulated in 1-MCP and ABA treatments. In the style–stigma plus stamen bud tissues *HrsACO* expression was mainly influenced by treatments at the end of the time course (9 h). After 6 h *HrsACO* was up-regulated in style–stigma plus stamen tissues in ABA-treated buds, while it was down-regulated in all treatments after 9 h. In the ovaries, on the other hand, the *HrsACO* expression changes were mainly observed after 3 h and 6 h. In the ovaries of buds treated with ACC and ABA, the *HrsACO* transcripts were lower than in the controls after 3 h, while they were up-regulated after 6 h in all treatments.

The gene expression of *HrsETR* in the petals of buds was up-regulated in all treatments after 1 h and with ACC and ABA treatments after 3 h and 9 h. The *HrsETR* in style–stigma plus stamens was up-regulated in all treatments after 3 h and 6 h. In the ovaries, the gene expression of this receptor was down-regulated by ACC and 1-MCP after 1 h and 9 h, while it was up-regulated after 3 h in the 1-MCP treatment and after 6 h in the ACC treatment. In the ABA-treated flowers *HrsETR* expression was up-regulated after 1 h and 6 h, and down-regulated after 9 h as observed in the ACC and 1-MCP treatments. The *HrsERS* expression in petals was up-regulated in all treatments after 1 h, and in ACC and ABA treatments after 3 h. On the other hand, after 6 h *HrsERS* was down-regulated in 1-MCP- and ABA-treated buds. In the style–stigma plus stamen tissues, the *HrsERS* transcripts were lower in all treatments, except after 6 h in the ABA-treated buds. In the ovaries, significant variations in *HrsERS* gene expression were observed after 3 h. The *HrsERS* expression was lower in the ovaries of buds treated with ACC and ABA.

Expression of HrsACS, HrsACO, HrsETR, *and* HrsERS *genes in open flowers after ACC, 1-MCP, and ABA treatments*

The ethylene biosynthetic and perception pathway was also investigated at a molecular level in open flowers (Fig. 8; Supplementary Tables S2, S4, S6 at *JXB* online). In petals, the *HrsACS* gene expression was mainly down-regulated in all treatments except after 3 h in ABA-treated flowers. The ACC treatment lowered the *HrsACS* transcript abundance after 3–9 h. The 1-MCP treatment after 3 h significantly down-regulated the *HrsACS* gene expression in petals. The ABA treatments increased the *HrsACS* expression level after 3 h, while they repressed the transcripts of this gene after 6 h and 9 h. In the style–stigma plus stamen tissues, *HrsACS* gene expression was only up-regulated following 1 h and 9 h of ACC treatment. Instead ABA repressed *HrsACS* transcript accumulation after 1 h and 3 h of the time course. The 1-MCP treatment significantly lowered

HrsACS gene expression only after 3 h. In the ovaries, the *HrsACS* expression levels decreased after 1 h in all treatments and with 1-MCP and ABA treatments after 6 h. The *HrsACS* gene was only up-regulated in the ACC treatment after 6 h.

The *HrsACO* gene in petals of open flowers was up-regulated as soon as after 1 h of ACC and ABA treatments.
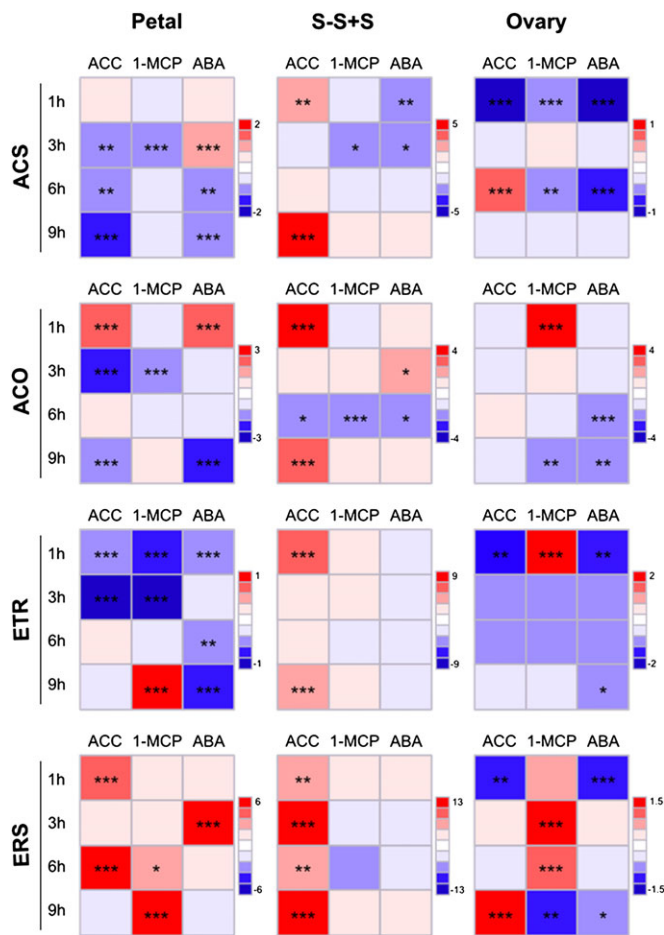

Fig. 8. Effects of ACC, 1-MCP, and ABA treatments on the expression of genes involved in ethylene biosynthesis and perception. Open flowers were treated with 0.1 mM ACC, 500 ppb 1-MCP, and 0.1 mM ABA. Gene expression levels were determined by qRT-PCR, and are expressed as fold change relative to the transcript abundance in control flowers. The flower tissues examined were: petals, style–stigma plus stamens (S–S+S), and ovaries. Values are the means of three biological replicates and are displayed as a row of coloured boxes. Each timing treatment is represented by a row of coloured boxes, and each exogenous treatment is represented by a single column. Effects of chemicals shown as red to brighter red and blue to brighter blue indicate significant up-regulation or down-regulation of gene expression, respectively. The maximum value for image contrast was determined for each gene assessed. The *, ** and *** symbols in each coloured box indicate significant differences in gene expression (related to the controls) at $P <0.05$, $P <0.01$, and $P <0.001$, respectively, assessed by a Bonferroni post-test.

The *HrsACO* in petals from ACC-treated flowers was down-regulated after 3 h and 9 h, in 1-MCP-treated flowers after 3 h, and in ABA-treated flowers after 9 h. In style–stigma plus stamen tissues the *HrsACO* mRNAs were enhanced by ACC treatment after 1 h and 9 h, and by ABA after 3 h. In contrast, *HrsACO* gene expression was down-regulated after 6 h in all treatments. In the ovaries, *HrsACO* up-regulation was initially only observed following 1 h of 1-MCP treatment. The same treatment showed the opposite effect after 9 h. The ACC treatment did not affect this gene, while ABA repressed the transcript accumulation after 6 h and 9 h.

The two isolated genes involved in ethylene perception were influenced in different ways by the treatments. The *HrsETR* mRNAs were lower than the controls after 1 h in all treatments. The same results were observed after 3 h in ACC and 1-MCP treatments. The inhibition of *HrsETR* gene expression was also observed after 6 h and 9 h following ABA treatment.

In the style–stigma plus stamen tissues, the *HrsETR* gene expression was only influenced by ABA, which increased the transcript after 1 h and 9 h compared with the controls. In the ovaries the effect of the treatments on *HrsETR* was observed after 1 h. ACC and ABA inhibited *HrsETR* transcript accumulation, while 1-MCP treatment had the opposite effect. The effect of ABA treatment was also detected after 9 h.

HrsERS gene expression was up-regulated in all treatments but at different time points. ACC affected *HrsERS* immediately after 1 h and 6 h. In contrast, 1-MCP influenced *HrsERS* after 6 h and 9 h. ABA increased the *HrsERS* transcripts only after 3 h. In the style–stigma plus stamens in ACC-treated flowers, *HrsERS* was up-regulated throughout the whole experimental period. In the ovaries, *HrsERS* gene expression was down-regulated after 1 h following ACC and ABA, but up-regulated after 9 h in ACC-treated flowers. The 1-MCP treatment increased the expression of *HrsERS* after 3 h and 6 h, but inhibited the expression after 9 h as observed in the ABA treatment.

Discussion

Gene expression of HrsACS, HrsACO, HrsETR, *and* HrsERS, *putative ethylene biosynthetic and receptor genes in* H. rosa-sinensis, *is involved in flower development and senescence*

The isolation of components involved in ethylene biosynthesis and perception in *H. rosa-sinensis*, and the characterization of these genes, in natural senescence conditions or through the use of external hormonal stimuli, enabled the examination of the regulatory mechanism underlying the ethylene-sensitive process of flower senescence in depth.

Hibiscus flowers showed the first morphological–structural changes, from anthesis to the first senescence symptom characterized by petal in-rolling after 9 h. ACS and ACO genes are encoded by a multigene family, which exhibits

differential tissue specificity at various stages of flower life and/or differential regulation by environmental or hormonal stimuli (Yang and Hoffman, 1984; Kende, 1993).

In the present study the expression analysis of both *HrsACS* and *HrsACO* genes revealed a high differential expression in *H. rosa-sinensis* tissues at the bud and open flower stages. These results suggest that at the early flower development stage, ACS and ACO genes are likely to be involved in the regulation of ethylene biosynthesis transcriptionally. This is because the results positively correlated with ethylene levels in isolated *H. rosa-sinensis* floral tissues, which showed a considerable ethylene production in style–stigma plus stamen and ovary tissues (Fig. 5B, C). In addition, in bud flowers, *HrsACS* transcript accumulation was greatly enhanced at an early stage in style–stigma plus stamen and ovary tissues (Fig. 2).

Similar evidence has been reported in carnation and petunia (Tang *et al.*, 1994; Jones, 2002), and the present results may indicate that ethylene plays a role in the reproductive process of *H. rosa-sinensis* during the development of flowers. In open flowers, the results showed that ethylene production was effectively enhanced following detachment and was highest in ovaries, then style–stigma plus stamens, and lastly in petals (Fig. 5D–F).

Previous studies on rose cv White Sim and carnation have shown a positive regulation through an increase in both ethylene production and ACS expression as the flowers matured to senescence (Wang *et al.*, 2004; Tanase *et al.*, 2008). In contrast to these findings, in *Hibiscus* the *HrsACS* gene did not increase in the tissues examined, as senescence advanced (Fig. 3). It is reasonable to assume that ethylene biosynthesis is transcriptionally regulated at ACO levels during *H. rosa-sinensis* flower senescence, without the requirement for ACS gene transcription, as reported previously by Wang *et al.* (2002).

In fact, *HrsACO* showed an increase in its expression levels in different tissues as the ageing process advanced, and this was generally positively correlated with the increase in ethylene production. In other plant species such as *Alstroemeria*, a marked enhancement of ACO gene expression has been observed, rather than ACS, at the last stages of development (Wagstaff *et al.*, 2005), and, in some rose cultivars, no changes in ACS transcripts during senescence have been reported (Müller *et al.*, 2000b; Tanase *et al.*, 2008). These discrepancies concerning the molecular regulation of the ethylene biosynthetic pathway among different plant species might be related to the fact that ACS and ACO enzymes are encoded by multifamily genes, with differential regulation in each tissue and in response to different stimuli.

During flower development, the expression of ethylene receptors seems to be involved in the regulation of flower tissue development. Hall and Bleeker (2003) reported several flower abnormalities and defects in Arabidopsis *etr1-ers1* mutants. It is widely accepted that ethylene receptors regulate ethylene responses negatively, thus determining an inverse relationship between the transcript abundance of ethylene receptors and ethylene sensitivity (Hua and Meyerowitz, 1998; Bleecker, 1999). Transgenic tomato with reduced levels of *LeETR4* were found to exhibit an acceleration of flower senescence, and the overexpression of Never ripe (*Nr*) gene determined a phenotype insensitive to ethylene (Ciardi *et al.*, 2000; Tieman *et al.*, 2000). Moreover, in *Dianthus* and *Oncidium*, the levels of *DcERS2* and *OgERS1* decreased during flower senescence, causing an enhancement of ethylene sensitivity (Shybuya *et al.*, 2002; Huang *et al.*, 2007). Several studies have also reported the expression of ethylene receptors to be up-regulated during flower senescence or in response to external stimuli (Vriezen *et al.*, 1997; Müller *et al.*, 2000a; Kuroda *et al.*, 2003; Ma *et al.*, 2006; Mutui *et al.*, 2007).

In this study, although a decreased level in petal and ovary tissues at the open flower stage was observed 3 h after flower detachment (Fig. 3), the transcript accumulation of *HrsERS*, in both bud and open flower stages, was regulated in a tissue-specific manner, through a positive correlation with the progression of senescence, especially in petals and style–stigma plus stamens. In contrast, the *HrsETR1* expression level decreased inversely with the concomitant climacteric peak in ethylene production shown by the petals of open flowers (Figs 3, 5D).

These results indicate that the *HrsERS* and *HrsETR* genes are differentially regulated by ethylene. However, it remains to be resolved whether the abundance (decrease or increase) of receptors can cause the inactivation of CTR components and the effective activation or inactivation of downstream components.

Ethylene controls flower senescence in Hibiscus

In the present study, in accordance with previous results (Woodson *et al.*, 1985), ACC and 1-MCP treatments were found to accelerate and prevent petal in-rolling, respectively (Fig. 4). In-rolling characterizes the rapid and drastic senescence process in *H. rosa-sinensis* ethylene-sensitive flowers (Hoyer, 1996). To examine how ethylene influences the expression of ethylene biosynthetic genes in three different floral tissues at bud and open flower stages, the expression of *HrsACS* and *HrsACO* genes and ethylene production after ACC or 1-MCP treatment were determined. It is widely accepted that ethylene biosynthesis in ripening fruits and senescing flowers is subject to both positive and negative feedback regulation (Kende, 1993). A positive feedback regulation, through an increase in ethylene production triggered by exogenous ethylene, with the activation of ACS and/or ACO has been reported for petunia (Tang and Woodson, 1996), carnation (Jones and Woodson, 1997), and orchid (O'Neill *et al.*, 1993). A negative feedback regulation has been shown for transgenic petunia flowers (Wilkinson *et al.*, 1997), persimmon fruit (Zheng *et al.*, 2006), and ripening tomato and banana (Nakatsuka *et al.*, 1999; Inaba *et al.*, 2007).

In this study, ethylene biosynthesis was found to be under both positive and negative feedback regulatory

mechanisms in a tissue-temporal specific manner during flower development and senescence after treatments with ACC and 1-MCP. In petal and ovary tissues of open flowers, ethylene production (Fig. 5D, F) and the expression of ethylene biosynthetic genes after 3 h of ACC treatment and after 3 h and 9 h of 1-MCP treatment were under negative and positive feedback regulation, respectively (Fig. 8). On the other hand, in bud flowers tissues, this regulatory mechanism was diametrically opposed. A similar control mechanism between bud and open flower stages was also observed for style–stigma plus stamen tissues, but it was in contrast to previous results obtained for petals and ovaries. In fact, in style–stigma plus stamens from ACC-treated open flowers, ACC enhanced the transcript abundance of *HrsACO* after 1 h (Fig. 8) with an increase in ethylene production (Fig. 5E). On the other hand 1-MCP down-regulated the transcript abundance of *HrsACO* in style–stigma plus stamens after 6 h (Fig. 8), but did not inhibit ethylene production. In fact it had a stimulating effect at the end of the experimental time frame (Fig. 5E).

In petals and ovaries from open flowers, the reduction in gene expression of *HrsACS* under ACC treatment was a particularly intriguing result. It opened up the possibility that ACC content could exert an inhibitor-like effect on the regulation of ACS gene expression. Differences in *HrsACS* and *HrsACO* gene expression in response to ACC and 1-MCP treatments between buds and open flowers may modulate the differential sensitivity to ethylene, which is a consequence of unknown tissue-specific and developmental factors.

Similar to the present findings, others studies on climacteric fruit have reported a differential feedback regulation between pre-climacteric and ripening fruits and also between different tissues. In tomato pre-climacteric fruit, ethylene production is negatively feedback regulated by *LeACS* and *LeACO* genes. In addition, with an increase in ethylene production during the onset of the climacteric stage, the expression of *LeACS* and *LeACO* was markedly enhanced as a result of a positive feedback regulation (Nakatsuka *et al.*, 1999). Inaba *et al.* (2007) showed that ethylene biosynthesis in banana fruit was under a negative feedback regulation mechanism in the pulp, whereas a positive feedback system operated in the peel.

In the perception regulatory machinery that causes ethylene responses, *Hibiscus* bud flowers treated with ACC and 1-MCP showed a complex regulation of ethylene receptor expression among different tissues. ACC treatment on bud flower tissues has led to an increase in ethylene production (Fig. 5A–C), confirming the presence of a very active ethylene-forming enzyme system at this early developmental stage (Woodson *et al.*, 1985), and determining an overaccumulation of both *HrsETR* and *HrsERS* transcriptional levels in petals (Fig. 7). Petals and ovaries from ACC-treated open flowers showed an earlier (after 1 h) down-regulation of *HrsETR* gene expression (Fig. 7). Thus, the regulation of *HrsETR* under ACC treatment may have the purpose of increasing tissue sensitivity by reducing the number of its receptors (Bleecker, 1999).

When open flowers were treated with 1-MCP, the treatment increased *HrsETR* transcripts after 1 h, and *HrsERS* transcripts between 3 and 6h in the ovary tissues and after 9 h in the petals (Fig. 8), indicating a tissue-temporal specific reduction in ethylene sensitivity. These results are consistent with the standard model of ethylene signal transduction, which postulates that an increase in receptor abundance would result in a decreased sensitivity to ethylene (Bleeker, 1999). However, in the present study *HrsETR* and *HrsERS* expression patterns in style–stigma plus stamens strongly increased after ACC treatment (Fig. 8), showing that the regulation of ethylene receptors positively correlated with the accelerated senescence process of whole flowers. These results showed that possibly an ACC-induced and presumably ethylene-dependent senescence signal can be triggered by a positive increase in ethylene sensitivity through an enhancement of transcript abundance of both ethylene receptors in style–stigma plus stamen tissues.

Is ABA directly involved in Hibiscus *flower senescence*?

In *H. rosa-sinensis* open flowers, exogenous ABA may control the biosynthesis of ethylene via a feedback mechanism, as shown by the deferred decrease in ethylene production in all open flower tissues (Fig. 5D–F). This is further confirmed by the modulation of transcription activity of ethylene biosynthetic genes, which was consistent with the inhibition of ethylene biosynthesis (Fig. 8). Although ABA has been previously shown to enhance senescence symptoms by acting as a promoting stimulus through an increase in ethylene production (Panavas *et al.*, 1998; Aneja *et al.*, 1999; Müller *et al.*, 1999), according to the findings presented here, the application of ABA promoted flower senescence and suppressed ethylene production in rose flowers (Mayak and Havely, 1972).

These observations suggest that the involvement of these two hormones in flower senescence is via the same signal transduction pathway. In addition, in *H. rosa-sinensis*, ABA may control ethylene biosynthesis and/or signalling in order to amplify its antagonistic effect on flower senescence. This antagonism between ABA and ethylene, which reciprocally regulate each other's metabolism and signalling pathway, is in keeping with a recent study on the antithetic cross-talk of these two hormones upon seed germination and early seedling growth in *Arabidopsis* (Cheng *et al.*, 2009).

The role of ABA in the gynoecium as a trigger of flower senescence has been previously described as likely to be an accumulation of this hormone before the climacteric increment in ethylene production (Onoue *et al.*, 2000). According to these results, in *H. rosa-sinensis* it appears that ABA accumulated early (3 h post-detachment) in the ovary and style–stigma plus stamen tissues (Fig. 6E, F). This took place before the climacteric increase in ethylene (Fig. 5E, F), and exogenous ABA treatment also affected the transcript abundance of ethylene biosynthetic and receptor genes early (Fig. 8). This would seem to support

the key role played by the gynoecium as a trigger of flower senescence (Shybuya *et al.*, 2000).

The endogenous concentration of ABA in *H. rosa-sinensis* during natural senescence was recently reported to be relatively low during early flower development, then peaked at the open flower stage and finally decreased during senescence (Trivellini *et al.*, 2011). In this study, ABA content does not appear to change in open flowers over the 9 h period. However, when exogenous treatments with a promoter or inhibitor of senescence are imposed an alteration in ABA concentration was observed. In this study 1-MCP treatments determined a rapid change in ABA content, which led to a sequential increase, first in style–stigma plus stamens, then in petals (Fig. 6D, E), while in the ovary (Fig. 6F) it seems to decrease with time throughout the experiment. However in the latter stages, ABA content and flower longevity were respectively reduced (Fig. 6D–F) and enhanced (Fig. 4). The increase in 1-MCP-induced flower longevity might result from an initial tissue-specific counterbalance effect of ABA content and its successive decline in all flower tissues, thus confirming a possible role for ABA in flower longevity by regulating the ethylene perception pathway.

In conclusion, it is believed that the most important finding of the present study is that ABA has a regulatory effect on the ethylene biosynthetic and perception machinery at a physiological and molecular level. The findings would seem to show a refined regulatory mechanism of flower senescence in *H. rosa-sinensis*, where both ABA and ethylene are spatially and temporally involved in the coordination of differential ageing rates in flower tissues: (i) in ABA-accelerated flower senescence, both ethylene production and the transcriptional activity of ethylene biosynthetic genes decreased, showing that ethylene may be antagonized by ABA; (ii) its effects are most likely to target the ovary tissues by the prompt regulation of ethylene biosynthetic and receptor genes; (iii) 1-MCP appears to exert its positive effect on flower life through the up-regulation of *HrsETR* first in the ovary and later in petals with a consequent decrease in tissue sensitivity to ethylene and through the decline in ABA content in all flower organs; (iv) the accelerated flower senescence observed following ACC treatments may be derived from the simultaneous strong enhancement of both ethylene biosynthetic and receptor gene expression and ethylene production, in style–stigma plus stamens; and (v) ABA accumulation was substantially decreased in petals and ovaries at a later stage, thus showing that ABA may be antagonized by ethylene action.

Further research is needed, especially to determine the involvement of other plant hormones that could be spatially and temporally involved in the coordination of differential flower ageing rates. Currently, the distribution and action of cytokinins are under investigation in this species. Moreover, the isolation and characterization of the 9-*cis*-epoxycarotenoid dioxygenase (NCED) genes in *H. rosa-sinensis* might improve the understanding of the ABA–ethylene cross-talk during flower senescence.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Time-dependent expression of ethylene biosynthetic genes (*HrsACS* and *HrsACO*) and ethylene perception genes (*HrsETR1* and *HrsERS1*) after *Hibiscus rosa-sinensis* bud flower removal from the plant at stage –1.

Table S2. Time-dependent expression of the ethylene biosynthetic genes (*HrsACS* and *HrsACO*) and ethylene perception genes (*HrsETR1* and *HrsERS1*) in *Hibiscus rosa-sinensis* opened flowers removed from the plant at stage 0.

Table S3. Effects of ACC, 1-MCP, and ABA treatments on the expression of genes involved in ethylene biosynthesis and perception in bud flowers. Gene expression levels were determined by qRT-PCR, and expressed as fold change relative to the transcript abundance in the control flowers. Values are the means of three biological replicates.

Table S4. Effects of ACC, 1-MCP, and ABA treatments on the expression of genes involved in ethylene biosynthesis and perception in open flowers. Gene expression levels were determined by qRT-PCR, and expressed as fold change relative to the transcript abundance in the control flowers. Values are the means of three biological replicates.

Table S5. Statistical analysis for Fig. 7 (two-way ANOVA).

Table S6. Statistical analysis for Fig. 8 (two-way ANOVA).

Table S7. Time-dependent expression of ethylene biosynthetic genes (*HrsACS* and *HrsACO*) and ethylene perception genes (*HrsETR1* and *HrsERS1*) in *Hibiscus rosa-sinensis* bud flowers and open flowers. The relative expression level for all genes in petal, style–stigma plus stamens (S–S+S), and ovary is expressed as fold changes to the expression level of all genes at 1 h, which is defined as 1.0. Values are the means of three biological replicates.

Table S8. Statistical analysis for Fig. 2 (two-way ANOVA).

Table S9. Statistical analysis for Fig. 3 (two-way ANOVA).

Acknowledgements

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